The impacts of commonly used medications on the trans-sulphuration pathway

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

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30 August 2024

COVID-19 statement

This research was funded by Jazan University, Saudi Arabia, under the supervision of Dr Peter Rose and Dr Simon Welham. Our original hypothesis was that some commonly-used medications can affect vitamin B_6 status among the elderly population in homecare in the UK, leading to alteration of the trans-sulphuration pathway (TSP) metabolites. However, this research was started just one month before the COVID-19 pandemic caused disruption. In March 2020, the UK was in "Lockdown" and restrictions on meeting people were in place for seven months due to government guidelines, as well as university restrictions. As such, we considered the resources available at the time to quickly adapt in order to preserve the research. Therefore, we had to do a secondary analysis to assess vitamin B_6 status among the UK population using commonly-available medications, as well as conducting a scoping review to assess the impact of medications on the TSP metabolites in humans. Following that, the difficulty in getting ethical approval to conduct our original study had led us to conduct a series of cell culture work in the lab, to further understand the impact of medication on some TSP metabolites. However, access to the lab was restricted, which was slightly eased in October 2020. However, laboratory work was further disrupted due to reduced lab availability (two hours per day); these restrictions alongside the university restrictions, such as reduced personnel, were not removed until August 2021. Furthermore, we had to close the labs and relocate them to a new building, which caused additional disruptions to work between March and July of 2021. The start of lab work was further delayed as a result of the new facilities flooding, causing a disruption that lasted for ten weeks. Additionally, obtaining culture media and reagents for cell culture has been difficult for us during this research and there were shortages of CO_2 nationwide. During the research, I had COVID-19 in June 2022, which kept me from the lab for 10 days (following the national restriction guidelines). The need to alter the method of original research was evident; however, despite these unexpected events, we had to mitigate the impact of COVID-19 on the research.

ABSTRACT

Worldwide life expectancy is predicted to increase, which can lead to an increased reliance on medications to manage multimorbidity. Self-medication and cost of living crisis has increased this pattern. The trend towards increased medication use can increase the possibility of having a larger population at risk of micronutrient deficiencies / inadequacy in users. Despite the widespread use of medications, studies on drug-nutrient interactions remain limited. This is particularly concerning for critical metabolic processes like the trans-sulphuration pathway (TSP) that depend on vitamin B6 for its flux. This work employed a multi-faceted approach aimed at providing crucial insights into how commonly prescribed drugs influence the TSP.

We conducted a scoping review to assess current evidence on the impacts of medications on TSP metabolites in humans using paracetamol (APAP) as a model therapeutic. Eight out of 13 studies showed that, despite a lack of risk factors for dysregulate glutathione (GSH) homeostasis among participants in this review, significant reduction in GSH upon short-term use (≤ 4 days) of APAP treatment was reported. No definitive conclusion could be made regarding other TSP metabolites assessed due to lack of human studies. While we acknowledge these metabolic targets are largely identified as GSH related due to the known detoxification pathway for APAP. This review raised a gap of knowledge which would be interesting to investigate; **i)** advancing age coupled with malnutrition with low protein intakes and other specific dietary patterns with low SAAs (such as vegetarian) are reported in animal models as risk factors for APAP-induced GSH depletion leading to toxicity; whether or not this phenomena and other health consequences *viz.* sarcopenia would mirror such studies in humans needs further investigation, and **ii)** previous *in vitro* work showed that GSH depletion can lead to H2S production reduction, we hence raise a

hypothesis that APAP-associated cardiovascular risk may be mediated through reduced H_2S production resulting from GSH depletion.

Secondary, reductions in dietary vitamin B_6 and pyridoxal 5′-phosphate (PLP) levels could affect the flux of TSP and are associated with increased relative risk of age-related diseases. In regards to this, we carried out a secondary analysis using the National Diet and Nutrition Survey Rolling Programme (NDNS) to assess dietary intake of vitamin B_6 and plasma PLP among the UK population (aged ≥ 19 years) and to investigate the impact of common medications on vitamin B_6 status. Results showed that median dietary vitamin B_6 intake of UK population met the reference nutrient intake (RNI) reaching 1.7 mg day^{-1} and the median plasma PLP in the entire population was 42.8 nmol L^{-1} and were higher than the threshold for vitamin B_6 deficiency. However, data showed that both plasma PLP concentrations and dietary vitamin B_6 intake tend to decline with age (P < 0.001). The NDNS data set included twelve reported therapeutic drugs. Of these, only antidepressant was associated with low dietary vitamin B_6 (P = 0.007, R^2 = 0.15). Seven drug classes were associated with plasma PLP concentrations reduction namely lipid-lowering drugs, analgesics, antibacterials, antidiabetics, antidepressants, Ca^{2+} blocker and prescribed asthma.

Further, we validated and developed a human hepatoma HepG2 model to assess the cytotoxicity profile of two drugs, APAP and sulphasalazine (SSZ). Results showed that both APAP and SSZ exhibit cytotoxicity in HepG2 cells. Mechanistic findings showed possible mechanisms of cytotoxicity **i)** inducing early oxidative markers; ROS formation, lipid peroxidation and mitochondria depolarisation (P < 0.01, for both drugs) and **ii)** inducing later markers of apoptotic cascade; DNA damage, LDH leakage and PARP cleavage. Both drugs are partially caspase-dependent, however, caspase-3 plays a more prominent role in the apoptotic cascade induced by SSZ. Both drugs

significantly reduce intracellular GSH levels in a concentration-dependent manner at 24 hours ($P < 0.001$, for both drugs). Interestingly, APAP at low concentrations (≤1 mM) significantly reduced cellular GSH levels without immediate cell death. However, this GSH depletion increased cell vulnerability to additional oxidative stress i.e. DNA damage.

In the second set of our i*n vitro* studies, we assessed the impact of APAP and SSZ on hydrogen sulphide (H_2S) and it key enzymes (CBS and CSE), alongside polysulphide (H_2S_n) levels. We showed that APAP could indirectly affect H_2S production in HepG2 cells by significantly reduce the expressions of CBS ($P < 0.01$) and CSE ($P = 0.023$), however, no significant direct reduction in H_2S was noticed upon short time APAP exposure (up to 4 hours). SSZ showed interesting findings as CSE expression was significantly elevated when cells were treated for 24 hours with low concentrations (0.12 mM and 0.25 mM; ($P = 0.034$, $P = 0.049$), respectively). At higher concentrations, CSE expression started to decrease, though not statistically significantly. A concentration-dependent reduction in CBS expression, however, not statistically significant, was noticed when cells treated with SSZ. In this chapter, we also assess the impact of H_2S and H_2S_n donors (NaHS and Na_2S_2, respectively) on APAP- and SSZ- induced oxidative stress markers. Our results showed both NaHS and Na_2S_2 had protection effects as shown by reduction in ROS formation, lipid peroxidation, mitochondria depolarisation, LDH leakage, DNA damage, cleaved PARP as well as preserving GSH levels. But also, both donors can act as direct scavengers as both NaHS and Na_2S_2 exhibit antioxidant properties, detected by the ABTS and copper reduction assays.

In conclusion, multi-faceted investigation provides evidence for medication-induced dysregulation of the TSP metabolites, enzymes and vitamin B_6 cofactor. However,

further work is needed to measure TSP metabolites in humans using medications such as Tau and H_2S and whether or not their reductions contributed to some side effects of drugs. A causal link between medication use, vitamin B_6 inadequacy and changes in TSP metabolites in humans, an interesting area to be explored in the future.

ACKNOWLEDGEMENTS

Most of all, I would like to express my sincere gratitude to my supervisor, Assistant Professor Peter Rose, who provided me with a great environment, incredible support and valuable advice during my PhD journey. This thesis would have not been possible without his thoughtful guidance, encouragement and willingness to help. That he has gone beyond what might be expected, whenever required, is genuinely appreciated. My grateful thanks also go to my second supervisor, Simon Welham, and to the technicians team.

These acknowledgments would not be complete without mention of my gratitude to my parents, Mansour and Zakiah, and to my siblings, who have supported me throughout my journey. My special thanks go to my brother, Nawaf, who has been my rock throughout.

Finally, I thank Jazan University and the Saudi Ministry of Higher Education for funding my PhD studies.

PUBLICATIONS AND PRESENTATIONS

Research publications

1. **Alsaeedi, A.,** Welham, S., Rose, P. and Zhu, Y.Z., 2023. The Impact of Drugs on Hydrogen Sulfide Homeostasis in Mammals. *Antioxidants*, 12(4), p.908.
2. **Alsaeedi, A.,** Welham, S. and Rose, P., 2023. Impact of lifestyle factors on dietary vitamin B_6 intake and plasma pyridoxal 5′-phosphate level in UK adults: National Diet and Nutrition Survey Rolling Programme (NDNS)(2008–2017). *British Journal of Nutrition*, pp.1-13.

Paper in preparation for publication

1. Polysulphide supplementations protect against paracetamol- and sulphasalazine-induced oxidative stress damages in HepG2 cells.
2. Polypharmacy and micronutrient status: a narrative review.

Oral presentation

1. **Alsaeedi, A.,** Welham, S. and Rose, P., 2023. Impact of lifestyle factors on dietary vitamin B_6 intake and plasma pyridoxal 5′-phosphate level in UK adults: National Diet and Nutrition Survey Rolling Programme (NDNS) (2008–2017). *The European Society for Clinical Nutrition and Metabolism (ESPEN)*, 11 – 14 September 2023, Lyon, France.
2. **Alsaeedi, A.,** Welham, S. and Rose, P., 2021. The effects of Paracetamol on Sulphur metabolism in humans: a systematic review. *Nutrition Futures*, 7-8 September 2021, London, United Kingdom.

Paper presented at scientific conferences / meetings

1. **Alsaeedi, A.,** Welham, S. and Rose, P., 2022. Paracetamol-induced cytotoxicity in HepG2 cells. *Postgraduate Bioscience Researchers Symposium 2022 Programme*, 6 - 8 July 2022, University of Nottingham, United Kingdom.

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LIST OF ABBREVIATIONS

TSP	Trans-sulphuration pathway
SAAs	Sulphur amino acids
APAP	Paracetamol
SSZ	Sulphasalazine
NAC	N-Acetylcysteine
GSH	Glutathione
Cys	Cysteine
Tau	Taurine
CBS	cystathionine beta synthase
CSE	Cystathionine gamma-lyase
H_2S	Hydrogen sulphide
CoL	Cost of living
OTC	Over the counter
RNI	Reference Nutrient Intake
RDA	Recommended daily allowances
Hcy	Homocysteine
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
TM	*Transmethylation*
RM	Remethylation
SAHH	S-adenosylhomocysteine hydrolase
HNMT	Histamine-N-methyltransferase
COMT	Catechol-O-methyltransferase
MS	Methionine synthesis
THF	Tetrahydrofolate
5-MTHF	5-methyltetrahydrofolate
MTHFR	Methylenetetrahydrofolate reductase
CDO	Cysteine dioxygenase
AST	Aspartate aminotransferase
HTAU-DH	Hypotaurine by hypotaurine dehydrogenase
CSAD	Cysteine sulfinic acid decarboxylase

DHLA	Dihydrolipoic acid
Trx	Thioredoxin
3-MP	3-mercaptopyruvate
CAT	Cysteine aminotransferase
3-MST	3-mercaptopyruvate sulphurtranspherase
GS	Glutathione synthesis
NHS	National Health Service
WHO	World Health Organisation
DDD	Defined daily doses
RAS	Renin-angiotensin system
PPI	Proton pump inhibitors

Chapter 1 Introduction

1 Introduction

1.1 Overview

Global demographic trends indicate significant changes in population dynamics and healthcare needs. The United Nations estimates the world's population will reach 9.7 billion by 2050, with global life expectancy at birth increasing from 72.6 years in 2019 to 77.1 years in 2050 (UN, 2019). Concurrently, the proportion of the global population over 65 years is expected to rise from one in eleven in 2019 to one in six by 2050 (UN, 2022). These trends, while reflecting advancements in healthcare and public policy, have led to an increased reliance on medications to manage health conditions and maintain quality of life. Over the past five years, there has been a 14% increase in worldwide medication use (IQVIA, 2024), highlighting a growing global dependence on pharmaceutical intervention. However, long-term medication use can inadvertently affect micronutrient levels, potentially inducing malnutrition among users (Ramgoolie and Nichols, 2016). Factors such as self-medication, the global cost of living crisis and limited access to healthcare in some countries have contributed to increased use of over-the-counter (OTC) drugs, raising concerns about polypharmacy (using ≥ 5 drugs / day) and its health implications (Meadows et al., 2024).

Despite the widespread use of medications, studies on drug-nutrient interactions remain limited (Mohn et al., 2018). This is particularly concerning for critical metabolic processes like the trans-sulphuration pathway (TSP). TSP depends on pyridoxal 5'-phosphate (PLP), the active form of vitamin B_6, and converts dietary sulphur amino acids (SAAs) to essential compounds such as glutathione, taurine, sulphate and the novel gaseous signalling molecule hydrogen sulphide (H_2S) (Olson, 2018). Precise regulation of TSP is crucial for maintaining redox status, cellular signalling

homeostasis and detoxification processes (Pizzorno, 2014; Zhang et al., 2019; Averill-Bates, 2023).

Given the critical role of TSP in maintaining health and the increasing global reliance on medications, there is a pressing need for comprehensive studies assessing the impact of commonly-used drugs on the flux of this pathway. As such, this thesis attempted to better understand the relationship between medication use-induced micronutrient deficiencies in relation to TSP. The current chapter involved two key aspects, covering; **1)** the trend of medication use, polypharmacy and micronutrient deficiency**; 2)** the current knowledge and research behind the significance of TSP in human, dietary SAA sources, TSP dysregulation and the significant role of H_2S in mammals.

1.2 Medication, polypharmacy and micronutrients

1.2.1 Medication usage and emergent trends in routes of drug access

Use of pharmacological agents is the cornerstone of treatment for many chronic diseases, such as diabetes, arthritis and hypertension and in the management of inflammation or pain, both acute and chronic, that may afflict individuals (Alorfi, 2023). Global drug use has grown by 14% in the last five years, with further increases predicted to be in the range of an additional 12% growth by 2028 (IQVIA, 2024). It is currently estimated that 3.8 trillion defined daily doses (DDD) are applied annually across the globe (IQVIA, 2024). This trend is mirrored in the overall rates of medication usage in England and Wales, which increased by 42.6% between 2004 and 2019 (Naser et al., 2022). For the previous five years, the amount spent globally on medications at list prices has increased by 35% and, by 2028, it is expected to rise by 38% (IQVIA, 2024). In the UK, the combined costs of medications prescribed in hospitals is £9.45 billion for 2022 - 2023. This represents 49.2% of the total expenditure of medications used nationally, the remainder including therapeutics prescribed and dispensed in the community or by dentists (£32.4 million), in prisons and other medical support systems (£24.9 million), respectively (National Health Service Business Services Authority [NHSBSA], 2023). **Table 1.1 and 1.2** summarise the top ten medications in the UK stratified respectively by cost and volume (i.e. quantity of prescriptions issued) dispensed in 2019 (Bennett Institute for Applied Data Science, 2020). The statistics from both tables suggest that the UK healthcare system is facing a significant burden from chronic diseases, with substantial economic impacts associated with medication use. Indeed, in 2019, in National Health Service (NHS) primary care, there was a wide spread of several medications (**Tables 1.1 and 1.2**), with three drug classes emerging as being noteworthy. These classes are among the

most widely-used drugs in terms of both volume and cost, namely: analgesics (23,196,389 items; £213,975,882); antidiabetic medications (41,654,329 items; £545,347,171); and renin-angiotensin system (RAS) drugs (65,424,446 items; £188,452,348). These statistics also show how common health conditions like pain, diabetes and hypertension and heart diseases are in NHS primary care and how much money is allocated to managing these conditions (**Table 1.1 and 1.2**). Regardless of cost, the data highlighted the most prescribed medication in the UK's NHS primary care in 2019 as being lipid-regulating medication, prescribed for people with high cholesterol (77,997,663 items). This high prescription rate aligns with the prevalence of cardiovascular diseases (CVDs) (7.6 million) among the UK population (British Heart Foundation, 2024). The second most-prescribed medication was RAS (for hypertension and heart diseases) (65,424,446 items – see above), followed by proton pump inhibitors (PPIs) (for indigestion and heartburn) (62,236,186 items); Ca^{2+} blocker (for hypertension) (42,419,537 items); antidiabetics (for diabetes) (41,654,329 items – see above); selective serotonin reuptake inhibitors (for depression) (40,254,277 items); beta blockers (for hypertension) (39,505,912 items); non-opioid analgesics (for pain relief) (34,487,135 items); and antiplatelets (for heart attack and stroke) (33,308,350 items). Given the high prescription volumes across multiple drug classes, many patients may be taking multiple medications, which could indicate a trend towards polypharmacy (using ≥ 5 drugs / day). In fact, polypharmacy is common in UK primary care, with ≈ 17% of adults (≥ 20 years) using 4 - 9 medications and ≈ 5% even using ≥ 10 medications (Payne et al., 2014).

Table 1.1 Top 10 spend on therapeutic classes of drugs in 2019 in NHS Primary Care (Bennett Institute for Applied Data Science, 2020).

Drug Class	Prescription Items	Cost	Use
Corticosteroids (Respiratory)	21,099,043	£548,653,646	Inhalers for lung conditions
Antidiabetic Drugs	41,654,329	£545,347,171	Diabetes
Oral Anticoagulants	18,486,373	£541,626,860	Blood thinners - to prevent clots / strokes
Insulin	7,538,314	£332,531,643	Diabetes
Control of Epilepsy	28,704,569	£278,841,283	Epilepsy
Enteral Nutrition	3,725,985	£236,304,624	Malnutrition
Analgesics	23,196,389	£213,975,882	Pain relief
Renin-Angiotensin System Drugs	65,424,446	£188,452,348	High blood pressure - heart disease
Diabetic Diagnostic and Monitoring Agents	7,145,514	£155,910,265	Blood sugar test for diabetes

Table 1.2 Top 10 therapeutic classes, as represented by absolute volume prescribed in 2019 in NHS Primary Care (Bennett Institute for Applied Data Science, 2020).

Drug Class	Prescription Items	Cost	Use
Lipid-Regulating Drugs	77,997,663	£116,000,000	Statins-high cholesterol
Renin-Angiotensin System Drugs	65,424,446	£188,000,000	High blood pressure - heart disease
Proton Pump Inhibitors	62,236,186	£98,727,913	Indigestion or stomach ulcers
Calcium-Channel Blockers	42,419,537	£105,000,000	High blood pressure - heat disease
Antidiabetic Drugs	41,654,329	£545,000,000	Diabetes
Selective Serotonin Re-Uptake Inhibitors	40,254,277	£66,154,129	Depression
Beta-Adrenoceptor Blocking Drugs	39,505,912	£67,928,111	High blood pressure - heart disease
Non-Opioid Analgesics and Compound Preparations	34,487,135	£128,000,000	Pain Relief
Antiplatelet Drugs	33,308,350	£66,684,077	Reduce the risk of heart attacks and stroke

Moreover, data from various sources indicate that certain medication classes are commonly used across different countries. For instance, the Centres for Disease Control and Prevention (CDC) reported that analgesics, antihyperlipidemic, antidepressants and antidiabetic agents were among the most commonly used medications in the US population in 2019 (Santo and Kang, 2023). Similarly in Saudi Arabia, analgesics, antibiotics, PPIs and antidiabetic agents were the most commonly used medications between 2010 and 2015 (AlKhamees et al., 2018). In Canada, antihypertensives, lipid-lowering drugs and antidepressants were commonly used from 2016 to 2019 (Statistics Canada, 2021). In Australia, lipid-lowering drugs, PPIs, cardiovascular medications and antidiabetic agents were among commonly used drugs between 2017 - 2018 and 2022 - 2023 (Australian Institute of Health and Welfare (AIHW), 2024). Despite the global variations in healthcare policies, clinical guidelines, cultural practices and medication accessibility, these data suggest that there are global trends in the wide spread of medication use to manage the prevalence of chronic diseases such as diabetes, hyperlipidaemia, depression, CVDs and chronic / acute pain conditions. However, in most countries, over the counter (OTC) medications (e.g. analgesics), can be purchased from non-pharmacy outlets, such as supermarkets, convenience stores, petrol stations or online (Oleszkiewicz et al., 2021). This suggests that these data may have become skewed as OTC cannot be reliably captured (AIHW, 2024). This emergent pathway of drug use is now becoming a worldwide problem due to inadequate medical provisions in some countries (Bennadi, 2013) and financial constraints caused by the global cost of living (CoL) crisis (Meadows et al., 2024). This potentially increases the self-medication rate (Tavares, Ferreira and Cavadas, 2022).

According to the World Health Organisation (WHO) (2000), self-medication refers to the utilisation of medications by the patient to address illnesses or symptoms that they

have independently diagnosed, or the continuous or sporadic administration of a prescribed medication for chronic symptoms. Across medications widely used, paracetamol is one of the most-used self-medicated drugs (Tariq and Din, 2017; George and Meldrum, 2020). One of the biggest risks associated with self-medication is polypharmacy (Oleszkiewicz et al., 2021), which will be discussed in **section 1.2.2** below. Unfortunately, the trend towards increased medication use can also increase the possibility of having a larger population at risk of micronutrient deficiencies / inadequacy in users (Kiani et al., 2022), especially when medications are taken long-term (Mohn et al., 2018) or in combination with other drugs (Long et al., 2012).

1.2.2 Polypharmacy

Polypharmacy refers to the concurrent use of multiple medications by an individual to manage one or more health conditions. While definitions vary, the most commonly-accepted criterion is the use of five or more drugs per day (Masnoon et al., 2017). Frequent and simultaneous use of multiple medications often becomes necessary due to the high burden of co-occurring chronic diseases, a condition known as "multimorbidity" (Muth et al., 2019). Multimorbidity may occur as a result of extended life expectancy in the general population (Sergi et al., 2011). The projected increase in life expectancy worldwide is predicted to be 4.5 years, from 73.6 years in 2022 to 78.1 years in 2050 (Institute for Health Metrics and Evaluation (IHME), 2024). Over the past 20 years, polypharmacy has become a significant global public health concern, particularly among the elderly population (Bardel, Wallander and Svärdsudd, 2000; Charlesworth et al., 2015). Indeed, polypharmacy (≥ 5 drugs) has been shown to affect a range between 19% and 68% of elderly individuals (≥ 60 years) in several developed countries; these incidents have been noted in the UK (Rawle et al., 2018); the US (Young et al., 2021); Denmark (Jørring Pallesen et al., 2022); Sweden (Zhang

et al., 2020); Canada (Harris et al., 2022); Australia (Wylie et al., 2020); and Korea (Shin, Go and Kim, 2024). Although it is more common and well-documented in the elderly, research has also documented its occurrence among young adults (Haider et al., 2014; Lunsky and Modi, 2018).

In fact, polypharmacy often presents with a complex range of health challenges that can have a significant impact on patient outcomes. The relationship between negative health outcomes and polypharmacy, regardless of drug number classification, is prevalent among older individuals (≥ 55 years) and includes: impairment in physical performance (≥ 10 drugs / day) (Sganga et al., 2014); depression (≥ 5 drugs / day) (Öztürk, Ganidağlı and Öztürk, 2023); drug adverse reaction (≥ 5 drugs / day) (Mabuchi et al., 2020); hospitalisation (≥ 5 drugs / day) (Pascual et al., 2020); dementia (≥ 5 drugs / day) (Cheng et al., 2018); and higher risk of mortality or incident disability (Odds Ratio (OR) 5.3) and hospitalisation (OR 2.3) (≥ 5 drugs / day) (Bonaga et al., 2018). These studies emphasise the association between medication usage and a wide range of adverse health outcomes.

1.2.3 The impact of medication / polypharmacy on micronutrient status

1.2.3.1 Micronutrient – an overview

Micronutrients are needed from our diet in smaller quantities than other nutrients, such as carbohydrate, fat and protein (macronutrients) (Espinosa-Salas and Gonzalez-Arias, 2023). However, they have a vital and critical influence on the body's functioning, development and growth (Shenkin, 2006; Cena and Calder, 2020). The main classifications of micronutrients are vitamins and minerals. The former are organic micronutrients and categorised as water-soluble (B vitamins and vitamin C) or fat-soluble (vitamins A, E, D and K) (Espinosa-Salas and Gonzalez-Arias, 2023). Minerals, on the other hand, are inorganic, and further classified as macrominerals

(calcium, phosphorus, magnesium, sodium, potassium, chloride and sulphur) or microminerals (iron, copper, zinc, selenium and iodine).

An adequate micronutrient intake is necessary for supporting physiological processes and critical metabolic pathways. Indeed, these substances have varying important functions in human health, each having unique implications. **Table 1.3** shows examples of selected micronutrient functions in the human body, including acting as cofactors for enzyme activity in vital biochemical reactions (Huskisson, Maggini and Ruf, 2007); participating in DNA synthesis (Green et al., 2017; Hariz and Bhattacharya, 2019); supporting hormone production (Shergill-Bonner, 2013); facilitating blood formation (Wishart, 2017); energy metabolism (Tardy et al., 2020); modulating gene transcription (Allgood and Cidlowski, 1992); enhancing immune function (Gombart, Pierre and Maggini, 2020); and protecting cells from peroxides and free radicals as antioxidants (Sies, Stahl and Sundquist, 1992; McDowell et al., 2007). Furthermore, insufficient intake of micronutrients can lead to deficiency, a micronutrient deficiency being the absence of at least one of the micronutrients needed for optimum human health (Godswill et al., 2020). In fact, 2 billion people globally suffer from micronutrient deficiencies (Lowe, 2021). These can lead to negative health conditions (Engle-Stone et al., 2019) and vary according to the specific nutrient(s) involved. **Table 1.3** shows examples of differing micronutrient deficiency significance. For instance, deficiency in B vitamins can lead to neurological disorders (Hanna et al., 2022); CVDs (Wang, 2024); and liver damage (Yan et al., 2018); while magnesium deficiency can lead to abnormal heart rhythms and muscle cramps (National Institute of Health (NIH), 2022); iron deficiency causes iron deficiency anaemia (NIH, 2023); and hypothyroidism can occur as a result of iodine deficiency (NIH, 2024). In general, long-term micronutrient insufficiency can lead to complex metabolic disorders (Gröber, Schmidt and Kisters,

2020) and increase potential risk of chronic diseases, such as CVDs, osteoporosis and cancer (Tulchinsky, 2010).

Table 1.3 Function of selected micronutrients in human body, dietary sources, deficiencies and biomarkers for assessing status.

Micronutrient	Function	Food source	Deficiency symptom	Biomarker	Reference
Vitamin B_6 (pyridoxine)	• The active, pyridoxal 5'-phosphate (PLP), serve as a cofactor for a large number of enzymes (> 140). • Brain function (neurotransmitter production). • Immune system. • Red blood cells (RBCs) synthesis. • Regulates hormones. • Carbohydrate, fat and protein metabolism.	Meat, poultry, fish, eggs, wide range of fruit and vegetables such as banana, avocado and green paper.	Marginal vitamin B_6 deficiency: oral stomatitis, depression, confusion and irritability. Sever vitamin B_6 deficiency: dermatitis, microcytic anaemia and seizures.	Plasma PLP.	(British Nutrition Foundation (BNF), 2023; Brown et al., 2017; DoH, 2008)
Vitamin B_9 (folate)	• DNA synthesis, stability and repair. • RBCs. • Cell division and growth. • Regulate homocysteine. • Neurological function. • Pregnancy and fetal development.	Green leafy vegetables, fruits (orange and berries), peas, beans and fortified breakfast cereals.	Megaloblastic anaemia, causing headache, concentration difficulties, short of breathing and weaknesses.	Concentration in plasma, serum and erythrocyte.	(BNF, 2023; Sarwar, Sarwar and Sarwar, 2021; DoH, 2008)
Vitamin B_{12} (cobalamin)	• RBCs. • Nervous system. • DNA synthesis. • Amino acid metabolism. • Faty acid metabolism.	Meat, fish, shellfish, milk, cheese, eggs and fortified breakfast cereals.	Megaloblastic anaemia (large abnormal RBCs), depression and neurological damages.	Serum / plasma vitamin B12, Holotranscobalamin (holoTC), Methylmalonic acid (MMA) and Homocysteine.	(BNF, 2023; Sarwar, Sarwar and Sarwar, 2021; DoH, 2008)

1.2.3.2 Micronutrient deficiency risk factor

Many factors can affect micronutrient status, such as poor diet (Kiani et al., 2022); special dietary patterns (e.g. vegan) (Craig, 2009); some health conditions, *viz.* Crohn's disease (Weisshof and Chermesh, 2015); genetic disorders (Tummolo et al., 2023); Godswill et al., 2020); age-related factors (Hoffman, R., 2017); socioeconomic status (Zhu et al., 2020); and lifestyle factors, e.g. alcohol and smoking (Eichholzer, 2003). Of significant importance in relation to the topic of this thesis is that the use of certain medications can lead to deficiency or suboptimal levels of micronutrients (Sayedali, Yalin and Yalin, 2023). This will be covered in **section 1.2.3.4.**

1.2.3.3 Assessment of micronutrient status

According to European Society for Clinical Nutrition and Metabolism (ESPEN) guidelines, assessing micronutrient deficiency involves both dietary assessment and biochemical testing (Berger et al., 2022). The UK's Committee on Medical Aspects of Food and Nutrition Policy (COMA) established dietary reference values in 1991, which have since been updated by the Scientific Advisory Committee on Nutrition (SACN) (Powers, 2021). Nutrient requirements are assumed to be normally distributed, with the Estimated Average Requirement (EAR) meeting the needs of 50% of healthy individuals; intake below the EAR suggests potential inadequacy. The Lower Reference Nutrient Intake (LRNI), set two standard deviations below the EAR, is sufficient for only 2.5% of individuals (Powers, 2021), indicating they are unlikely to meet their requirement. Conversely, the Reference Nutrient Intake (RNI), which is two standard deviations *above* the EAR, meets the needs of 97.5% of individuals, suggesting adequacy if intake exceeds this level. Biological measures are essential for confirming nutrient deficiency (Powers, 2021), with modern techniques allowing for the assessment of micronutrient levels in plasma, serum, blood cells, urine or faeces

(Jackson, 1999; Picó et al., 2019). **Table 1.3** provides examples of biomarkers used to assess micronutrient status in humans.

1.2.3.4 Medication- / polypharmacy-induced micronutrient depletion

The medical and pharmaceutical world have historically paid little to no attention to interactions between medications and micronutrients. There are relatively few research studies looking at possible drug-nutrient interactions (Mohn et al., 2018). Since almost all physiological processes in the body are mediated by one of these metabolic processes controlled by enzymes (Gröber, Schmidt and Kisters, 2020), alterations in micronutrient status can lead to severe metabolic dysfunctions (Berger et al., 2022).

Mechanism of action

Medicines can either directly or indirectly influence a patient's micronutrient status (the indirect mechanism will be detailed later in this chapter). Drug-induced micronutrient depletions can occur directly by altering the pharmacokinetic properties of micronutrients - namely their absorption, distribution, metabolism or excretion (ADME) (Prescott et al., 2018). This can occur since both, medications and micronutrients, might utilise the same metabolic and transport pathways in the human body. This implies that there is always a chance of drug interactions affecting nutritional status when taking medications. **Table 1.4** showed representative micronutrients affected by commonly-used medications. A more comprehensive table of micronutrient deficiency induced by common drugs is presented in **Appendix 1.1**. The literature shows several micronutrient-related deficiencies have been associated with usage of various drug classes, e.g. antiepileptic, antibiotic, anti-inflammatory, antidiabetic, antidepression, Xanthine (for asthma), diuretic, angiotensin-converting enzyme (ACE) inhibitor, oral contraceptive, acid suppressing drugs, corticosteroid and lipid-lowering medications.

These agents have been shown to cause diminished levels of several micronutrients, namely B vitamins (vitamin B_1, vitamin B_6, vitamin B_9, vitamin B_{12}), vitamin C, calcium, magnesium, selenium, potassium, sodium, zinc and iron, in users via a range of mechanisms (summarised in **Appendix 1.1**). These depleting mechanisms are diverse and complex, ranging from: reduced absorption (which can occur through decreases in micronutrient solubility) (Henry et al., 2005); impaired intestinal absorption (Lechner, 2015; Damião et al., 2016); reduced cellular uptake (Kurstjens et al., 2017; Bouras et al., 2020); lowered bioavailability (McColl, 2009); impaired absorption of essential complexes, such as the intrinsic factor-B_{12} complex, which is necessary for vitamin B_{12} absorption (Damião et al., 2016); elevated excretion (resulting from impaired reabsorption processes (Spital, 1999); increased elimination (Friedman and Bushinsky, 1999); altered metabolism (inhibition of micronutrient synthesis) (Heller and Friedman, 1983); elevated catabolism rates (NIH, 2023); impaired metabolic processes (Shojania, 1982; Shere et al., 2015); changes in gene expression and transporter activity (William and Danziger, 2016); interference with micronutrient function (which can manifest as competitive inhibition of enzymes) (Lechner, 2015); and reduction in the availability of active forms through oxidation processes (Ubbink et al., 1990). These multiple mechanisms underscore the complex and multifactorial relationship between medication use and micronutrient deficiency.

Classic examples of drug-induced deficiencies are reported for the B vitamins, like B_6, B_9 and B_{12}. These micronutrients are known to play critical roles in many enzymatic reactions, such as the trans-sulphuration pathway (Olson, 2018) and methionine cycle (Lyon et al., 2020), and are essentially for proper function of the cardiovascular, neurologic and hemopoietic systems (Sbodio, Snyder and Paul, 2019; Hanna et al., 2022). Importantly, these vitamins are critical in maintaining metabolic systems

associated with cellular redox signalling systems, drug metabolism and the synthesis of endogenous gaseous signalling molecules like hydrogen sulphide (H_2S) (Snyder and Paul, 2019; Mukherjee, Banerjee and Singh, 2023).

Using the case of vitamin B_{12} as an example, uptake requires the presence of secreted hydrochloric acid in the stomach, to liberate vitamin B_{12} from food matrices, and intrinsic factor (IF), also secreted in the stomach, that chelates B_{12} in the duodenum to aid its stability and transport into the small intestine. Once in the small intestine, the IF-vitamin B_{12} complex facilitates absorption into the bloodstream (Ankar and Kumar, 2022). Critically, a lack of either of these components impairs vitamin B_{12} status in individuals and can cause metabolic abnormalities, such as insulin resistance and defective neurotransmitter synthesis (Lam et al., 2013; Calderón-Ospina and Nava-Mesa, 2020; Zhu, et al., 2023). Several therapeutics are known to reduce IF and stomach acids, leading to diminished vitamin B_{12} absorption, such as acid-suppressing medications like histamine-2 receptor antagonists and proton pump inhibitors (PPIs) (**Table 1.4**).

Magnesium homeostasis is similarly impacted by drug usage (**Appendix 1.1**). Again using this micronutrient as an example, poor absorption is reported in patients who take PPIs (> 1 year usage) (Gommers, Hoenderop and de Baaij, 2022). Magnesium is an essential micronutrient, serving as a cofactor in over 300 enzymatic reactions, and is needed for proper nerve transmission, energy production, temperature regulation, muscle activation and development of healthy bones (NIH, 2022). Deficiency in magnesium increases the relative risk of cardiovascular diseases, such as hypertension, stroke, heart attack and atherosclerosis (NIH, 2022). This is likely reflective of the important metabolic roles this nutrient plays in the cardiovascular system.

Table1.4 Representative micronutrients affected by pharmacological interventions and their associated depletion mechanisms.

Micronutrient	Drug class	Mechanism	Reference
Vitamin B_1 (Thiamine)	Diuretic	↑ Urinary loss of vitamin B_1	(Rieck et al.,1999)
Vitamin B_6 (Pyridoxine)	Diuretic	↑ Vitamin B_6 excretion	(Mydlík, Derzsiová and Žemberová, 1999)
	Antiepileptic	↑ Catabolism rate of vitamin B_6	(NIH, 2023)
	Xanthine (for asthma)	↑ Pyridoxal (a form of vitamin B_6) oxidation, lead to ↓ the availability of PLP **(*)**	(Ubbink et al., 1990)
	Antidepression	↓ PLP synthesis	(Heller and Friedman, 1983)
Vitamin B_9 (Folate)	Anti-inflammatory	↓ Folate absorption, competitively inhibits the enzyme dihydrofolate reductase (DHFR) **(**)**	(Lechner, 2015)
	Oral contraceptive	Impair folate metabolism	(Shere et al., 2015; Shojania, 1982)
	Antibiotic	Inhibit the DHFR **(**)** enzyme	(Lechner, 2015)
Vitamin B_{12} (Cobalamin)	Antidiabetic	Calcium-dependent endocytosis **(***)** in the gut is disrupted by metformin. The absorption of Intrinsic Factor-B_{12} complex is impaired as a result, which lowers the absorption of vitamin B_{12}	(Damião et al., 2016)
	Acid suppressing drug	↓ Absorption of Intrinsic Factor-B_{12} complex ↓ Gastric acid secretion	(Valuck and Ruscin, 2004)

(*) PLP (pyridoxal-5′-phosphate) is the active form of vitamin B_6.

(**) DHFR is an enzyme needed for converting folate to its active form (tetrahydrofolate).

(***) Calcium plays a crucial role in the uptake of the B_{12}-intrinsic factor complex in the terminal ileum.

Furthermore, therapeutics may indirectly impact an individual's micronutrient and overall nutritional status in several ways. They may cause: **i)** changes in body composition, such as weight loss; **ii)** induce gastrointestinal side effects like nausea, vomiting and diarrhoea; or **iii)** lead to other side effects, such as impaired cognition or vision that can affect a patient's ability to obtain, prepare and eat food (Prescott, Drake and Stevens, 2018). These effects can potentially drive malnutrition in medication users (Ramgoolie and Nichols, 2016), an area often overlooked by researchers.

Malnutrition, as defined by ESPEN, is "a state resulting from lack of intake or uptake of nutrition that leads to altered body composition (decreased fat free mass) and body cell mass leading to diminished physical and mental function and impaired clinical outcome from disease" (Cederholm et al., 2015). The mechanisms by which medications can trigger malnutrition are illustrated in **Figure 1.1**. Many medications can reduce appetite (Kesari and Noel, 2022), typically as a manifestation of negative drug effects. For instance, the impact of certain medications on appetite suppression or changes to flavour perception can result in reduced food consumption (D'Alessandro et al., 2022). This decreased intake can affect micronutrient absorption and bioavailability, potentially leading to malnutrition and poor health outcomes (D'Alessandro et al., 2022). When combined with the direct impacts on micronutrient deficiencies discussed earlier, these factors together can increase the risk of malnutrition in medication users (Ramgoolie and Nichols, 2016). This is particularly critical among the elderly due to, firstly, their sensitivity to disparate nutrient intakes caused by changes to food intake, flavour perception, textural changes, chewing issues, satiety levels and changes in mobility that dictate energy expenditure (Rolls, 1993; Jeon et al., 2021); secondly, the risk of chronic diseases like CVDs, delayed wound healing and decreased muscle function can be triggered by malnutrition in this

vulnerable group (Saunders and Smith, 2010; Corcoran et al., 2019). Several studies of older people have found a direct correlation between malnutrition and polypharmacy (Ramgoolie and Nichols, 2016; Kok et al., 2022; Zanetti et al., 2023). For example, a recent Japanese cross-sectional study focused on elderly people in day-care facilities (mean age 79 years) showed that the severity of malnutrition correlated with rates of polypharmacy in this group with key drivers noted as: ≥ five medications, particularly PPIs, anti-constipation and antihypertensive drugs, and disability in activities of daily living (Nakamura et al., 2021). Moreover, this issue (medication-induced malnutrition) is significant among individuals in hospitals, as malnutrition is already common among hospitalised patients, with reported global prevalence rates ranging from 13% to 78%, depending on the condition, whether surgical or medical inpatients (Kubrak and Jensen, 2007).

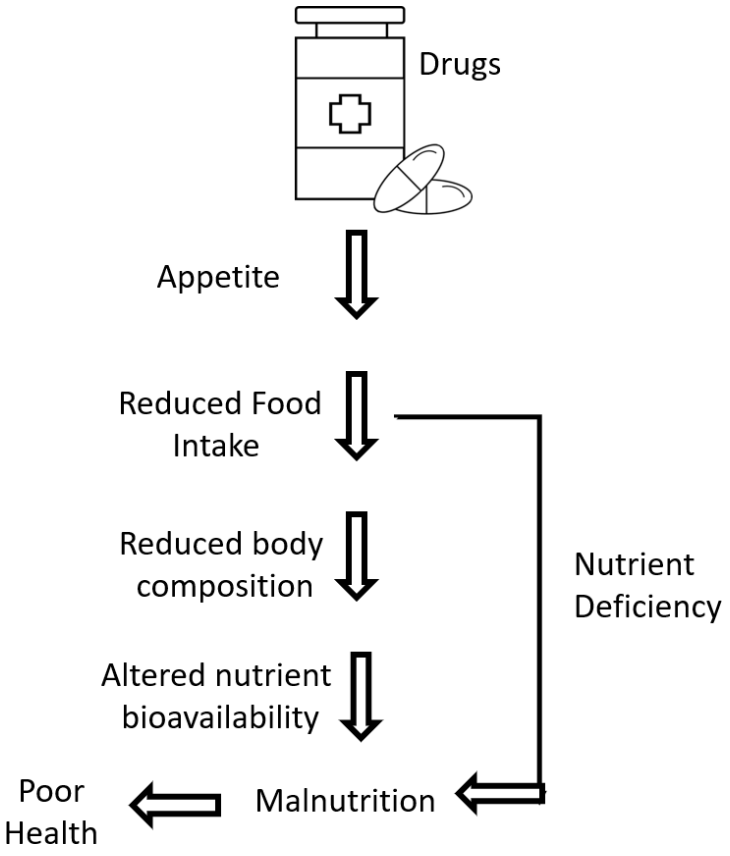


Figure 1.1 Generalised hypothetical summary of indirect mechanism of drug-induced malnutrition in humans. Drug-induced changes in appetite lead to reduced food intake and body consumption (weight loss), which can affect the micronutrient bioavailability. This in turn drives malnutrition and overall poor health (adapted from D'Alessandro et al. 2022).

Clearly, all evidence points to the fact that medication usage can have direct or indirect impacts on micronutrient status and overall health. While current recommendations are to utilise supplements (Kiani et al., 2022), micronutrient deficiencies are often initially unseen, but becoming more apparent with long-term use or polypharmacy.

One key focus of our research that spans decades has been the trans-sulphuration pathway (TSP) and its role in regulating cell signalling molecules critical for health. TSP depends on the active form of vitamin B_6, pyridoxal-5-phosphate (PLP), for its flux (Olson, 2018). Therefore, we were curious as to the impact of common medication on this system and some of its metabolites, namely glutathione (GSH), hydrogen sulphide (H_2S) (and related sulphur species) and its key enzymes. This area will be addressed below.

1.3 The trans-sulphuration pathway (TSP)

It is clear that medication usage can drive micronutrient deficiency in humans. One of the important themes in the literature was the notable changes that can occur in the levels of B vitamins and how this can impact on metabolic pathways linked to the cardiovascular, neurological and other organ systems, such as the liver. In the following sections we will describe the functional role of the TSP and its requirement for producing key metabolites required in GSH homeostasis, in the production of the novel gaseous signalling molecule H_2S, and other metabolites. The TSP is widely recognised as playing important roles in all organ systems and tissues and, as such, changes in its function are linked to poor health and disease (Sbodio, Snyder and

Paul, 2019). For the remaining text, we will focus our narrative on the TSP and the impact of changes in its metabolic function. TSP requires dietary sulphur amino acids (SAAs), mainly cysteine and methionine, to function properly. An understanding of the metabolic fate of these molecules and its physiological significance in relation to health and medication use was covered in our recent work and will be covered in brief below (Alsaeedi et al., 2023).

1.3.1 Sulphur amino acids (SAAs)

Four amino acids contain sulphur, namely methionine (Met), cysteine (Cys), homocysteine (Hcy) and taurine (Tau) (**Figure 1.2**); only the first two of these are incorporated into proteins (Brosnan and Brosnan, 2006). Met and Cys are the primary source of SAAs found in animal protein products like meat, fish, poultry, eggs and dairy products (Rehman et al., 2020; Rose et al., 2021) and range from between 3% and 6% of the total amino acids in proteins (Nimni et al., 2007). Hcy is generated in the body as an intermediate metabolite during the metabolism of methionine and is a recognised diagnostic marker for cardiovascular disease severity, since elevated levels drive free radical production and endothelial damage (Rehman et al., 2020). Tau is a conditionally-essential sulphur-containing amino acid derived from meat and seafood (Santulli et al., 2023) and can be supplemented in certain energy drinks (Clauson et al., 2008); the reason behind its inclusion in these commercially-available drinks may be due to Tau's ability to regulate cardio myocellular contraction (van de Poll, Dejong and Soeters, 2006).

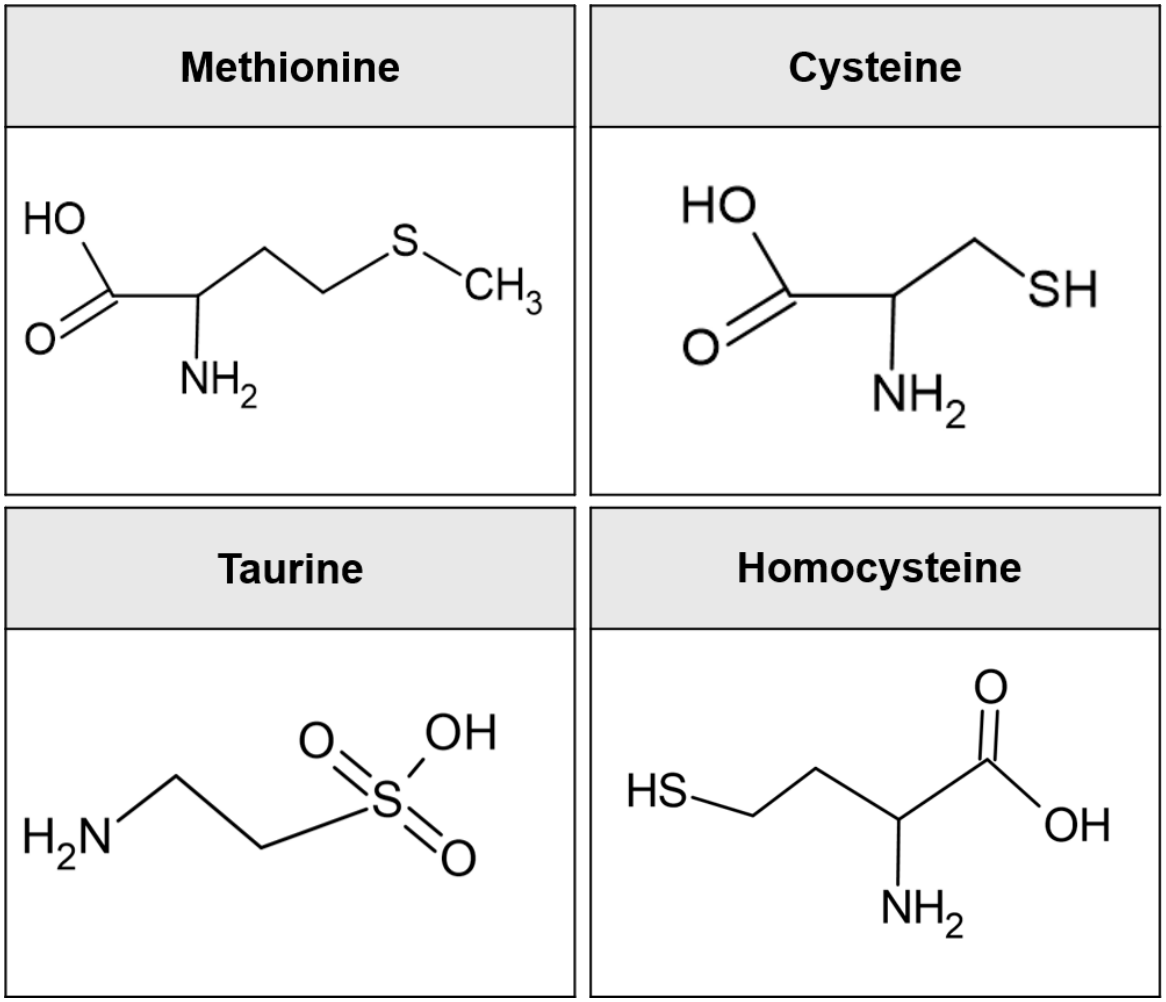


Figure 1.2 Common sulphur amino acids (SAAs) methionine, cysteine and taurine are usually found in foods (animal protein products), while homocysteine is produced as an intermediate metabolite during the metabolism of methionine in our body.

SAA intakes are not subject to specific RNI values in the UK, as is the case for other vitamins and minerals (Food Standards Agency, 2003). Rather, they are predicted based on protein consumption (Tesseraud et al. 2008). The RNI for protein for adults aged ≥ 19 years is 0.75 g / kg / day (Committee on Medical Aspects of Food Policy (COMA), 1991; BNF, 2021). In the US, the recommended daily allowances (RDA) for SAA Met and Cys, provided by the Food and Nutrition Board of the National Academies for adults aged ≥ 19 years is 19 mg / kg / day (to be sufficient to fulfil the

nutritional needs of nearly all healthy adults, specifically 97% to 98%) (Institute of Medicine, 2005), with 12.2 mg / kg / day for Met and 6.6 mg / kg / day for Cys (Dong et al., 2020). The EAR for SAAs is set at 15 mg / kg / day for adults, which satisfies the needs of 50% of the population of healthy adults (Institute of Medicine, 2005). Both EAR and RDA requirements are impacted by a person's age, sex and stage of life (**Table 1.5**). Deficiencies in dietary SAAs impact on Met and Cys homeostasis (Jones et al., 2011); increase oxidative stress (Song et al., 2021); and can impact on health (Navik et al., 2021). For example, reduction in dietary SAAs, like Met, drives bone-related disorders (Huang et al., 2014); immune function impairment (Bangyuan et al., 2012; Wu et al., 2013; Wu et al., 2018); inflammation-related diseases (Sutjiati, Wirjatmadi and Kalim, 2018); and liver damage (Matthews et al., 2021).

Table 1.5 Recommended Daily Allowances (RDA) and Estimated Average Requirement (EAR) for sulphur amino acids (SAAs) (Met + Cys) across different age groups and sex, as established by the Food and Nutrition Board of the National Academies (Institute of Medicine, 2005).

Age	RDA (mg / kg / day)		EAR (mg / kg / day)	
	Female	Male	Female	Male
Infants (0 – 6 months)	*	*	*	*
Infants (7 - 12 months)	43	43	30	30
Children (1 - 3 years)	28	28	22	22
Children (4 - 8 years)	22	22	18	18
Children (9 - 13years)	21	22	17	18
Children (14 - 18years)	19	21	16	17
Adults ≥ 19 years	19	19	15	15
Pregnancy	25	-	20	-
Lactating	26	-	21	-

RDA, sufficient to fulfil the nutritional needs of nearly all healthy adults, specifically 97% to 98%; **EAR,** sufficient to fulfil the nutritional needs of 50% of the population of healthy adults. **(*)**, there is no RDA or EAR for Met and Cys for this age group due to insufficient evidence; however, the Adequate Intake (AI) for this group is 59 mg / kg / day. AI is a value that, in situations where an RDA cannot be established, is based on estimated nutrient intake by a group (or groups) of healthy individuals, either through observation or experimentation.

1.3.2 Absorption

Met, Cys and Tau are primarily absorbed in the small intestine, with the majority of absorption occurring in the jejunum and ileum (Broer, 2008). This absorption process is highly efficient, as protein digestion products are absorbed with approximately 95 - 99% efficiency across the intestinal epithelium (Stipanuk, 2004). These SAAs are taken up into enterocyte cells (the intestinal epithelial cells) primarily via active transport mechanisms (transcellular transport) (Becquet et al., 2023). From the enterocytes, they are then transported across the basolateral membrane into the bloodstream (**Figure 1.3**).

Dietary **Met** is primarily carried by the neutral amino acid transporters B^0AT1 (SLC6A19), LAT1 (SLC7A5), LAT2 (SLC7A8) and LAT4 (SLC43A2), potentially ASCT2 (SLC1A5) and the neutral / cationic amino acid transporter $ATB^{0,+}$ (SLC6A14) (Broer, 2008; Becquet et al., 2023). Dietary **Cys** is absorbed as both Cys and cystine (CySSCy, the oxidised form of Cys). Cys transport is mediated by the neutral amino acid transporters B^0AT1 (SLC6A19), ASCT2 (SLC1A5) and the system L transporters, including LAT1 (SLC7A5) and LAT2 (SLC7A8). CySSCy uptake is predominantly facilitated by $b^{0,+}$ transporters ($b^{0,+}$AT/rBAT, SLC7A9/SLC3A1). The antiporter system x_c^- (SLC7A11) facilitates the exchange of extracellular CySSCy for intracellular glutamate in various cell types (Sbodio, Snyder and Paul, 2019). This transport mechanism may contribute to intestinal CySSCy absorption (Burdo, Dargusch and Schubert, 2006). Dietary **Tau** transport is predominantly facilitated by the Tau transporter TauT (SLC6A6) and the proton-coupled amino acid transporter PAT1 (SLC36A1) (Becquet et al., 2023; Broer, 2008). Localisation of these transporters in the intestine enterocyte is presented in **Figure 1.3**.

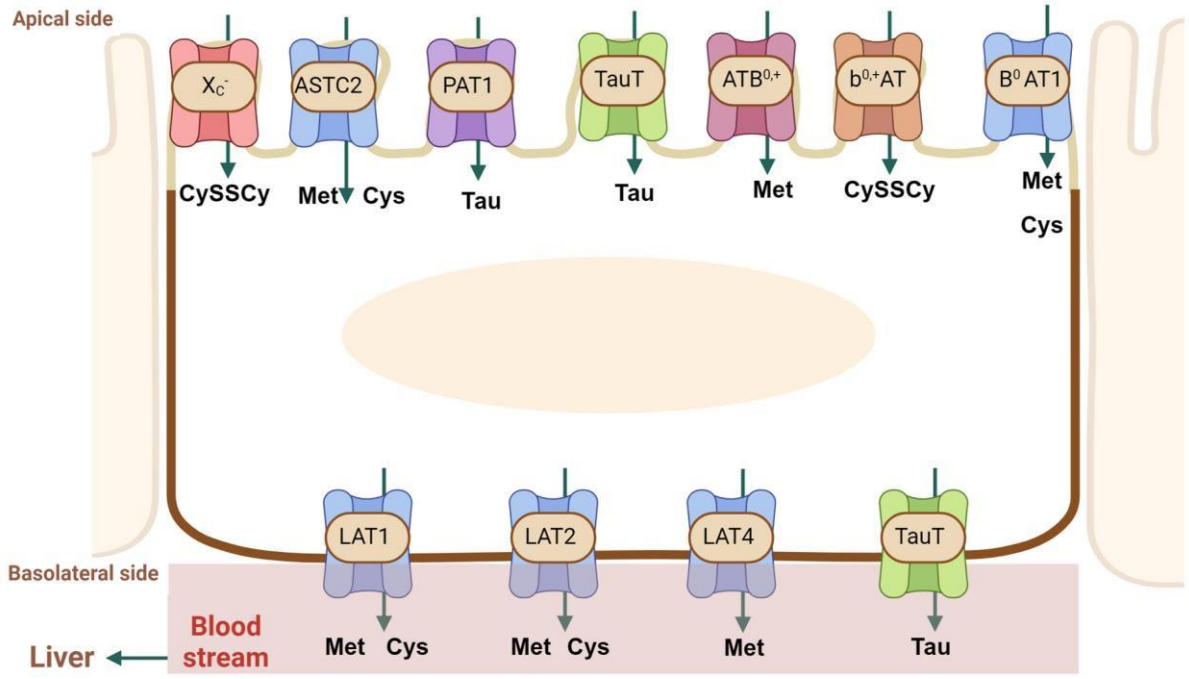


Figure 1.3 Schematic representation of the localisation of the key SAAs (Methionine [Met], cysteine [Cys] and taurine [Tau]) transporters involved in the absorption and transport Met, Cys and Tau in the intestinal enterocyte and blood stream. Cys is absorbed as both the reduced form (Cys) and the oxidised form cystine (CySSCy). Once inside the cell, CySSCy is rapidly reduced to two molecules of Cys due to the cell's reducing environment and then transported out of the cell into the bloodstream as Cys, as indicated in the figure. Apical (luminal) transporters are shown on the upper membrane, while basolateral transporters are depicted on the lower membrane. Arrows indicate the direction of transport. These transporters are **neutral amino acid transporters** (B^0AT1, ASCT2, LAT1, LAT2 and LAT4), **cationic amino acid transporters** ($b^{0,+}AT$), **taurine transporters** (TauT), **proton-coupled transporters** (PAT1) and **other unique transporters** (X_C^- and $ATB^{0,+}$). The figure is modified from Becquet et al. (2023) and generated with (BioRender.com).

Once absorbed, Met, Cys and Tau enter the portal circulation and are transported to the liver and subsequently distributed to various tissues for metabolic utilisation (Stipanuk, 2004). The liver is the primary organ used for the metabolism of SAAs, via three phases of SAA metabolism: **1)** Transmethylation; **2)** Remethylation; **3)** Trans-sulphuration (TSP) pathways. Transmethylation and remethylation processes have

been comprehensively covered previously in really good reviews (Blom and Smulders, 2011; Koklesova et al., 2021). The TSP, which is crucial for the synthesis of important antioxidants like GSH and the gasotransmitter H_2S, is detailed below and presented in **Figure 1.4**.

1.3.3 Trans-sulphuration pathway

Following uptake and metabolism, SAAs enter the TSP. This metabolic pathway is responsible for producing Cys for use in protein synthesis, for the production of redox molecules like GSH and for the generation of endogenous signalling molecules such as H_2S (Sbodio, Snyder and Paul, 2019). The many steps involved in the TSP system are catalysed by enzymes that depend on pyridoxal-5-phosphate (PLP), the active form of vitamin B_6, as a coenzyme like cystathionine β-synthase (CBS) (EC 4.2.1.22) that condenses Hcy with serine to generate cystathionine and cystathionine γ-lyase (CSE) (EC 4.4.1.1) needed to produce Cys and H_2S (Sbodio, Snyder and Paul, 2019), as shown in **Figure 1.4**. In addition, the redox molecule GSH is produced via γ-glutamyl cysteine synthetase (γ-GCS) (EC 6.3. 2.2), that converts Cys into γ-glutamyl cysteine, which will be converted further by glutathione synthetase (GS) (EC 6.3.2.3) into GSH. Tau is produced from Cys via a multistep process requiring cysteine dioxygenase (CDO) (EC 1.13.11.20), hypotaurine dehydrogenase (HTAU-DH) (EC 1.8.1.3) and cysteine sulphinic acid decarboxylase (CSAD) (EC 4.1.1.29) enzymes. Cys produces sulphate via the action of CDO and aspartate aminotransferase (AST) (EC 2.6.1.1) and sulphite oxidase (SO) (EC 1.8. 3.1). Interestingly, H_2S is largely generated in mammalian systems through the TSP pathway (**Figure 1.4**), or via a coupled reaction involving the mitochondrial enzymes cysteine aminotransferase (CAT, EC 2.6.1.3) and 3-mercaptopyruvate sulphurtranspherase (3-MST, EC 2.8.1.2) (Kimura, 2011; Olson and Straub, 2016).

Collectively, many TSP metabolites are necessary for detoxification, osmotic regulation, cellular signalling homeostasis, and for nervous system and antioxidative processes (Stipanuk et al., 2002; Zhang et al., 2019). The important roles for several of these metabolites are summarised in **Table 1.6**. Critically, diminished levels of these compounds are reported in conditions such as alcoholic and non-alcoholic fatty liver diseases (Medici et al., 2010; Werge et al., 2021); Parkinson's disease (Corona-Trejo et al., 2023); neurodegenerative diseases (Zhou and Wang, 2023); autism disorder (Han et al., 2015); chronic kidney diseases (Busch et al., 2010); hypertension (OGAWA et al., 1985); chronic obstructive pulmonary disease (COPD) (Zinellu et al., 2002); and metabolic changes, like increased oxidative stress (Preiser, 2012) and decreased tissue GSH levels (Ballatori et al., 2009).

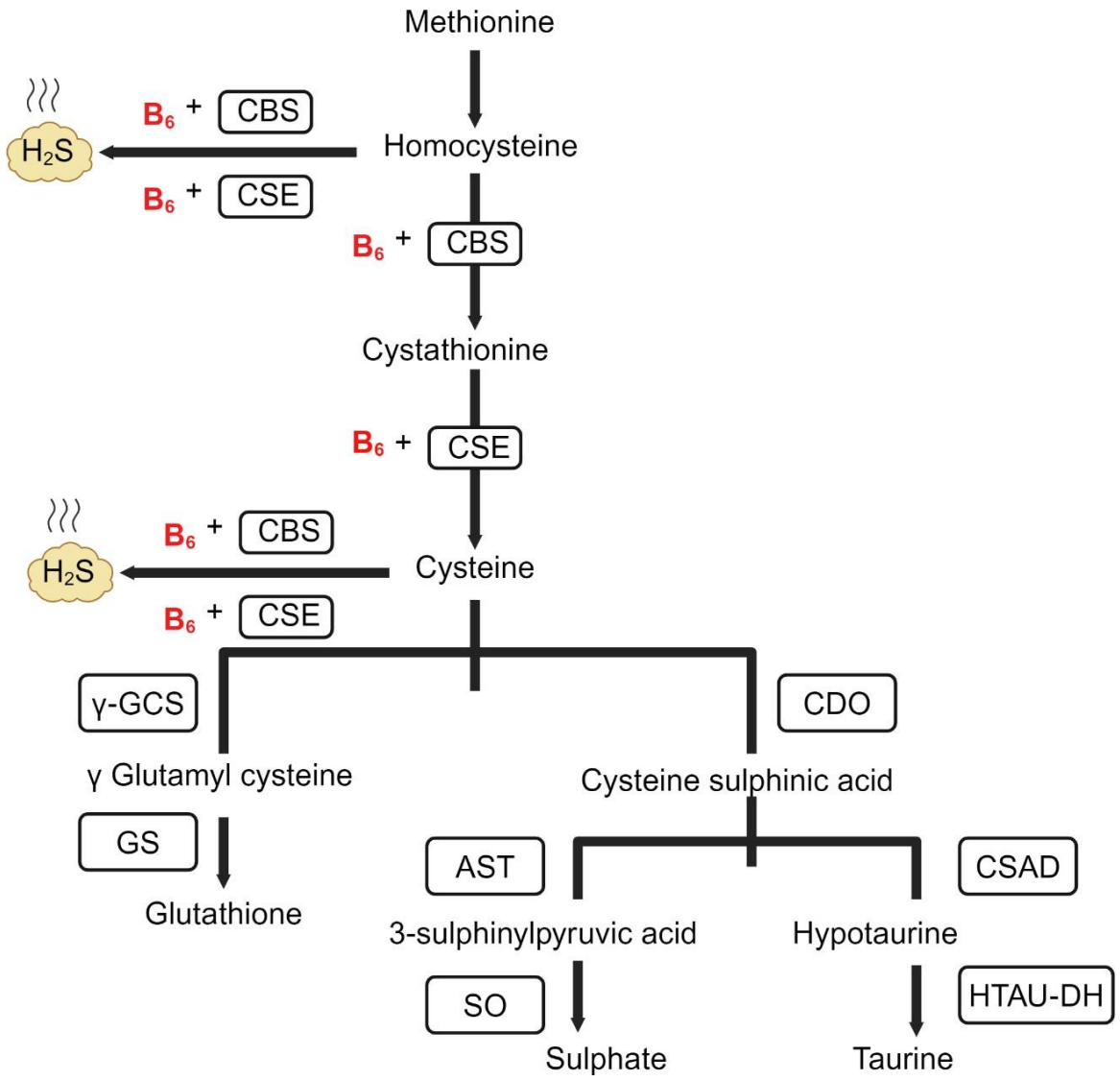


Figure 1.4 An outline of the trans-sulphuration pathway (TSP) and related metabolic routes in humans. This figure highlights the involvement of the active form of vitamin B_6 in many steps of TSP as a cofactor for the key enzymes; CBS and CSE to produce crucial molecules including glutathione, taurine, sulphate and H_2S. **Abbreviations**: **CBS**, cystathionine β-synthase (EC 4.2.1.22); **CSE,** cystathionine γ-lyase (EC 4.4.1.1); **γ-GCS**, γ-glutamyl cysteine synthetase (EC 6.3. 2.2); **GS**, glutathione synthetase (EC 6.3.2.3); **CDO**, cysteine dioxygenase (EC 1.13.11.20); **CSAD**, cysteine sulfinic acid decarboxylase (EC 4.1.1.29); **HTAU-DH**, hypotaurine dehydrogenase (EC 1.8.1.3); **AST**, aspartate aminotransferase (EC 2.6.1.1); **SO**, sulphite oxidase (EC1.8. 3.1); **H_2S**, hydrogen sulphide. Illustration created with (BioRender.com).

Table 1.6 Important roles of TSP metabolites

TSP metabolite	Function	Reduction linked with disease / condition
Cysteine	• In the immune system, enhance muscle function, positively linked with bone mineral density (Baines et al., 2007). • A building block for approximately 2 % of proteins in the body (Rehman et al., 2020). • Maintain redox homeostasis (Fra, Yoboue and Sitia, 2017).	• Immune dysfunction (Ghezzi et al., 2019). • Ageing (Dröge, W., 2005). • Huntington's disease (Paul et al., 2014). • Growth retardation (Lieberman et al., 1996).
Glutathione	• Free radical scavenging (Baba and Bhatnagar, 2018; Fang, Yang and Wu, 2002). • Detoxification of xenobiotic (Pizzorno, 2014). • Controls cytokine production (TNF-α and NF-κβ) (MASELLA and MAZZA, 2009). • A cofactor for antioxidant enzymes like glutathione peroxidases and glutathione S-transferases (Farhat et al., 2018).	• CVDs (Shimizu et al., 2004) and atherosclerosis (Ceballos-Picot et al., 1996). • Intensifying oxidative stress among patients with cystic fibrosis (Dickerhof et al., 2017). • Ageing (Sekhar et al., 2011). • Neurodegenerative disorders (Liu et al. 2005. • Liver disease (Bianchi et al., 2000).
Taurine	• Cellular homeostasis and reducing oxidative stress (Jangra et al., 2024). • Control osmotic pressure (Hussy et al., 2000). • Stabilising cell membranes (Wenting et al., 2014). • Controlling calcium signalling (Schaffer, Solodushko and Kakhniashvili, 2002). • Antioxidative (Parvez et al., 2008), anti-inflammatory (Wójcik et al., 2013) and anti-atherosclerosis (Zulli et al., 2009).	• Oxidative stress (Jong, Azuma and Schaffer, 2012). • Cellular senescence and DNA damages (Rinaldi et al., 2024). • Age-related diseases (Singh et al., 2023).
Sulphate	• Detoxification (den Bakker et al., 2024). • Bone and cartilage health (Pfau et al., 2023). • Regulating cellular function (Markovich, 2001).	• Many organs, tissues, and / or biological processes may be adversely affected by inadequate sulfonation (Langford, Hurrion and Dawson, 2017) such as affected neurodevelopment, endocrinopathies, and skeletal abnormalities (den Bakker et al., 2024).
H_2S	• Cryoprotection (Johansen, Ytrehus and Baxter, 2006). • Cellular antioxidant function (Kimura and Kimura, 2004; Whiteman et al. 2004). • Inflammation and inflammatory signalling (Li et al., 2005). • Directly activates K_{ATP} channels in vascular smooth muscle cells (Tang et al., 2005), causing vasorelaxation (Zhao and Wang, 2002).	• CVDs (Yan, Du and Tang, 2004). • Sexual dysfunction (Srilatha, Adaikan and Moore, 2006). • Neurodegenerative conditions such as Alzheimer disease (Eto et al., 2002), dementia (Zhang, Jiang and Liu, 2009), and Parkinson's disease (Hu et al., 2010). • Chronic kidney diseases (Aminzadeh and Vaziri, 2012).

Abbreviations: **TNF-α**, Tumor Necrosis Factor-α; **NF-κB**, Nuclear Factor; kappa B; **Nrf2**, Nuclear factor erythroid 2-related factor 2 .

1.3.4 Dysregulation in the TSP pathway

It is becoming clearer that TSP shows great plasticity in its metabolic function. Current evidence shows that nutritional status and genetic factors have a significant influence on the levels of TSP-related metabolites. For example, dietary SAA restriction (Lyons et al., 2000; Jones et al., 2011; Olsen et al., 2024); vitamin B_{-6} deficiency and other micronutrient deficiencies (Kuzniar et al., 2003; Ozturk et al., 2003; Choi and Cho, 2009; DeRatt et al. 2014; Wu, Xu and Huang, 2016); restrictive, e.g. vegetarian, diets (McCarty, 2004); and ingestion of anti-nutrients like the non-protein amino acid 1-amino D-proline (1ADP) (Mayengbam et al., 2016): all promote diminished levels of tissue and / or plasma Cys, GSH or H_2S levels. Critically, dietary interventions such as vitamin B_6 usage (Zhang et al., 2009) or sufficient SAA intakes (Margaritelis et al., 2021) can reverse such dysregulation, as observed in users of the DASH diet (Dietary Approaches to Stop Hypertension) (Pirouzeh et al., 2020), which is a SAA-adequate diet. Moreover, several common polymorphisms in TSP enzymes also impact on the levels of TSP metabolites. Polymorphisms in CBS and CSE have been described and these have been linked to various pathophysiological conditions in humans (Wang and Hegele, 2003; Beard and Bearden, 2011). Genetic deficiency or mutation alter enzyme activity (Meier et al., 2003), tissue expression and enzyme cofactor dynamics (Jakubowski, 2020). To date, genetic polymorphisms are linked to increased risk of stroke (Ding, Lin and Chen, 2012); essential hypertension (Yun et al., 2008; Shi et al., 2015); and premature coronary artery disease (Tsai et al., 1996). They are also associated with elevated levels of homocysteine in the plasma and diminished levels of cystathionine and cysteine (Wang et al., 2004; Amaral et al., 2017; Rose, Moore and Zhu, 2017). Some studies have also pointed to reduced levels of the signalling

molecule H_2S, in plasma and serum, following dietary restriction (Olsen et al., 2024) or genetic polymorphisms (Yang et al., 2008).

1.3.5 TSP and hydrogen sulphide (H_2S)

Researchers now recognise H_2S as the third gaseous signalling molecule in mammals, joining nitric oxide (NO) and carbon monoxide (CO) (Rose, Moore and Zhu, 2017). This molecule has been demonstrated over the last ten years to be synthesised by a variety of tissues, where it serves as a signalling molecule with unique biological effects, a point considered in greater detail later. It has been demonstrated that the majority of H_2S's biological impacts occur through its interactions with the sulphur atoms in cysteine residues found on regulatory proteins (known as protein S- sulphhydration) (Olson, 2018). Interestingly and, considering the current evidence, one of the most critical chemical characteristics of H_2S is its capacity to form polysulphide species (H_2S_n, n > 2). Indeed, H_2S_n can be generated from mitochondrial oxidation of H_2S by sulphide-quinone oxidoreductase (SQR) (Kimura, 2021), an H_2S catabolic enzyme. H_2S_n are reactive sulphur species (RSS) that play important roles in cellular signalling and redox balance. H_2S_n, common in nature, exhibit diverse biological activities. In fact, current data suggest that H_2S_n compounds may be responsible for many effects previously attributed to H_2S (Kimura, 2015; Olson, 2018).

1.3.6 Biochemistry of hydrogen sulphide (H_2S) and polysulphide (H_2S_n)

Hydrogen sulphide (H_2S) H_2S is the sulphur compound with the lowest oxidation state of - 2 (Giuffrè and Vicente, 2018). At room temperature, H_2S exists as colourless gas with a strong, unpleasant and pungent rotten-egg smell (Polhemus et al., 2014; Andrés et al., 2023). H_2S is soluble in water (40 g / L) and is highly volatile, with a vapour pressure of 1740 kPa at 21°C (Olson, 2018). When dissolved in water, H_2S is

a weak acid, where it splits into two dissociation states: into H^+ and HS^- and further to S^{2-}, as follows: $\mathbf{H_2S \rightleftharpoons HS^- + H^+ \rightleftharpoons S^{2-} + 2H^+}$, with the $pK_{a1} \sim 6.8$ and $pK_{a2} > 12$ at 37 °C (Olson, 2005; Hughes, Centelles and Moore, 2009). In an aqueous solution with a physiological pH of 7.4, roughly 18.5% of the total H_2S is present as the undissociated acid and the remaining 81.5% as the HS^- and S^{2-} (Munteanu et al., 2022). Hence, it is currently unknown whether the biological effects are the result of H_2S gas alone or whether they are also mediated by a combination of species (HS^- and S^{2-}), given the existence of both species under physiological conditions. Moreover, dissolved $H_2S \approx HS^-$ at pH 7.0, while S^{2-} is frequently considered to be negligible. The HS^- / H_2S ratio in cells can vary from 0.006 in acidic lysosomes (pH 4.7) to 12.6 in the mitochondrial matrix (pH 8.0) (Olson and Straub, 2016). Lipophilic in nature, dissolved H_2S easily diffuses across membranes (Mathai et al., 2009), effectively establishing pH-dependent equilibria on both sides of these barriers, while ionised species (HS^- and S^{2-}), on the other hand, are more chemically reactive.

Polysulphide (H_2S_n) Inorganic H_2S_n and their organic counterparts (RSnH, RSnR) can contain varying numbers of sulphur atoms, with up to 8 being common. Most biologically-active polysulphides typically contain 2 - 5 sulphur atoms. These include disulphanes (S2), trisulphanes (S3), tetrasulphanes (S4) and pentasulphanes (S5) (Olson, 2018). As the number of sulphur atoms in a polysulphide increases, both pK_{a1} and pK_{a2} values decrease (Kamyshny et al., 2003), indicating increased acidity and a higher tendency to exist in anionic forms at physiological pH.

1.3.7 Biological and physiological role of H_2S and H_2S_n

Prior to the mid-1990s, H_2S was primarily regarded as a toxic substance for humans (Andrés et al., 2023). However, now H_2S, alongside H_2S_n, exert diverse effects across

mammalian cells and tissues, influencing various physiological processes (**Figure 1.5**). They play crucial roles as important modulators of mitochondrial function (Elrod et al., 2007; Uba et al., 2021), providing cellular protection against stress (Johansen, Ytrehus and Baxter, 2006; Yang et al., 2014; Sun et al., 2021); modulating inflammatory responses and signalling pathways (Li et al., 2005; Morsy et al., 2010; Jiang and Chen, 2022); alleviating insulin resistance (Chen et all., 2017; Kowalczyk-Bołtuć et al., 2022); and promoting tissue regeneration and wound repair (Wallace et al., 2007; Fan et al., 2022). Furthermore, these endogenous sulphur compounds have been implicated in the regulation of multiple organ systems, including the cardiovascular (Li et al., 2008; Xiong et al., 2023) [e.g. regulating blood pressure (Liang et al., 2017) and exhibiting anti-atherosclerotic activity (Liu et al., 2013)]; nervous (Abe and Kimura, 1996; Sun et al., 2021; Sharif et al., 2023); and renal systems (Tripatara et al., 2008; Sun et al., 2021). Their functions extend to the gastrointestinal (Distrutti et al., 2006; Magierowski et al., 2013), as well as the respiratory (Fu et al., 2008; Jiang and Chen, 2022) and reproductive systems (Wang et al., 2013; Dutta, Sengupta and Samrot, 2024), notably affecting erectile function (d'Emmanuele di Villa Bianca et al., 2009). Additionally, H_2S and H_2S_n have been associated with metabolic processes related to obesity (Comas and Moreno-Navarrete, 2021; Kowalczyk-Bołtuć et al., 2022) and have shown potential in influencing longevity and age-related disorders (Qabazard et al., 2014; Kowalczyk-Bołtuć et al., 2022).

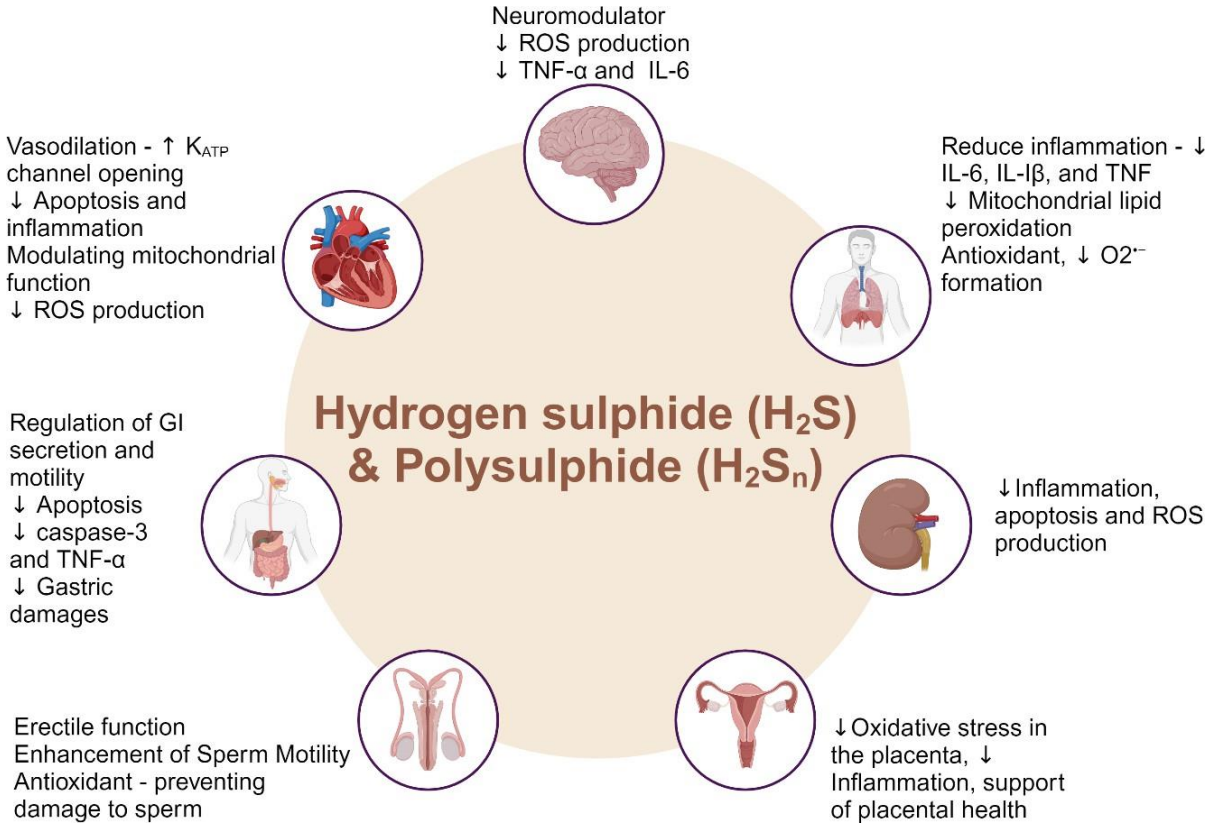


Figure 1.5 In mammalian systems, hydrogen sulphide (H_2S) and polysulphides (H_2Sn) are essential for both biological and physiological processes. The TSP is the main source of H_2S in cells and tissues. Two critical enzymes are involved in this process: cystathionine γ-lyase (CSE) (EC 4.4.1.1) and cystathionine β-synthetase (CBS) (EC 4.2.1.22). After it is generated, H_2S can produce more sulphur species, such as polysulphides. These sulphur-containing compounds cause a variety of physiological and biochemical reactions in cells. Their effects are especially important for preserving homeostasis in the cardiovascular and brain systems, among others. (Illustration created with (BioRender.com).

1.4 Work conducted in this thesis

This thesis presents a four-chapter investigation into medication-induced alterations of the TSP, advancing from population-level analyses to targeted cellular studies. Despite the ubiquitous use of medications in modern healthcare, studies on drug-nutrient interactions remain scarce (Mohn et al., 2018). While individual studies have examined the effects of specific drugs on vitamin B_6 status (Heller and Friedman, 1983; Ubbink et al., 1990; NIH, 2023), there is a lack of comprehensive population-level data assessing the impact of a wide range of commonly-used medications on vitamin B_6 status. This broader analysis is critical, as it could provide a more complete picture of how various drugs affect vitamin B_6 levels, potentially impacting the TSP, a key metabolic process influenced by these levels. We also conducted a scoping review to examine the broader implications of medication use, using paracetamol as a therapeutic model, on TSP metabolites.

Our focus then narrowed to two widely-prescribed medications: paracetamol (an analgesic) and sulphasalazine (an anti-inflammatory agent) (**Figure 1.6** and **Table 1.7**). While the hepatotoxicity of these drugs is well-documented (**Table 1.7**), their specific effects on TSP metabolite (i.e. H_2S and H_2S_n) production in liver cells remain largely unexplored (Alsaeedi et al., 2023). This gap in the literature is particularly significant, given the liver's central role in both drug metabolism and TSP activity. Drug selection was based on their known effects on thiol status related to the TSP. Paracetamol has been demonstrated to affect levels of GSH, a key intracellular antioxidant, in humans (Jannuzzi, Kara and Alpertunga, 2018). Sulphasalazine, on the other hand, influences the cystine transporter (X_c^-) (**Figure 1.3**) (Shukla et al., 2011), which plays a crucial role in cellular cysteine uptake and subsequent GSH status. Existing research, primarily conducted in animal models and cell cultures, has

provided preliminary insights into the effects of these drugs. Studies have reported reductions in H_2S levels in the brain (Wiliński et al., 2011); decreased hepatic CBS (Elshazly El-Moselhy and Barakat, 2014) and CSE levels (Li et al., 2019); and reduced CBS expression in the brain (Wang et al., 2021). However, these studies did not directly quantify H_2S and H_2S_n levels in liver cells, underscoring the need for further investigation into the impact of paracetamol and sulphasalazine on H_2S / H_2S_n production in hepatic cells. This could potentially reveal previously unrecognised consequences of drug use on liver metabolism.

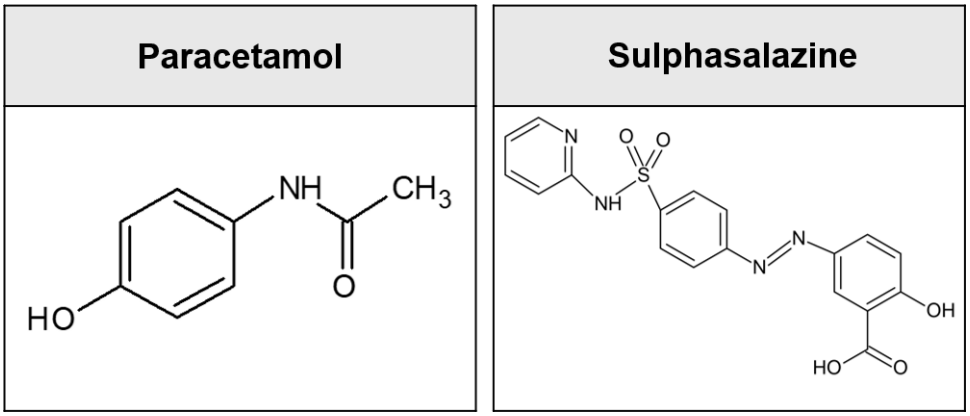


Figure 1.6 Drugs used in the thesis studies as models to study TSP, namely paracetamol and sulphasalazine

Table 1.7 Medication used in the current work

Drug	Indication	Recommended dose	Toxicity	Form	Reference
Paracetamol	Relief mild to moderate pain.	4 g in 24 hours (two 500 mg tablets four times), with four hours between doses	Cardiovascular, gastrointestinal events and liver cancer	Liquid formulations, oral tablets, and capsules, as well as rectal suppositories.	(Ayoub, 2021) (Roberts et al., 2016)
Sulphasalazine	Used in the management of rheumatoid arthritis and inflammatory bowel diseases.	The doses will be adjusted individually, but generally, for example in maintaining ulcerative colitis cases, 2 g / day is considered as a satisfactory dosage with eight hours intervals	Renal and liver disease	tablets, liquid and rectal suppositories.	(Choi and Fenando, 2020). (Heidari et al., 2016) (DeMichele, Rezaizadeh and Goldstein, 2012) (Molnár et al., 2010)

1.4.1 Thesis aims

We employed a multi-faceted approach aimed at providing crucial insights into how commonly-prescribed drugs influence this vital metabolic pathway. Thesis layout and research aims were as follows:

- To investigate the impact of commonly used medications on TSP metabolites in humans, using paracetamol as a therapeutic model: **a scoping review.**
- To assess dietary vitamin B_6 and plasma pyridoxal-5′-phosphate (PLP) in the UK population alongside determining the impact of commonly-used medications on their levels: **a secondary analysis.**

- To validate and develop the HepG2 cell as a cellular model of oxidative stress and apoptosis, to aid in the assessment of medications by measuring GSH levels and other oxidative markers: **a cell culture-based study.**
- To evaluate the impact of medications on H_2S, H_2S_n and its related enzymes, and to further assess H_2S and H_2S_n donors on cell cytotoxicity and oxidative stress markers: **a cell culture-based study.**

1.4.2 Thesis hypothesis

We hypothesise that common medications, exemplified by paracetamol and sulphasalazine, will reduce the TSP metabolites (H_2S, H_2S_n and GSH) and decrease the expression of the key enzymes (CBS and CSE) in HepG2 cells

Chapter 2 Materials and Methods

2 Materials and methods

2.1 Materials

2.1.1 Chemicals

Dulbecco's Modified Eagle Medium (DMEM) (D6429), Phosphate Buffered Saline (PBS) (P4417), Bovine serum albumin (BSA) (9048-46-8), Crystal Violet (548-62-9), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye (420200), 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) (D9542), 7-azido-4-methylcoumarin (AzMC) fluorogenic probe, Lipid Peroxidation malondialdehyde (MDA) Assay Kit (MAK085), Quantification kit for oxidised and reduced glutathione (38185), Paracetamol (PHR105), Hydrogen peroxide, Paraformaldehyde, Glycerol, Deoxycholate, Tris-Hydrochloride, Sodium Dodecyl Sulphat (SDS), Potassium chloride, D-(+)-Glucose (99.5 % GC), Sodium bicarbonate, Sodium phosphate dibasic, Ethylenediaminetetraacetic acid (EDTA), Egtazic aicd (EGTA), Sodium fluoride, Sodium pyrophosphate, Sodium orthovanadate, Sodium phosphate monobasic monohydrate were all obtained from Sigma-Aldrich (St Louis, USA). Sulfane Sulfur Probe 4 (SSP4) was purchased from Dojindo Molecular Technologies. Sulphasalazine was purchased from Acros Organics. Foetal Bovine Serum (FBS) (11550356), Triton X-100 (9002-93-1), Trypsin 0.25% EDTA (11570626), Dimethyl Sulfoxide (DMSO), Sodium chloride and Calcium chloride were from Fisher Scientific UK Ltd (Loughborough, UK). Penicillin-streptomycin (Pen/Strep) (P4333) was from Merck Life Science UK Ltd (Gillingham, UK). Methanol, Magnesium chloride hexahydrate and Isopropanol were from VWR Chemicals (Pennsylvania, USA). Protein inhibitor cocktail (04 693 124 001) was from Roche (Basel, Switzerland). Magnesium sulphate heptahydrate and Potassium phosphate monobasic came from Fisons (Glasgow, UK). CellROX® Deep Red reagent (C10422), MitoSOX™ Red indicator (M36008), Image iT

Lipid Peroxidation Kit (C10455), Cumene Hydroperoxide, CyQuant LDH cytotoxicity assay kit (C20301), Human poly-ADP ribose polymerase (PARP) 214 / 215 Enzyme-linked Immunosorbent Assay (ELISA) kit (KH00741) and enhanced chemiluminescence (ECL) (34075) were all from Thermo Fisher Scientific (Waltham, USA). 2', 7'-Dichlorofluorescein diacetate (DCF-DA) was from EMD Millipore Corp (Burlington, USA). Z-VAD-FMK was from APExBIO (Hsinchu City, Taiwan). Z-DEVD-FMK was from AdooQ Bioscience (Irvine, CA, USA). Finally, milk – Marvel Original Dried Skimmed Milk (< 1 % fat) – was obtained from a local shop.

2.2 Methods

2.2.1 General culture of Human hepatocellular carcinoma (HepG2) cell

2.2.1.1 Culturing HepG2 and media used

HepG2 cells obtained from ATCC (Virginia, USA) were maintained in DMEM supplemented with 100 mL / L FBS (10 %) and 10,000 units penicillin and 10 mg streptomycin / mL (1 %) (pH 7.4). Media was kept at 4°C and pre-warming in a bead bath (SUB Aqua 26 Plus) was carried out whenever the media were used, before application to the cells. HepG2 cells were maintained in T-75 cm^2 / T-225 cm^2 culture flasks in a humidified atmosphere of 5% CO_2 at 37°C (RS Biotech Incubator, 170-200P) until 70% confluence was reached.

For experimental treatments, FBS-free DMEM was used throughout the experiments, containing 10% distilled water (dH_2O) and 1% Penicillin-Streptomycin (pH 7.4). The reason for eliminating FBS from media during treatment is that FBS contains various growth factors that may interfere with or mask the cellular response to the experimental treatments. Therefore, serum deprivation helps synchronise cells to the same phase of the cell cycle prior to treatment.

2.2.1.2 Passage

Culture media was removed from flasks and washed with pre-warmed 10 mL of PBS before trypsinising them (2 mL) for three minutes in the incubator – Trypsin-EDTA (0.25%) helps to detach the cells from the surface of the flask – then culturing DMEM was added, before transferring the solution into a sterilised centrifuge tub and centrifuging at 1000 rpm for five minutes at room temperature (Eppendorf centrifuge, 5702 R). Supernatant was aspirated, then resuspension of cells was performed by adding fresh culturing DMEM media to the cell pellets. From this cell suspension, cells were transferred into flasks (3 mL for T-75 cm^2 flask and 9 mL for T-225 cm^2 flask), then fresh culturing media was added into the flasks (10 mL) and incubated in the incubator (5% CO_2 at 37°C). HepG2 cells were passaged three times per week.

2.2.1.3 Cell counting and seeding

To prepare cells for counting and plate seeding, the standard passaging procedure was conducted up to the point of cell resuspension. Subsequently, a 10 µl aliquot of the cell suspension was transferred to a hemacytometer (HAWKSLEY BS 748). Under the microscope (MicroPublisher 3.3 RTV, Canada), cells in the three observation squares of the hemacytometer were counted using a manual cell counter (Brannan, UK). Then the average of the cell number was taken and multiplied by 10^4 to get cell number per 1 mL. The total cell count was determined by multiplying the number of mLs of stock by the number of cells / mL. To determine the number of cells that were required for each plate, we multiplied the number of wells that were seeded by the number of plates and the number of cells that were desired for each well. The following formula was performed to determine the volume of cell suspension required for seeding density:

Volume of cell suspension needed (µl) =

(cell number needed) / (total cell number) x total volume of cell suspension (µl)

Next, we subtracted the seeding volume from the total volume of liquid required, to seed every plate. Each experiment conducted in 96-well plates was seeded with 100 µl, while experiments conducted in 6-well plates were seeded with 2 mL each and 10 mL for Petri dishes.

Regarding seeding for fluorescence imaging, as will be seen in CellROX® Deep Red reagent, MitoSOX™ Red indicator, Image-iT® lipid peroxidation and DAPI, before seeding cells, coverslips (22 mm^2, VWR International) were individually sterilised in 70% ethanol for 5 minutes and placed into each well of a 6-well plate. Then, plates were dried in a Microbiological Safety Cabinet (MSC) at room temperature. After that, 400,000 cells per well were seeded into the wells holding the coverslips. Cells were then incubated overnight in a humidified atmosphere of 5% CO_2 at 37°C, to encourage cell adherence.

2.2.1.4 Cryopreservation

The standard cell passaging procedure was conducted up to the point of cell resuspension. At this stage, instead of using regular culture medium, the cells were resuspended in 3 - 5 mL of cold freezing media (90% FBS and 10% DMSO) per T-75 cm^2 flask. 1 mL aliquots were placed into cryo-vials in a ‘Mr. Frosty’ container and placed in the freezer at -80°C overnight. After 24 hours of freezing, frozen HepG2 cells were kept in liquid nitrogen dewars until required (long-term storage).

2.2.2 Drug treatment preparation

Paracetamol (APAP) (Molecular weight [MW]: 151.163 g / mol) and sulphasalazine (SSZ) (MW: 398.394 g / mol) were dissolved in DMSO. Drug mixture was filtered, a final concentration of 10 mM of each drug was reached and then concentrations were

adjusted as needed by dilution in FBS-free DMEM media. Controls of these compounds contain < 0.1% DMSO. All reagents were freshly prepared for each individual experiment run on separate days.

2.2.3 Cell culture assays

Cell seeding densities and the number of experimental replicates are specified in the relevant chapters for each set of experiments.

2.2.3.1 Assessment of cell viability - crystal violet assay

The crystal violet method has been shown as an effective technique for evaluating viability in adherent cells (Elengoe and Hamdan, 2017). Crystal violet staining was employed in the current work to assess cell viability following the method outlined by Feoktistova, Geserick and Leverkus (2016). This dye permeates cell membranes and binds to deoxyribonucleic acid (DNA) and proteins. During the washing step, non-viable cells detach from the culture plate and are removed, resulting in a reduced amount of retained dye.

Briefly, after removing treatment media from clear flat-bottom 96-well plates (Costar Flat Bottom with Lid), cells were rinsed with warmed PBS (100 µl / well) (x 2) before adding 20 µl of 0.5% crystal violet solution to each well (prepared by diluting 0.5 g of crystal violet powder in 80 mL of dH_2O and 20 mL of methanol). Plates were then incubated at room temperature for 10 minutes. Subsequently, crystal violet solution was discarded and plates were rinsed with water and tap-dried to remove any residual stain. Methanol (200 µl / well) was used to solubilise the stain (Kamiloglu et al., 2020), allowing for optical density measurements. Absorbance was measured at 550 nm using a microplate reader (Bio-Rad – model 680 XR). Following Feoktistova, Geserick and Leverkus (2016), results were presented as percentage (%) of cell survival, which

was calculated from a comparison with the absorbance of control cells by the following equation:

Cell viability (% of control) =

(mean sample absorbance) / (mean control absorbance) x 100

2.2.3.2 Detection of intracellular reactive oxygen species (ROS) formation - 2', 7'-Dichlorofluorescein diacetate (DCFDA) assay

DCFDA is a fluorometric assay to evaluate intracellular ROS formation in cells. DCFDA is a cell-permeable probe that enters the cell and is then deacetylated by cellular esterases to form 2',7'-dichlorodihydrofluorescein (DCFH), which is non-fluorescent. In the presence of ROS, DCFH is rapidly oxidised to green-fluorescent 2',7'-dichlorofluorescein (DCF). The intensity of this green DCF fluorescence can therefore be used to quantify cellular ROS levels (Ng and Ooi, 2021).

In the current work, DCFDA assay was conducted following the method described by Vincent et al. (2004). Essentially, a working solution of 100 µM DCFDA was prepared and 50 µl was added to each well of a 96-well plate (black plate with clear bottom) (µCLEAR®, BLACK CellStar® F-Bottom). The cells were then incubated with the dye for 1 hour at 37°C in a 5% CO_2. Following incubation, the DCFDA solution was aspirated and cells were rinsed with Hank's Balanced Salt Solution (HBSS) (x 3). The HBSS composition included 140 mM sodium chloride, 5 mM potassium chloride, 1 mM calcium chloride, 400 µM magnesium sulphate, 500 µM magnesium chloride hexahydrate, 300 µM monosodium phosphate, 400 µM monopotassium phosphate, 6 mM D-glucose (dextrose) and 4 mM sodium bicarbonate.

Cells were exposed to individual drug treatments or 1 mM hydrogen peroxide (H_2O_2), all diluted in HBSS. H_2O_2 was used as a positive control for ROS generation (Sun et al., 2018). Fluorescence intensity was measured using a microplate reader (FLUOstar Omega - BMG Labtech) at excitation / emission wavelengths of 485 / 520 nm. Readings were taken at 0, 30, 60 and 120 minutes, in line with previous literature (Park, 2016). The fluorescence data was exported to Excel for analysis. Background fluorescence was subtracted from all fluorescence measurements and results are expressed in relative fluorescence units (RFU).

2.2.3.3 Measurement of lipid Peroxidation - malondialdehyde (MDA) assay

MDA is a product of lipid peroxidation (Ito, Sono and Ito, 2019) and a marker of oxidative stress, which was assessed in the current work using a commercial Lipid Peroxidation Assay Kit. This kit measures lipid peroxidation by quantifying the colorimetric product (detectable at 532 nm) formed when MDA reacts with thiobarbituric acid (TBA). The intensity of this product is directly proportional to the amount of MDA present.

Following the manufacturers' instructions, media was aspirated after treatment and cells were washed with cold PBS (x 3). Cells were scraped, transferred into autoclaved Eppendorfs and centrifuged for 5 minutes at 13,000 rmp. Supernatants were discarded and pellets were lysed on ice using the kit provided – MDA Lysis Buffer (300 µL) containing 3 µL of butylated hydroxytoluene (BHT) (100 x). Next, the samples were centrifuged at 13,000 x g for 10 minutes. Then, 200 µL of supernatant was transferred into autoclaved Eppendorfs to perform the assay. MDA standard solution, provided with the kit, was prepared and diluted in purified water to construct a standard curve; with concentration range of standards generated as follows: 20, 16, 12, 8, 4, 0 nmole / well.

600 µL of the TBA solution was added into each vial containing standard and sample. Vials were incubated at 95°C for one hour and cooled down to room temperature in an ice bath for 10 minutes. Then, 200 µl of standards and samples were added in clear flat-bottom 96-well plates (Costar Flat Bottom with Lid). MDA levels were detected by measuring the absorbance at 532 nm using a microplate reader (Bio-Rad – model 680 XR). Protein content of the samples was determined using Lowry assay (as will be detailed in **section 2.2.3.10**) to standardise the data. Results are presented as nmol MDA / mg protein

2.2.3.4 Assessment of mitochondrial membrane potential (Δ Ψ m) - JC-1 assay

Mitochondrial membrane potential was assessed using JC-1 dye. In mitochondria with high membrane potential (healthy), JC-1 accumulates and forms aggregates, emitting red fluorescence (excitation at 535 nm and emission at 590 nm), while, as the mitochondrial membrane potential decreases, JC-1 remains in its monomeric form, resulting in green fluorescence (excitation at 475 nm and emission at 530 nm). The ratio of red to green fluorescence thus serves as an indicator of changes in mitochondrial membrane potential.

This assay was carried out as described by Sivandzade, Bhalerao and Cucullo (2019). In short, culture media was discarded, cells were washed with pre-warmed PBS (x 2) and loaded with 2 µM of JC-1 in each well of a 96-well plate (black plate with clear bottom) (µCLEAR®, BLACK CellStar® F-Bottom) for 30 minutes. Cells were washed with pre-warmed PBS (x 3) and were treated with the drugs or 1 mM H_2O_2 concentration diluted in HBSS. H_2O_2 at 1 mM concentration was seen to induce mitochondrial membrane depolarisations in cell culture previously (Gonzalez et al, 2010); as such, it was used to serve as a positive control in the current work

After treatment, cells were washed with warmed PBS (x 3) and fluorescence measured using a FluoStar Omega plate reader. Background fluorescence was subtracted from all fluorescence measurements. Δ Ψ m was determined by the ratio of fluorescence intensity between 590 and 530 nm (red / green). Depolarised mitochondria are indicated by decreased Δ Ψ m, as previously described (Sivandzade, Bhalerao and Cucullo, 2019).).

2.2.3.5 Visualising oxidative stress markers

Four fluorescence-based methods were used to visualise several oxidative stress markers (**Table 2.1**), including: CellROX® Deep Red reagent to assess ROS formation in the cytoplasm; MitoSOX™ Red indicator to assess ROS generation in the mitochondria (superoxide [$O_2^{\bullet-}$]); Image-iT® to visualise lipid peroxidation (LPO); and 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) staining to assess DNA damages.

CellROX® Deep Red reagent is a novel fluorogenic probe designed to detect ROS production levels in cells. It is a cell-permeant dye and its signal is localised in the cytoplasm with excitation / emission maxima at 640 / 665 nm. The reagent itself is not a fluorescent; however, upon oxidation with ROS, CellROX® exhibits bright red fluorescence. The fluorescence intensity of this probe increases proportionally with increasing ROS levels. On the other hand, MitoSOX™ Red reagent is a fluorogenic dye used to detect mitochondrial superoxide ($O_2^{\bullet-}$). It is selectively targeted mitochondrial and, once this probe is in the mitochondria, it is readily oxidised by $O_2^{\bullet-}$ and generated red fluorescence with excitation / emission maxima of approximately 510 / 580 nm. Image-iT®, on the other hand, is a selective fluorescent reagent for lipid peroxidation based on C-11 BODIPY. When lipids are oxidized, the peak emission shifts from red (~ 590 nm) to green (~ 510 nm). Lastly, DAPI is a DNA-specific

fluorescent probe used to label DNA by entering the cell membrane, which emits blue fluorescent light at 461 nm, indicating cell damage.

A 1 mM H_2O_2 diluted in sterile Earle's balanced salt solution (EBSS) served as a positive control in these experiments, due to its ability to induce ROS formation and apoptosis (Liu et al., 2014; Rao et al., 2013). The EBSS composition included 1 M calcium chloride, 810 µM magnesium sulphate heptahydrate, 5 M potassium chloride, 26 mM sodium bicarbonate, 117 mM sodium chloride, 1 mM sodium phosphate monobasic monohydrate and 6 mM D-glucose (dextrose). The solution's pH was adjusted to 7.4 prior to use. For studies visualising LPO, cumene hydroperoxide at 100 µM concentration diluted in PBS was used as positive controls to generate lipid peroxidation, as per the manufacturer's recommendations.

After the designated time point treatment, media was aspirated and cells were washed with warmed PBS (x 2). In DAPI and CellROX experiments, adherent cells were first fixed by applying 1 ml of 4% paraformaldehyde (PFA) to each well and incubated at 37°C in 5% CO_2 for 10 minutes. After the fixation step, PFA was aspirated from each well and the cells were rinsed three times with PBS. In DAPI experiments, cells were further permeabilised by applying applied 1 ml of Triton X-100 to each well and incubated at 37°C in 5% CO_2 for 30 seconds. Fluorescent probes were added at the recommended manufacturer's concentrations (**Table 2.1**) and samples incubated in the dark in a humidified atmosphere of 5% CO_2 at 37°C for the recommended incubation period (**Table 2.1**). Cells were then washed again with warmed PBS (x 2) to remove residual probe prior to visualisation. To prevent dehydration, 1 mL of PBS was added to each well. Each coverslip was then individually removed and placed on a slide for imaging. Images were captured using an EVOS microscope (Life Technology, AMF4300) at relevant fluorescence mode (**Table 2.1**). The settings were

kept consistent for all images and across all three independent replicates for each assay.

Table 2.1 Fluorescence-based methods used in the current work to visualise oxidative markers in HepG2 cell line

Specificity	**CellROX®**	**MitoSOX™**	**Image-iT®**	**DAPI**
	ROS in cytoplasm	ROS in mitochondria	Lipid peroxidation	DNA damage
Supplier	Thermo Fisher Scientific	Thermo Fisher Scientific	Thermo Fisher Scientific	Sigma-Aldrich
Applied concentration	5 µM (diluted in PBS)	5 µM (diluted in HBSS)	10 µM (diluted in PBS)	300 nm (diluted in PBS)
Probe incubated time (minute)	30	10	30	5
Ex / Em (nm)	640 / 665	510 / 580	581 / 590 (red) 488 / 510 (green)	359 / 461
Colour	Red (RFP filter)	Red (RFP filter)	Red (RFP filter) green (GFP filter)	Blue (DAPI filter)
Fixation	4 % PFA (10 mins)	-	-	4 % PFA (10 mins)
Permeabilisation	-	-	-	0.2 % Triton X-100 (≤1 mins)

Abbreviations: **DAPI**, 4′,6-diamidino-2-phenylindole; **ROS**, Reactive oxygen species; **PBS**, Phosphate buffered saline; **HBSS**, Hanks' Balanced Salt Solution; **Ex / Em**, Fluorescence excitation / emission; **PFA**; Paraformaldehyde

2.2.3.6 Determination of intracellular glutathione level

Intracellular glutathione level was quantified using a commercial assay kit (Sigma-Aldrich, 38185). The assay was performed in accordance with the manufacturer's instructions.

After treatment, cells were washed with cold PBS (x 3) and collected by scraping, the suspension then being centrifuged at 200 x g for 10 minutes at 4°C. The resulting supernatant was removed, and the cell pellet was washed with PBS before repeating the centrifugation step. Subsequently, 80 µl of 10 mM HCl was added to the pellet. Cell lysis was achieved through two cycles of freeze-thawing. After cell lysis, 20 µl of 5% sulfosalicylic acid (SSA) was added to the sample. The mixture was then centrifuged at 8,000 x g for 10 minutes. The resulting supernatant was collected and transferred to a new Eppendorf tube. Then dH_2O was added to reduce SSA concentration to 0.5 %. A series of GSH standard solutions was prepared using the kit-provided standards. These were diluted in 0.5% SSA to create a concentration range of 50, 25, 12.5, 6.25, 3.13, 1.57, and 0 µmol / L GSH. In a clear flat-bottom 96-well plate (Costar Flat Bottom with Lid), 40 µl of each standard and sample was added, followed by 120 µl of buffer solution. The plate was then incubated at 37°C for 1 hour. Following the incubation period, each well received 20 µl each of substrate working solution, coenzyme working solution and enzyme working solution. The plate was then incubated for an additional 10 minutes at 37°C. Absorbance was measured at 415 nm using a FluoStar Omega plate reader. GSH concentrations in the samples were calculated using the standard curve equation ($y = mx + c$), as per the manufacturer's instructions. Protein content of the samples was determined by using the Lowry assay

(as will be described in **section 2.2.3.10**) to standardise the data. Results are presented as nmol GSH / mg protein.

2.2.3.7 Detection of lactate dehydrogenase (LDH) leakage

Membrane integrity was assessed by measuring the leakage of LDH using a commercially available and validated assay kit; the CyQUANT™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific) was used.

LDH is a cytoplasmic enzyme found in all cells and is released in cell culture media when the plasma membrane is damaged (Kumar, Nagarajan and Uchil, 2018). The assay detects LDH activity when it converts lactate to pyruvate, producing reduced nicotinamide adenine dinucleotide (NADH). This NADH then reduces tetrazolium salt to a pink formazan. The intensity of the pink colour, measured by a plate reader, indicates the amount of LDH released from damaged cells.

Following the protocol provided by the manufacturer, after the treatments, 10 µl of 10 X lysis buffer was added to designated maximum-LDH-activity wells. The plate then underwent a 45-minute incubation at 37°C in 5% CO_2. Aliquots of 50 µl from each sample were transferred to a clear flat-bottom 96-well plate (Costar Flat Bottom with Lid). Subsequently, for each sample, 50 µl of Reaction Mixture was added, mixed well and the plate was kept out of the light for an additional 30 minutes of incubation at 37°C in 5% CO_2.The reaction was terminated by adding 50 µl of Stop Solution to each sample well. Bubbles were punctured using a needle to ensure accurate readings. A BioRad plate reader was then used to measure absorbance at wavelengths of 415 nm and 655 nm. The absorbance data was transferred to Microsoft Excel for analysis. The 655 nm readings were subtracted from the 415 nm readings for each sample. Then

total LDH activity for each sample was determined and presented as % LDH leakage as the following equation:

% of LDH leakage =

sample's absorbance value / maximum LDH value x 100

2.2.3.8 Assessment of caspases involvement in cell death mechanism - caspase inhibitor

The caspase inhibitors Z-VAD-FMK (pan-caspase inhibitor) and Z-DEVD-FMK (caspase-3 inhibitor) were utilised, following the protocol outlined by Rose et al. (2003). Prior to drug treatment, cells were incubated for 1 hour with 75 µM in a clear flat-bottom 96-well plate (Costar Flat Bottom with Lid) of either the pan-caspase inhibitor or the caspase-3 inhibitor. Following this pre-treatment, the cells were exposed to the drugs under investigation. Cell viability was assessed after 24 hours using the previously described crystal violet method (**sections 2.2.3.1**).

2.2.3.9 Assessment of poly-ADP ribose polymerase (PARP) involvement in cell death – ELISA

PARP cleavage was evaluated in cell lysates using a commercial ELISA kit (Thermo Fisher Scientific). The assay was performed in accordance with the protocol provided by the manufacturer. Following PBS washing, cells were scraped and resuspended in 200 µl of fresh PBS. This suspension was then centrifuged at 8,000 x g for 10 minutes. The supernatant was subsequently discarded, retaining the cell pellet, which was resuspended in a lysis buffer and incubated on ice for 30 minutes. This buffer comprises of 10 mM Tris (pH 7.4), 100 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 1 mM sodium fluoride, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 % Triton X-100, 10 % glycerol, 0.1 % SDS and 0.5% deoxycholate. Following lysis,

the samples were centrifuged at 13,000 x g for 10 minutes at 4°C. The resulting supernatant was collected and stored at - 80 °C for further analysis. Cleaved PARP standards were prepared in a series of dilutions ranging from 10 ng / ml to 0 ng / ml, according to the manufacturer's protocol. Cell lysate samples were diluted (1:5), and 50 µl of each diluted sample and standard was added to wells of the coated ELISA plate. Cleaved PARP detection antibody was then applied to each well. The plate was incubated at room temperature for 3 hours on an orbital shaker operating at 500 rpm.

Following several aspirations, anti-rabbit IgG HRP was added and the plate was allowed to incubate for an additional 30 minutes at room temperature. The plate underwent multiple aspirations once more, then stabilized chromogen was added to each well. The plate then underwent a 30-minute dark incubation at room temperature. Stop solution was subsequently added to terminate the reaction. Absorbance was measured at 450 nm using the BIORAD plate reader (680XR Microplate reader). Background values were subtracted from both standards and samples and a 4-parameter algorithm was used to fit the standard curve. Sample cleaved PARP concentrations were derived from the standard curve and adjusted for the 5-fold dilution. These values were subsequently normalised to protein content as determined by the Lowry assay (detailed in **section 2.2.3.10**). Results are expressed as cleaved PARP / mg protein.

2.2.3.10 Western blot analysis

The workflow of western blot process used in this thesis is depicted in **Figure 2.1**.

Protein extraction

After cell treatments, the media was taken out and washed three times in 1x PBS. Then 200 µl of freshly made lysis buffer was added. This buffer contained 50 mM Tris

HCl, 150 mM sodium chloride, 0.1% SDS, 1% Triton X-100, and 1x / 10 mL protein inhibitor cocktail (Roche cOmplete Tablets, Mini EASYpack-04693124001). Cells were scraped, transferred into autoclaved Eppendorfs and allowed to lyse for 10 minutes on ice. A 10 µl sample was examined under a haemocytometer to confirm that the cells had fully lysed. After centrifuging the cell lysates for 15 minutes at 4°C at 15,000 rpm, the supernatant containing soluble protein was transferred to autoclaved Eppendorfs and kept at - 80°C until needed, as detailed in Bio-Rad (2012).

Protein quantification assay

By using the Lowry assay as detailed (Lowry et al., 1951), the protein content of cell extracts was determined. The composition of reagent 1 and reagent 2 used in this assay is presented in **Table 2.2**.

BSA was used and prepared in dH_2O to construct a standard curve; with concentration a range of standards was generated, as follows: 1.2, 1, 0.8, 0.6, 0.4, 0.2 to 0 mg / ml. Then, standards (50 µl) and 50 µl of a 1:5 dilution of the protein extract samples were added in triplicate in a clear flat-bottom 96-well plate (Costar Flat Bottom with Lid). Next, 150 µl of 0.1 mM NaOH and 50 µl of reagent 1 (**Table 2.2**) were added to each well. The plate was tapped to ensure even distribution and allowed to incubate for five minutes at room temperature. Following the 5-minute incubation period, 50 µl of reagent 2 (**Table 2.2**) was added to each well and the plate was left to incubate for an additional 20 minutes. The BIORAD plate reader (680XR Microplate reader) was then used to read the plate at 655 nm.

Table 2.2 Composition of Lowry reagent 1 and reagent 2

Reagent 1 component	Volume (mL)
2 % sodium carbonate (Na2Co3) in 0.1 M of NaOH	5
1 % Copper sulphate (CuSO4)	0.5
2 % potassium; sodium (KNa) tartrate	0.5
Reagent 2 component	**Volume (mL)**
Folin–Ciocalteu phenol reagent	0.5
0.1 M NaOH	5

Western blot analysis of cell lysates

Protein concentrations in cell extracts were normalised to ensure consistent sample loading, and western blotting was performed in accordance with Bio-Rad (2012) instructions. Cell extracts were prepared for electrophoresis by mixing extracts (2 mg / ml protein concentration [30 µl of total volume]) with 10 µl of a 2 x SDS- Dithiothreitol (DTT). The stock solution of 2 x SDS-DTT (10 ml) consists of 2 ml glycerol, 1 M Tris / HCl, 10 % SDS, 0.154 g DTT and a small amount of bromophenol blue. To denature the proteins, the mixture was boiled at 95°C for 5 minutes. Following this, we centrifuged the samples at 3000 rpm for 3 minutes (Biofuge 13, Heraeus Instruments) to remove any remaining cellular fragments. 30 µl of each prepared sample was loaded into individual wells of a 12% SDS-PAGE gel (CriterionTM TGX Stain-FreeTM Precast Gel from BIORAD). This was immersed in 1x running buffer, which contained 100 ml of 10 x Tris / Glycine / SDS running buffer (National Diagnostics) and 900 mL of MilliQ water. To estimate protein sizes, 5 µl of AmershamTM ECLTM RainbowTM Marker- High range (PRN756E) was used as a protein ladder. All gels were run at 200 V for 45 minutes at room temperature.

To transfer proteins from the gels onto polyvinylidene difluoride (PVDF) membranes (Amersham™ Hybond™ 0.2 µm, Cat.No 10600021) that had been pre-soaked in

methanol, we assembled the transfer cassettes and placed them in a tank (BIO-RAD TRANS-BLOT® CELL). The transfer cassettes include the following layers: sponge, blotting paper, PVDF membrane, gel, blotting paper and sponge (**Figure 2.1**), in which all the layers were pre-soaked in transferring buffer for 10 minutes. After that, the transfer cassette was kept in a transfer tank that filled with transfer buffer (composed of 30 g Glycine, 3 g Tris, 50 ml Isopropanol and 950 ml of MilliQ water). To prevent overheating during the transfer process, we added ice blocks around the tank. The transfer was run for two hours at a constant current of 350 mA. Upon completion, the PVDF membrane was removed from the transfer apparatus and, to verify successful protein transfer, the membrane was stained with Ponceau stain.

After rinsing off the stain, the membrane was incubated with 5% milk dissolve in 1x TBST buffer, which contains 7.2 g sodium chloride, 16 ml 1M Tris (pH 6.5), 1 ml Tween-20 and 983 ml MilliQ water, for 1 hour on a rocker (Stuart See-saw rockers - SSL4) to block non-specific binding sites on the membranes. This blocking step preceded the application of the primary antibody. The membranes were then incubated with primary antibodies overnight on a rocker at 4°C, using the dilutions specified in **Table 2.3**. After the incubation, the membranes were washed in 1 x TBST (3 X) for 5 minutes. Then, the secondary antibody (either with anti-rabbit IgG horseradish peroxidase (HRP)-linked whole antibody [Cytiva – NA934] or anti-mouse IgG horseradish peroxidase-linked whole antibody [Cytiva – NXA931]) was applied and incubated the membranes at room temperature for 1 hours. Following this step, we repeated the washing process, again using 1x TBST (3 x) for 5 minutes. To detect the protein bands, we used ECL reagent (SuperSignalTM West Dura Extended Duration Substrate). After applying this substrate, the membrane was kept in dark conditions for 1 minute. We then imaged the chemiluminescent signals using a BIORAD ChemiDOC™ MP

Imaging System. For all western blot analyses, we used β-actin (A2066, Sigma) as loading controls.

To analyse relative protein expression, we conducted densitometric measurements of band intensities using ImageJ software (version 64, NIH). This analysis followed the protocol described by Stael et al. (2022). We then normalised the intensity of each target protein band to its corresponding β-actin signal.

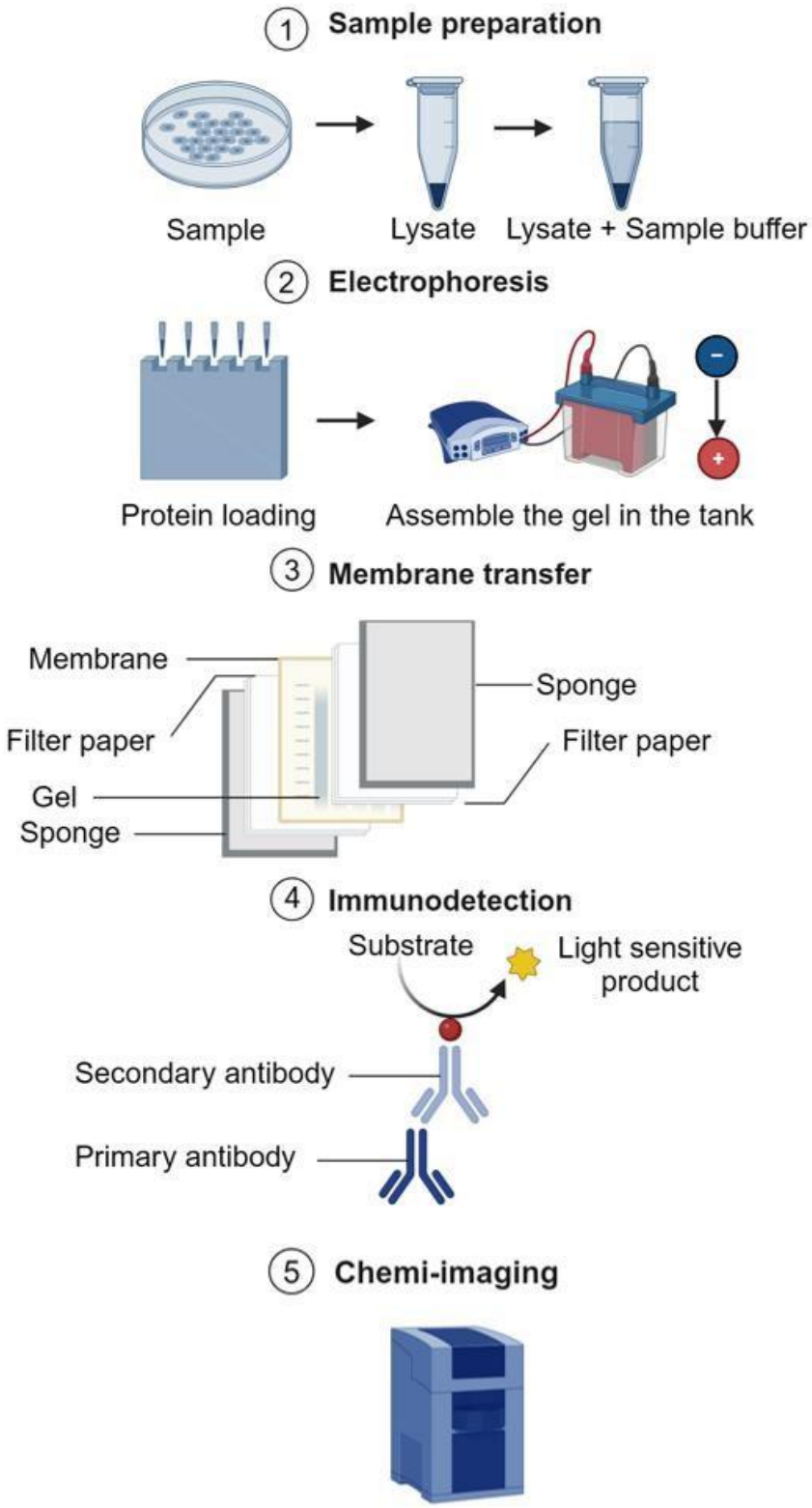


Figure 2.1 Western blot workflow used in the current work (illustration created with BioRender.com)

Table 2.3 Antibody characteristics used for western blot in the current work

Primary antibody	Supplier	Product code	Dilution	Description	Predicted band size (kDa)
Anti-Actin	Sigma-Aldrich	A2066	1:1000	Rabbit	42
Anti-CBS	EMD Millipore Corp.	MABS518	1:2000	Mouse	61
Anti-CTH	Sigma-Aldrich	HPA023300	1:1500	Rabbit	45
Caspase-3	Cell Signal	D3R6Y	1:1000	Rabbit	35
Caspase-8	Cell Signal	D35G2	1:1000	Rabbit	57
Anti-Rabbit 2° (ECLTM Anti-rabbit IgG Horseradish Peroxidase Linked Whole antibody)	Amersham	NA934	1:5000	Donkey	-
Anti-Mouse 2° (Anti-mouse IgG Horseradish Peroxidase Linked Whole antibody)	Amersham	NXA931	1:5000	Sheep	-

2.2.3.11 Detection of hydrogen sulphide (H2S) and polysulphide (H2Sn) levels

Specific fluorophores, namely AzMC and SSP4, were used to assess H_2S and H_2S_n levels, respectively. AzMC is a fluorescent probe that selectively detects H_2S in biological samples. It operates through the H_2S-specific reduction of its azido group, releasing a fluorescent coumarin molecule. The resulting fluorescence intensity correlates directly with H_2S concentration, enabling quantitative analysis. SSP4 is a fluorescent probe specifically designed to detect sulphane sulphur species, including polysulphides (H_2S_n). It functions through a selective nucleophilic reaction between sulphane sulphur and SSP4's electrophilic centre, generating a fluorescent product. The resulting fluorescence intensity directly correlates with polysulphide concentration,

enabling quantitative measurements. The assays were conducted in line with previous work (Olson and Gao, 2019). Briefly, HepG2 cells were seeded in 96-well plates (black plates with clear bottom) at a cell density of 1×10^4 cell / well for 24 hours. HepG2 cells were loaded with 25 µM AzMC or 10 µM SSP4 in the dark for 30 minutes, washed with PBS twice and then treatment was applied. Tape was used to cover the plates to reduce volatilisation. Fluorescence measurement was performed using a FluoStar Omega plate reader. The excitation / emission of AzMC and SSP4 fluorescence was recorded at 365 / 450 nm and 482 / 515 nm, as per manufacturer's recommendations, respectively. Background fluorescence was subtracted from all fluorescence measurements. The PBS composition used was (in mM): 137 NaCl, 2.7 KCl, 8 Na_2HPO_4, 2 NaH_2PO_4; and the pH was adjusted to 7.4 using HCl or NaOH, as needed (Olson et al., 2019).

2.2.4 Statistical analysis

Data was entered into Microsoft® Excel® for Microsoft 365 for organisation purposes. Then analysis was performed using IBM SPSS statistics (version 27), and figures were drawn using GraphPad Prism 9 software.

Chapter 3 Impact of medications on the trans-sulphuration pathway metabolites in human: a scoping review using paracetamol as a therapeutic model

3.1 Abstract

The trans-sulphuration pathway (TSP) is a crucial metabolic pathway in mammalian systems, since its metabolites play important roles in controlling cellular redox status, in addition to roles in the nervous and cardiovascular systems, and functions in cellular and tissue detoxification processes. Dysregulation in the TSP occurs due to genetic factors like polymorphisms in key metabolic enzymes, poor sulphur amino acid (SAA) intakes, lifestyle factors and ageing. The aim of the current study was to assess the current evidence on the impacts of medications on TSP metabolites in humans, using paracetamol (APAP) as a model therapeutic. A scoping review was conducted in three databases, namely PubMed, Web of Science and ScienceDirect (up to April 2024), using key-term strategy. Strict inclusion criteria were employed to identify all relevant and available studies. Following selection, 13 studies were included in the current review. Eight showed significant reduction in glutathione (GSH) upon short-term use (≤ 4 days) of APAP treatment, whether measured in whole blood (% of changes ranged from - 2.55 to - 29.41% (n = 3)), in red blood cells (measurements after APAP treatment ranged from 2.44 ± 0.41 μmol / g Hb to 2.27 ± 1.06 μmol / g Hb (mean ± SD) (n = 1)), or in plasma GSH (measurements ranged from 0.8 μmol / L to 6.26 μmol / L (mean) (n = 4)). One out of two studies showed a reduction in median area under curve (AUC) of plasma cysteine (Cys) over three hours of 2 g APAP (5.1 $nmol \cdot ml^{-1} \cdot h$). Despite a lack of risk factors for dysregulate in GSH homeostasis among participants in the studies under review, significant GSH depletion was reported upon short-term APAP use. This reinforces the importance of nutritional assessment when prescribing APAP, especially among vulnerable populations, such as the elderly. This review raised two interesting questions which merited further investigation: **i)** advancing age, coupled with malnutrition with low protein intakes and other specific dietary patterns with low

SAAs, are reported in animal models as risk factors for APAP-induced GSH depletion leading to toxicity; and **ii)** the hypothesis that APAP-associated cardiovascular risk may be mediated through reduced H_2S production resulting from GSH depletion. Such information should influence clinical practice and prescribing guidelines. The current review raised a call for further human studies to be conducted, to assess whether or not APAP affects other TSP metabolites (e.g. H_2S) and whether their reduction contributed to any side effects of APAP.

Key words: Paracetamol, Trans-sulphuration pathway, Dietary sulphur amino acids, Ageing, Malnutrition.

3.2 Introduction

The trans-sulphuration pathway (TSP) is a crucial metabolic pathway that allows for the incorporation of sulphur derived from the amino acids methionine (Met) and cysteine (Cys) into other important sulphur-containing metabolites like glutathione (GSH), taurine (Tau) and hydrogen sulphide (H_2S) (Zhang et al., 2022). These metabolites play important roles in cellular redox status (Sbodio, Snyder and Paul, 2019), and in the nervous system (Corona-Trejo et al., 2023); cardiovascular physiology (Scammahorn et al., 2021); renal systems (Ngowi et al., 2020); inflammation (Murphy, Bhattacharya and Mukherjee, 2019); and in detoxification processes (Pajares and Perez-Sala 2018). A growing body of evidence shows that impaired function or genetic mutation in TSP genes, or changes in metabolite levels, can contribute to a spectrum of health conditions in humans, such as neurological conditions (Smith and Refsum, 2016); osteoporosis (Filip et al., 2024); cancer(s) (Zhang et al., 2015); inflammation (Wang et al., 2023); and other proinflammatory conditions (Rose, Moore and Zhu, 2017). The impairment of TSP can occur in some health conditions, for instance rheumatoid arthritis (Mangoni and Zinellu, 2024); Huntington's disease (Paul and Snyder, 2014); and autism (Han et al., 2015). Moreover, recent studies document an increased risk of drug-induced toxicity (Elkhateeb et al., 2023) and ageing (Sbodio, Snyder and Paul, 2019) in humans. From the standpoint of the diet, sulphur amino acid (SAA) intakes (Jones et al., 2011; Lyons et al., 2000) and B vitamin status (da Silva et al., 2013) can impact the TSP in humans, these factors being common in elderly individuals (Kjeldby et al., 2013; Volpi et al., 2013). SAAs are present in foods such as chicken, beef and fish (Nimni, Han and Cordoba, 2007). Currently, the Recommended Dietary Allowance (RDA) for SAAs for adults aged ≥ 19 years is 19 mg / kg / day (Meyers, Hellwig and Otten, 2006). However,

limited evidence is available as to lifestyle factors that may impact on the bioaccessibility of these nutrients in humans. Interestingly, ageing (Sbodio, Snyder and Paul, 2019), B-vitamin deficiency (Gregory et al., 2016) and smoking (Baines et al., 2007) have been reported to impact on this pathway; however, evidence is disparate. More recently, there has been some evidence reporting impacts of pharmacological drugs on the levels of various TSP metabolites in humans (Desouza et al., 2002; Dierkes, Luley and Westphal, 2007; Pickering et al., 2019; Zheng et al., 2023); in animals (Alsaeedi et al., 2023; Olatunji et al., 2024); and in cell models (Raza and John, 2012; Alsaeedi et al., 2023). Since the flux of TSP is critical for redox regulation and detoxification (Pajares and Perez-Sala 2018), changes in metabolite levels could have negative consequences on health. Metabolic assessment of the TSP profile could emerge as a predictor of oxidative stress and disease development (Mangoni and Zinellu, 2024). Therefore, we have systematically assessed current evidence on the impacts of therapeutic drugs on TSP metabolites in humans using paracetamol (Acetaminophen; APAP) as a therapeutic model, in view of recent evidence indicating prolonged impacts of APAP on the cardiovascular system and other organs (Roberts et al., 2016).

For more than 70 years, APAP has been marketed as an analgesic for mild to moderate pain. It has a reasonable safety profile when taken within the recommended dose: 4 g in 24 hours (500 mg - 1 g every 4 - 6 hours) (Ayoub, 2021). APAP was selected in the current work due to the wide usage of this drug in the general population and ease of access (McCrae et al., 2018). The current scoping review provides one of the first assessments of research seeking to highlight the impact of APAP on TSP metabolites in humans.

Aim and objective

The aim of the current chapter is to evaluate changes of TSP metabolites in users of APAP.

TSP metabolites assessed in the current review are:

- Methionine, cysteine, cystathionine, homocysteine, glutathione, taurine, sulphate, hydrogen sulphide and its related enzymes, *viz.* cystathionine beta synthase (CBS) and cystathionine gamma-lyase (CSE).

Hypothesis

Alterations in TSP metabolites can be induced upon APAP treatment in humans.

3.3 Methodology

3.3.1 Search strategy

This review was conducted according to the most updated checklist of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Extension for Scoping Reviews (PRISMA-ScR) (Tricco et al., 2018). A search strategy was developed to gather studies that measured TSP metabolites upon APAP treatment in different populations, to assess whether APAP affects TSP metabolites in humans. Relevant literature was identified by searching three databases, namely PubMed, Web of Science and ScienceDirect, up to April 2024. The terms used during the search process were 'paracetamol', 'APAP', 'acetaminophen', 'sulphur amino acid', 'sulfur amino acid', 'SAA', 'methionine', 'cysteine', 'cystathionine', 'homocysteine', 'glutathione', 'taurine', 'sulphate', 'sulfate', 'hydrogen sulphide', 'hydrogen sulfide', 'H_2S', 'cystathionine beta synthase', 'CBS', cystathionine gamma lyase' and 'CSE'. These terms were linked using Boolean search operators (AND, OR and NOT). Finding relevant articles also involved searching the original articles' reference lists. All references were managed and duplicates eliminated using the EndNote® reference manager.

3.3.2 Inclusion and exclusion criteria

Articles selected included human studies conducted to assess the impact of APAP on at least one of the interested outcomes and which met the following criteria: **(1)** published in the English language; **(2)** peer-reviewed articles with access to the full text; **(3)** all available published primary human studies; **(4)** any type of study design **(5)** reporting or not reporting SAA dietary intake; **(6)** age, APAP dose (therapeutic or supratherapeutic dosage [> 4 g / day]) and administration (orally or intravenously) were

not restricted. Studies were excluded if they: **(1)** were published in a language other than English; **(2)** conducted on animal or cell culture models **(3)** only the abstract was available; **(4)** toxicological studies with no outcomes measured; **(5)** reviews, letters, editorial or secondary papers.

3.3.3 Article selection

Titles and abstracts were evaluated during the first screening process. Then, full manuscripts were evaluated, after removing duplicates, in accordance with the inclusion criteria for the current review. Additional papers were also checked by reviewing the full texts. **Figure 3.1** shows the process of article selection included in the current scoping review.

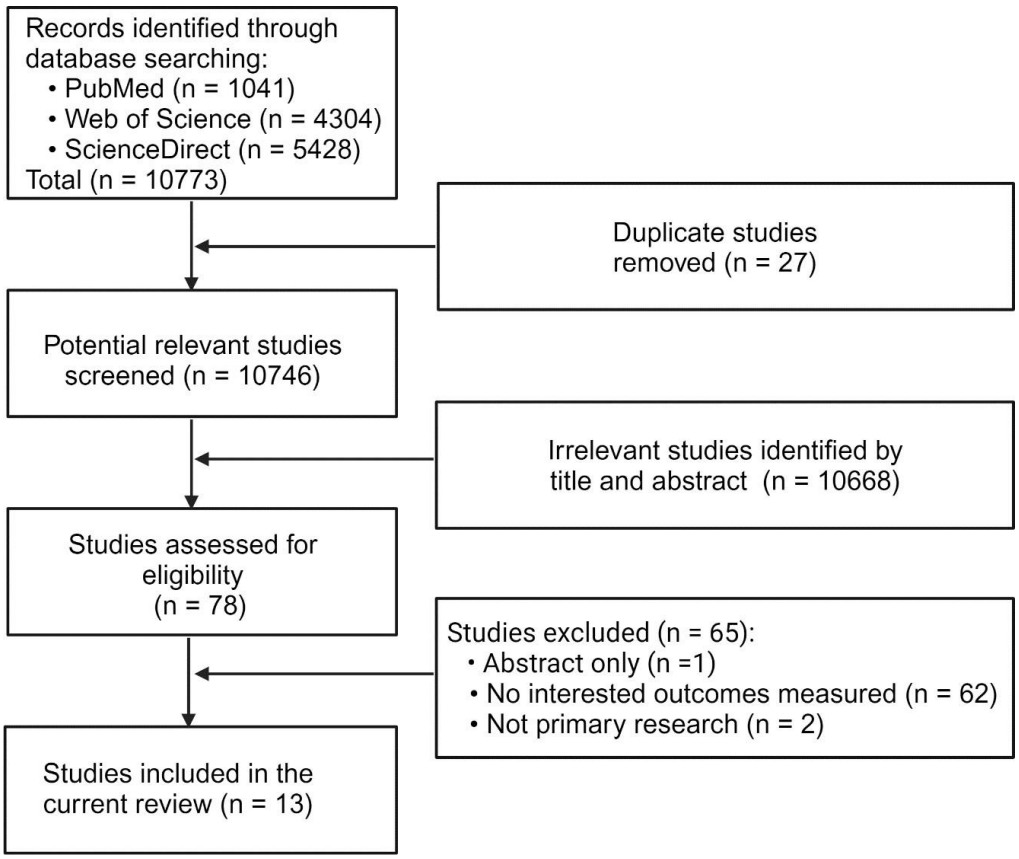


Figure 3.1 Flow chart depicting the process of paper selection included in the current scoping review following the interrogation of PubMed, Web of Science and ScienceDirect up to April 2024, using the PRISMA-ScR checklist (Tricco et al., 2018).

3.3.4 Data extraction and synthesis

Study and participant characteristics were extracted into **Table 3.1**, which included author(s) name and publication year; country in which the study was conducted; study design; study aim; sample size and subject conditions; age; body mass index (BMI); sex; APAP dosage and administration route; study duration; and, if applicable, other reported medications, dietary recommendations and other lifestyle factors. Interesting outcome measurements were also extracted and presented into **Table 3.2**, which included: values of measured outcomes that presented as mean ± standard deviation (SD) in μmol / g Hb or μmol /L or presented as percentage (%) of changes in comparison to baseline or to control groups, as will be indicated and clarified in the result section. *P* value was also extracted with consideration of a significance of $P \leq 0.05$. Data synthesis was performed on the extracted information from the included studies, combining the results into text alongside the tables.

3.4 Result

3.4.1 Study selection and characteristics

The literature searches identified 10,773 articles, which were subsequently condensed to 78 papers through abstract and title analysis after removing duplicates (n = 27) and irrelevant studies (n =10668). Additional examination by the screening of the full texts allowed the elimination of 65 papers because they were either toxicological studies with no outcomes measured (n = 62); full text was unavailable (n = 1); or they were secondary studies (n = 2); this left 13 relevant research articles. These papers were used for data extraction and final analysis, as presented in **Figure 3.1**.

Characteristics of the included studies are presented in **Table 3.1**. Of the included studies, seven were interventional (Lauterburg and Mitchell, 1987; Lauterburg and Velez, 1988; Burgunder, Varriale and Lauterburg, 1989; Kuffner et al., 2007; Mannery et al., 2010; Pickering et al., 2019; Alabdallat, 2021), with one of these (Kuffner et al., 2007) designed as double-blind randomised placebo-controlled trial and two (Mannery et al., 2010; Pickering et al., 2019) as randomised double-blind crossover-controlled trials. While the other six included studies were observational (Trenti et al., 1992; Kozer et al., 2003; Seifert and Anderson, 2007; Pickering et al., 2011; Pujos-Guillot et al., 2012; Hughes et al., 2015). Of the studies (n = 5) were undertaken in the United States of America (Lauterburg and Mitchell, 1987; Lauterburg and Velez, 1988; Kuffner et al., 2007; Seifert and Anderson, 2007; Mannery et al., 2010), (n = 3) in France (Pickering et al., 2011; Pujos-Guillot et al., 2012; Pickering et al., 2019) and one each (n = 5) in the United Kingdom (Hughes et al., 2015), Jordan (Alabdallat, 2021), Israel (Kozer et al., 2003), Switzerland (Burgunder, Varriale and Lauterburg, 1989) and Italy (Trenti et al., 1992).

Collectively, the total of subjects included in the identified studies was 443, stratified as: 135 healthy; 117 alcoholic; 27 with fever (> 38.5 ºC); 52 undergoing surgery; 10 suffering from arthritis; 79 newly-abstinent alcoholic and 23 with chronic headache. Four of the included studies were conducted on adults (mean ± SD ranged from 25 ± 4.4 years to 44 ± 8 years) (Kuffner et al., 2007; Mannery et al., 2010; Pickering et al., 2019; Alabdallat, 2021); one study focused on children (2 months - 10 years) (Kozer et al., 2003); one was conducted on the elderly (mean ± standard error of mean (SEM) was 74 ± 1.2 years) (Pujos-Guillot et al., 2012); and three studies had mixed-age groups (ranging from 21 years to > 65 years) (Seifert and Anderson, 2007; Pickering et al., 2011; Hughes et al., 2015); while four of the included trials did not report the participants' age (Lauterburg and Mitchell,1987; Lauterburg and Velez, 1988; Burgunder, Varriale and Lauterburg, 1989; Trenti et al., 1992). Moreover, although the sex of the participants was not applicable across four studies (Lauterburg and Mitchell, 1987; Trenti et al., 1992; Kuffner et al., 2007; Seifert and Anderson, 2007), the majority of participants in the current review whose sex was recorded were male (n = 131), with 46 participants being female. Only three studies (Kuffner et al., 2007; Mannery et al., 2010; Pujos-Guillot et al., 2012) reported on the BMI of participants, with the range of mean varying from healthy to overweight (23.8 kg / m^2 to 27.2 kg / m^2).

Furthermore, of total included participants, 80 were not taking APAP and served as controls, while 363 were on APAP; of these, 27 were taking APAP with either 600 mg / day N-acetylcysteine (NAC) (n = 24) – a Cys precursor used to treat APAP toxicity – or with 2 g / day NAC (n = 3) and served also as controls in their studies (Burgunder, Varriale and Lauterburg, 1989 and Pickering et al., 2019; respectively). The current review included varying doses of APAP ranging from 600 mg to $\geq$ 4 g per day. Most of the participants received the therapeutic dose of APAP (≤ 4 g / day and < 1 g / dose);

however, 20 received a supratherapeutic dose (> 4 g / day); and 101 had a single large dose of APAP > 1 g / dose. Only participants who underwent surgical procedures had APAP administered intravenously (IV) (n = 52) (Pickering et al., 2011; Hughes et al., 2015). The duration of APAP administration in the current review ranged from one experimental day to two weeks, with almost all the studies investigating the impact of short-term usage of APAP (≤ 5 days), except one trial which lasted for 14 days (Pujos-Guillot et al., 2012). No information on APAP administration route, dosage or APAP duration was clarified in one study, which referred only to "APAP users for long-term basis" (Trenti et al., 1992).

No dietary restriction / recommendation was acknowledged in the included studies; however, Mannery et al. (2010) provided the participants with three days of an equilibration diet (100 % RDA for SAAs), then two days with either 0 % or 100 % of RDA for SAAs prior to the experimental day of APAP treatment (2 g / dose). In Pujos-Guillot et al. (2012), no dietary recommendation was given to the participants; however, participants were requested to record their dietary intake in notebooks two days before the experiment and on the last day (14th day). Then food intakes were converted into nutrients by dieticians using Geni software. Similarly, in Pickering et al. (2011), SAA intake was calculated for each meal ordered by hospitalised patients. Moreover, in one trial, participants were asked not to change their diets during the study; however, no further details or results regarding diet (Pickering et al., 2019). Although most of the included studies did not mention the status of other lifestyle factors, such as smoking and alcohol consumption, of the total participants, 36 were not smokers (Mannery et al., 2010; Pickering et al., 2019) and 24 not alcoholic (Pickering et al., 2019). Apart from APAP treatment, most of the included trials did not report on whether participants were using other medications during the study period;

however, morphine was administered in Pickering et al. (2011). In Pujos-Guillot et al. (2012), half the participants were reported to have other medications (**Table 3.1**); however, they had been using them two months prior to the trial. Birth control pills were taken by all the in Mannery et al.'s (2010) study (n = 6).

Table 3.1 Characteristics of the included studies in the current scoping review, searching three databases; PubMed, Web of Science and ScienceDirect up to April 2024 (n = 13).

Author(s), year	Country	Study design	Study aim	Sample size / subject condition	Age	BMI (kg / m^2)	Sex	APAP	Duration	Other reported drugs	Diet / other lifestyle factor
Alabdallat (2021)	Jordan	Intervention study	To assess antioxidant properties of APAP	**n = 9**, healthy adults	30.6 ± 9.8 (mean ± SD)	N/A	**F** 3 **M** 6	Orally, **500 mg** twice a day	5 days	N/A	N/A
Pickering et al. (2019)	France	Intervention study (randomised double-blind, crossover, controlled trial)	To investigate if NAC prevent GSH depletion induced by therapeutic dose of APAP	**n = 24**, healthy adults	27.4 ± 6.2 (mean ± SD)	N/A	All **M**	Orally, **1 g** four times a day	Two sessions (4 days of APAP treatment each), with 14 days washout: [APAP + NAC (600 mg)] **or** [APAP only]	No analgesic or anti-inflammatory 7 days prior the study	Subjects were requested not to alter their diets, non-smokers or alcoholic
Hughes et al. (2015)	UK	Observational study	To assess if liver resection affecting APAP metabolism	**n = 22**, underwent liver resection [n = 11] and Ctrl (no liver resection or minimal, resection volume ≤ 10%) [n = 11]	**Liver patient** 67 [56-77] **Control** 62 [57-70] median [IQR]	N/A	**F** 4 **M** 18	IV, **1 g** / 6 hrs for 24. Then this dose was given orally through out the study period	4 post-operative days	Subjects were not treated with potential hepatotoxic drugs	N/A

Table 3.1 (continued) Characteristics of the included studies in the current scoping review, searching three databases; PubMed, Web of Science and ScienceDirect up to April 2024 (n = 13).

Author(s), year	Country	Study design	Study aim	Sample size / subject condition	Age	BMI (kg / m^2)	Sex	APAP	Duration	Other reported drugs	Diet / other lifestyle factor
Pujos-Guillot et al. (2012)	France	Observational study	To study how the elderly meet the increased need for SAAs induced by chronic APAP use	**n = 10**, elderly planning to be given APAP due to arthritis pain	74 ± 1.2 (mean ± SEM)	27.2 ± 1.1 (mean ± SEM)	**F** 5 **M** 5	Orally, 3 g / day	14 days	Half the participants had been on other drugs for 2 last months, no new drugs were reported. The drugs are: lansoprazole, pantoprazole, amlodipine, acénocoumarol, venlafaxine and pravastatin	No dietary recommend ation was given. Participants reported their diet 2 days before experiment and the last 4 days in notebook
Pickering et al. (2011)	France	Observational study	To assess APAP metabolism changes after surgery, and to assess the impact of age on APAP metabolism	**n = 30**, people underwent abdominal aorta by laparotomy surgery	65 ± 11 (mean ± SD)	N/A	**F** 2 **M** 28	IV, **1 g** / 6 hrs	4 days	Morphine	Oral food intake was gradually introduced, depending on the status. Calculation of SAAs intake was done by nutritionists

Table 3.1 (continued) Characteristics of the included studies in the current scoping review, searching three databases; PubMed, Web of Science and ScienceDirect up to April 2024 (n = 13).

Author(s), year	Country	Study design	Study aim	Sample size / subject condition	Age	BMI (kg / m^2)	Sex	APAP	Duration	Other reported drugs	Diet / other lifestyle factor
Mannery et al. (2010)	USA	Intervention study (randomised double-blind, crossover, controlled trial)	To examine the impact of APAP on plasma Cys and GSH with sufficient or insufficient SAAs	**n = 12**, healthy adults	**F** 25 ± 44 **M** 25 ±5.2 (mean ± SD)	**F** 23.8 ± 1.8 **M** 24.6 ± 0.78 (mean ± SD)	**F** 6 **M** 6	Orally, 0 or 15 mg / kg (two doses)	Four treatment periods, with one week apart; 3 days balance, 2 days on the assigned diet (0 % or 100 % SAAs), with or without APAP	Not on prescribed drugs, except women were all on birth control pills	SAA intake (0 or 100% of RDA), non-smokers, users of multivitamin supplement and APAP were asked to stop using it two weeks prior the study
Kuffner et al. (2007)	USA	Intervention study (double-blind randomised placebo-controlled trial)	To assess if plasma GSH is affected after 4 g APAP / day in newly abstinent alcoholic adult	**n = 79**, newly abstinent alcoholic adult [placebo (Ctrl) n = 23 and APAP n = 56]	**APAP** 43 ± 9 **Ctrl** 44 ± 8 (mean ± SD)	**APAP** 25 ± 5 **Ctrl** 25 ± 4, (mean ± SD)	N/A	Orally, **4 g** / day	3 days	Not taking APAP 4 days prior the study	N/A

Table 3.1 (continued) Characteristics of the included studies in the current scoping review, searching three databases; PubMed, Web of Science and ScienceDirect up to April 2024 (n = 13).

Author(s), year	Country	Study design	Study aim	Sample size / subject condition	Age	BMI (kg / m^2)	Sex	APAP	Duration	Other reported drugs	Diet / other lifestyle factor
Seifert and Anderson (2007)	USA	Observational study	To assess if people with chronic alcoholism are more vulnerable to APAP-induced toxicity	**n = 122**, [alcoholic n = 112 and healthy n = 10]	21 - 65 years	N/A	N/A	Orally, LDD: > **4 g** / day SLD: > **1 g** / dose TD: ≤**4 g** / day and ≤**1 g** / dose	Used APAP on a daily basis	Not using anticoagulation	N/A
Kozer et al. (2003)	Israel	Observational study	To assess changes in GSH among febrile children with repeated suprathera peutic APAP	**n = 51**, febrile children with therapeutic APAP [n=13] febrile children with suprathera peutic APAP [n=14] and Ctrl [n=24] febrile with no APAP	2 months - 10 years	N/A	**F** 26 **M** 25	Orally, (**30-75**) mg / kg / day and (**80-180**) mg / kg / day	Who received APAP for more than 72 hrs	N/A	Not chronic liver diseases or viral hepatitis

Table 3.1 (continued) Characteristics of the included studies in the current scoping review, searching three databases; PubMed, Web of Science and ScienceDirect up to April 2024 (n = 13).

Author(s), year	Country	Study design	Study aim	Sample size / subject condition	Age	BMI (kg / m^2)	Sex	APAP	Duration	Other reported drugs	Diet / other lifestyle factor
Trenti et al. (1992)	Italy	Observational study	To determine plasma GSH in participants use daily APAP on a long term	**n = 46**, Patient with chronic headache [n = 23] and Ctrl with no drug [n = 23]	N/A	N/A	N/A	N/A	Used APAP on a daily long-term basis	N/A	N/A
Burgunder, Varriale and Lauterburg (1989)	CHE	Intervention trial	To examine the effects of NAC on plasma GSH and Cys after APAP	**n = 9**, healthy volunteer receiving APAP only [n = 6] or APAP + NAC (2 g) [n = 3]	N/A	N/A	All **M**	Orally, **2 g** / dose	Single dose	N/A	N/A
Lauterburg and Velez (1988)	USA	Intervention trial	To assess plasma GSH among alcoholic after APAP	**n = 10,** [alcoholic n = 5 and healthy n = 5]	N/A	N/A	All **M**	Orally, **2 g** / dose	Single dose	N/A	N/A
Lauterburg and Mitchell (1987)	USA	Intervention study	To assess the impact of APAP on GSH / Cys turnover	**n = 19**, healthy volunteers	N/A	N/A	N/A	Orally, **600 - 1200 mg**	Single dose	Not having any drugs a week before the study	Following their regular diet and activities

Abbreviations: F, female; **M**, male; **n**, number of participant; **Ctrl**, control; **BMI**, body mass index; **APAP**, paracetamol; **IV**, intravenous; **SAA**, sulphur amino acid; **NAC**, N-Acetylcysteine; **GSH**, glutathione; **Cys**, cysteine; **LDD**, large daily dose (4 g / day); **SLD**, single large dose (> 1 g / dose); **TD**, therapeutic dose (≤ 4 g / day and ≤ 1 g / dose); **UK**, United Kingdom; **USA**, United State of America; **CHE**, Switzerland; **N/A**, not applicable. Age data is presented as a range or mean ± standard deviation (SD) or mean ± standard error of mean (SEM) or median ± interquartile range (IQR) values based on the original study reporting.

3.4.2 Measured outcomes from the included studies

Results from the included studies are summarised in **Table 3.2**. All the studies assessed the impact of APAP on GSH status. Of these, four measured GSH in whole blood cells (Pickering et al., 2011; Pujos-Guillot et al., 2012; Hughes et al., 2015; Pickering et al., 2019); two in red blood cells (Kozer et al., 2003; Alabdallat, 2021); and six in plasma (Lauterburg and Velez, 1988; Burgunder, Varriale and Lauterburg, 1989; Trenti et al., 1992; Kuffner et al., 2007; Seifert and Anderson, 2007; Mannery et al., 2010); while one study assessed the rate of GSH / Cys turnover after APAP treatment (Lauterburg and Mitchell, 1987).

Plasma Cys was measured in two studies (Burgunder, Varriale and Lauterburg, 1989; Pujos-Guillot et al., 2012) and taurine and sulphate in plasma were assessed in one trial (Pujos-Guillot et al., 2012). SAA dietary changes during APAP treatment were reported in two studies, with no dietary restrictions / recommendations given to the participants (Pickering et al., 2011; Pujos-Guillot et al., 2012); while the impact of 100 % RDA and 0 % RDA for SAA intake during APAP treatment was the focus of the Mannery et al. (2010) study. No other TSP metabolites were found in the current search that met the inclusion criteria with regard to APAP.

For clarification purposes, it is important to indicate that one of the included studies reported only the percentage of changes of whole blood GSH, with no actual quantitative values (Pickering et al., 2019). Therefore, whole blood GSH units were converted by the author from µmol / L to % of changes in the three other studies that measured GSH in whole blood, to ensure uniformity (Pickering et al., 2011; Pujos-Guillot et al., 2012; Hughes et al., 2015). Red blood GSH levels are presented in µmol / g Hb with units converted by the author from mg / g Hb to µmol / g Hb in one study

(Alabdallat, 2021) to ensure consistency for comparison. Plasma GSH, Tau and sulphate levels were all reported in µmol /L. However, it should be pointed that, in one study, plasma GSH and Cys levels were presented as median areas under curves (AUC) (nmol·ml^{-1}·h) of plasma concentration*time (Burgunder, Varriale and Lauterburg, 1989).

3.4.3 Review findings

3.4.3.1 GSH level

Because measuring human hepatic glutathione requires invasive tissue biopsy, tissue assessment is challenging. However, hepatic GSH stores are believed to be estimated from blood GSH concentrations (Burgunder and Lauterburg, 1987), but also by measuring GSH in plasma (Adams, Lauterburg and Mitchell, 1983) and GSH turnover (Hum, Robitaille and Hoffer, 1991) indicative of hepatic GSH levels.

Results from the current review collectively indicated that APAP negatively impacts on whole blood GSH levels, with percentages of changes ranging from - 2.55% to - 29.41%. Statistically-significant findings were reported, with the maximal therapeutic APAP (4 g) / day for four days in three out of four trials when compared to baseline (- 4.56 %, P = 0.03; - 18.2 %, P = 0.003; - 21.14, P = 0.0001) (Pickering et al., 2011; Hughes et al., 2015; Pickering et al., 2019), respectively. Moreover, the study by Pickering et al. (2011) compared the impact of APAP among participants aged < 65 years versus > 65 years on whole blood GSH, older group showing insignificant lower blood GSH compared to the younger group (P = 0.11), with both groups showing significant reductions in blood GSH levels compared to baselines (- 12.17% (P = 0.002) **vs** - 29.42% (P < 0.0001); < 65 years **vs** > 65 years, respectively). No significant reduction in blood GSH was observed in one trial, compared to baseline after ingestion

of 3 g / day of APAP for two weeks (- 2.55%) (Pujos-Guillot et al., 2012). This was perhaps due to the reported increase in SAA intake at the end of the study.

Furthermore, in the current review, red blood GSH level measurements after APAP treatment ranged from 2.44 ± 0.41 μmol / g Hb to 2.27 ± 1.06 μmol / g Hb (mean ± SD) across the included studies. Significant reduction was only seen when APAP was administered at supratherapeutic dosage. Indeed, one of the two studies that reported on red blood GSH levels showed significant reduction in GSH in red blood cells among febrile children after supratherapeutic dose of APAP treatment over a period of 72 hours (80 - 180 mg / kg / day), when compared to the control group (afebrile children with no APAP) (2.27 ± 1.06 μmol / g Hb **vs** 5.68 ± 1.88 μmol / g Hb; mean ± SD, P < 0.001, respectively) (Kozer et al., 2003). There was no significant reduction in red blood GSH levels when children had therapeutic dosage (30 - 75 mg / kg / day) compared to control (P = 0.071). However, the classification of APAP dosage (therapeutic or supratherapeutic) was calculated depending on parents' recall, which may lead to reporting bias. In the other study (Alabdallat, 2021), no significant reduction in red blood GSH was reported among APAP group (1 g / day, for 5 days) compared to baseline. This might be due to the fact that participants in this study used the minimum APAP dosage per day (1 g).

Plasma GSH measurements after APAP treatment ranged from 0.8 μmol / L to 6.26 μmol / L (mean) across the included studies. One study reported that median reduction in AUC of plasma GSH over three hours of a single large dose of APAP (2 g / dose) was 3.8 nmol·ml^{-1}·h (Burgunder, Varriale and Lauterburg, 1989). Three studies reporting on plasma GSH showed significant reductions in plasma GSH after APAP treatment in contrast to control (non-APAP users) (4.31±1.70 μmol / L **vs** 5.89±1.37 μmol / L, mean ± SD, respectively (P < 0.002)) (Trenti et al., 1992), when comparing

APAP + 0 % RDA for SAAs at the start and at the end of the study (1.3 μmol / L **vs** 0.8 μmol / L, mean, respectively, $P < 0.05$)), but not with APAP + 100 % RDA for SAAs (1.3 μmol / L **vs** 1.5 μmol / L, mean; at the start **vs** at the end of study, respectively) or to baseline (8.37 ± 2.65 μmol / L **vs** 6.26 ± 2.96 μmol / L (mean ± SD), $P < 0.05$) (Lauterburg and Velez, 1988). The latter study also compared plasma GSH level differences between healthy subjects using APAP and alcoholic subjects using APAP, finding significant reduction ($P < 0.05$).

On the other hand, the other two studies did not report significant reductions in plasma GSH when comparing alcoholic APAP users **vs** alcoholic non-APAP users (Seifert and Anderson, 2007) or when comparing between newly-abstinent alcoholic adults who were APAP users to newly-abstinent alcoholic adults non-APAP users (Kuffner et al., 2007). The rate of GSH / Cys turnover was significantly enhanced in the group treated with 1200 mg APAP compared to those who received 600 mg (0.1205 + 0.0273 h^{-1} **vs** 0.0445 + 0.0109 h^{-1}; mean ± SD, $P = 0.001$, respectively) (Lauterburg and Mitchell, 1987). This could be due to the increased need for GSH.

Together, regardless of where GSH is measured, the overall conclusion from these findings is that short-term use of APAP (≤ 4 days) can lead to depletion of GSH: *a)* at the maximal therapeutic dose of APAP (4 g / day) (Pickering et al., 2011; Hughes et al., 2015; Pickering et al., 2019); *b)* at a single large dose of APAP (2 g / dose) (Lauterburg and Velez, 1988; Burgunder, Varriale and Lauterburg, 1989; Mannery et al., 2010); and *c)* at the supratherapeutic APAP dose (> 4 g / day) (Kozer et al., 2003); while the minimal therapeutic dose of APAP (≈ 1 g / day) increased the rate of hepatic GSH / Cys turnover (Lauterburg and Mitchell, 1987). These significant reductions were reported among healthy subjects (n = 66), participants with headache (n = 23) and

fever (n = 14), people who underwent surgeries (n = 52) and alcoholic subjects (n = 5).

3.4.3.1 Cys, Tau and sulphate levels

Median reduction in AUC of plasma Cys over three hours of a single large dose of APAP (2 g / dose) was 5.1 nmol·ml^{-1}·h (Burgunder, Varriale and Lauterburg, 1989). Pujos-Guillot et al. (2012) noted insignificant reductions from 356 ± 23 µmol / L to 353 ± 17 µmol / L (mean ± SEM) (P > 0.05) for plasma Cys, while plasma taurine decreased from 59 ± 4 µmol / L. to 52 ± 3 µmol / L (mean ± SEM) (P > 0.05) and plasma sulphate reduced from 382 ± 45 µmol / L to 333 ± 32 µmol / L (mean ± SEM) (P > 0.05) at the end of using 3 g of APAP per day (measured on the 14th day), compared to baseline.

3.4.3.1 SAA intake during APAP treatment

Dietary SAAs were seen to increase in the elderly during APAP treatment (Pujos-Guillot et al., 2012), reaching 37 mg / kg / day (i.e. above the RDA). The researchers suggest that the high SAA intake was to meet the increased sulphur demand for paracetamol detoxification in older adults. Adults undergoing surgery, however, consumed a mean of 0.23 mg / kg / day of SAAs (Pickering et al., 2011), a negligible amount, during the post-surgery and study period (4 days). This may be explained by reduced appetite, post-surgical fasting (water only) and gradual reintroduction of limited food options. In Mannery et al.'s study (2010), APAP reduced plasma GSH levels only in a scenario of SAA inadequacy (0 % RDA for SAAs), as reported above.

Table 3.2 Impact of APAP on TSP metabolites in human studies included in the current scoping review, searching three databases: PubMed, Web of Science and ScienceDirect up to April 2024 (n = 13).

First author (year)	Interested outcome	Outcome	*P* value
Alabdallat (2021) (*)	• Red blood GSH	**Baseline** (mean ± SD, µmol / g Hb): 2.37 ± 0.37 **After APAP** (mean ± SD, µmol / g Hb): 2.44 ± 0.41	> 0.05
Pickering et al. (2019)	• Whole blood GSH	**APAP + Placebo** (% of change): - 4.56 **APAP + NAC** (% of change): + 0.33	0.03
Hughes et al. (2015) (*)	• Whole body GSH	**Control** (% of change): -18.2 **Liver resection** (% of change): - 28	**Compared to baseline:** *P* = 0.003, both groups **Control vs liver resection:** *P* = 0.43
Pujos-Guillot et al. (2012) (*)	• Alteration in SAAs diet during APAP treatment • Whole blood GSH • Plasma Tau • Plasma Cys • Plasma sulphate	**Dietary SAAs** intake increased reaching 37 mg / kg / day after APAP treatment **Whole blood GSH** (% of change): -2.55 **Plasma Cys** (mean ± SEM, µmol / L): **Baseline:** 356 ± 23 **At day 14:** 353 ± 17 **Plasma Tau** (mean ± SEM, µmol / L): **Baseline:** 59 ± 4 **At day 14:** 52 ± 3 **Plasma sulphate** (mean ± SEM, µmol / L): **Baseline:** 382 ± 45 **At day 14:** 333 ± 32	> 0.05 for all measured biomarkers
Pickering et al. (2011) (*)	• Dietary SAAs changes • Whole blood GSH • Impact of age on APAP metabolism	**Dietary SAAs:** A mean of 0.23 mg / kg / day of SAAs, a negligible amount, during the study period (4 days of post-surgery). **Whole blood GSH:** **At the end of the study compared to baseline (% of change):** - 21.14 **Impact of age on GSH after APAP:** **< 65 year:** **At the end of the study compared to baseline (% of change):** -12.17 **>65 year:** **At the end of the study compared to baseline (% of change):** - 29.41	**Compared to baseline:** 0.0001 **< 65 years:** 0.002 **> 65 years:** < 0.0001

Table 3.2 (continued) Impact of APAP on TSP metabolites in human studies included in the current scoping review, searching three databases: PubMed, Web of Science and ScienceDirect up to April 2024 (n = 13).

First author (year)	Interested outcome	Outcome	*P* value
Mannery et al. (2010)	• Plasma GSH	**APAP + 0 % SAAs** (mean, µmol / L): **Before APAP:** 1.3 **After APAP**: 0.8 **APAP + 100 % SAAs** (mean, µmol / L): **Before APAP:** 1.3 **After APAP**: 1.5	< 0.05
Kuffner et al. (2007)	• Plasma GSH	**APAP** (mean ± SD, µmol / L): **Baseline:** 2.17 ± 0.97 **After APAP:** 2.27 ± 0.85 **Placebo** (mean ± SD, µmol / L): **Baseline:** 1.90 ± 0.68 **After APAP:** 2.02 ± 0.74	> 0.05
Seifert and Anderson (2007)	• Plasma GSH	**LDD** (mean ± SD, µmol / L): 2.3 **SLD** (mean ± SD, µmol / L): 2.8 **TD** (mean ± SD, µmol / L): 2.7 **Control** (mean ± SD, µmol / L): 2.6	> 0.05
Kozer et al. (2003)	• Red blood GSH	**Control** (mean ± SD): 5.68 ± 1.88 µmol / g Hb **Therapeutic APAP** (mean ± SD): 4.43 ± 1.54 µmol / g Hb **Supratherapeutic APAP** (mean ± SD): 2.27 ± 1.06 µmol / g Hb	**Control vs supratherapeutic:** < 0.001 **Control vs therapeutic:** *P* = 0.071
Trenti et al. (1992)	• Plasma GSH	**APAP** (mean ± SD, µmol / L): 4.31±1.70 **Control** (mean ± SD, µmol / L): 5.89±1.37	< 0.002
Burgunder, Varriale and Lauterburg (1989)	• Plasma GSH Plasma Cys	**GSH**: (median AUC: nmol·ml^{-1}·h): 3.8 **Cys** (median AUC: nmol·ml^{-1}·h): 5.1	N/A
Lauterburg and Velez (1988)	• Plasma GSH	**Control** (mean ± SD, µmol / L): **Baseline:** 8.37 ± 2.65 **After:** 6.26 ± 2.96 **Alcoholic** (mean ± SD, µmol / L): **Baseline:** 4.66 ± 1.87 **After:** 2.40 ± 1.36	< 0.02 < 0.05

Table 3.2 (continued) Impact of APAP on TSP metabolites in human studies included in the current scoping review, searching three databases: PubMed, Web of Science and ScienceDirect up to April 2024 (n = 13).

First author (year)	Interested outcome	Outcome	*P* value
Lauterburg and Mitchell (1987)	• Rate GSH / Cys turnover	**APAP (600 mg) (mean ± SD):** 0.0445 + 0.0109 h^{-l} **APAP (1200 mg) (mean ± SD):** 0.1205 + 0.0273 h^{-l}	0.001

Abbreviations: APAP, paracetamol; **SAAs**, sulphur amino acids; **NAC**, N-Acetylcysteine; **GSH**, glutathione; **Cys**, cysteine; **Tau**, taurine; **LDD**, large daily dose (4 g / day); **SLD**, single large dose (> 1 g / dose); **TD**, therapeutic dose (≤ 4 g / day and ≤ 1 g / dose); **AUC**, area under curve; **(*)** units for these studies were converted by the author for uniformity purposes. $P < 0.05$ considered statistically significant.

3.5 Discussion

Paracetamol (APAP) is one of the most commonly-used analgesics worldwide; it is included on the essential medicines list of the World Health Organisation (WHO) for relief of fever and mild to moderate pain (WHO, 2021). APAP-induced toxicity, such as hepatotoxicity, is reported in many countries worldwide, including the UK (Craig et al., 2011). The standard treatment for APAP toxicity is NAC, a precursor of Cys, when introduced within 8 hours of APAP administration (Ershad, Naji and Vearrier, 2019). Despite this, APAP causes 50% of all therapeutic poisonings in the UK and is a significant issue (Townsend et al., 2001). Affordability, ease of use and access can drive this toxicity problem. The licensed oral paracetamol dosage for adults in the UK is 4 g / day, divided into 0.5 - 1 g doses every 4 - 6 hours (National Institute for Health and Care Excellence [NICE], 2024). Although the general consensus is that therapeutic doses of APAP are not toxic, regular use of APAP increases the incidence of cardiovascular and gastrointestinal events (Roberts et al., 2016) and liver cancer (Tian et al., 2024) in users. Moreover, with the ease of access to this therapy, it is likely that many users failed to comply with the recommendations of usage and clearly more research is needed in this area. Importantly, and rather surprisingly, few human studies have been conducted into whether APAP affects TSP metabolites. Maintaining optimal cellular function and redox homeostasis require precise control of this pathway. Dysregulation in TSP can occur due to genetic factors (Elkhateeb et al., 2023), poor SAA intake (Lyons et al., 2000; Jones et al., 2011) or ageing (Sbodio, Snyder and Paul, 2019), all of which could lead a person to be more vulnerable to the harmful effects of this medication. This scoping review provides one of the first assessments to scope for the deleterious effects of APAP on TSP metabolites in

humans; although we acknowledge these metabolic targets are largely identified as GSH-related, due to the known detoxification pathway for this compound.

The majority of human-based evidence suggests that APAP usage significantly induces GSH depletion. Indeed, the reductions reported in the current review were upon short-term usage (≤ 4 days) regardless of APAP dose. This result mirrored the findings from animal studies that showed GSH levels in liver or serum significantly reduced after short-term APAP treatment at the therapeutic dose (60 mg / kg / day) (Sinthorn et al., 2016); at a single large dose (3 g / kg) (Fakurazi, Hairuszah and Nanthini, 2008); and at supratherapeutic dose (500 mg / kg / day) (Lotfy et al., 2020). Reduction in GSH not only affects the redox system (Lauterburg and Mitchell, 1987), but it can also drive negative-health conditions such as liver abnormalities (Townsend, Tew and Tapiero, 2003); immune dysregulation (Zhang et al., 2017); worsening diabetes complications (Ballatori et al., 2009); and cardiovascular diseases (Rybka et al., 2011). In fact, recent findings indicate that inhibition of GSH synthesis can lead to decreased H_2S production in cells (Olson and Gao, 2019). This observation raises an intriguing hypothesis regarding the potential mechanisms underlying paracetamol's reported cardiovascular effects, such as stroke, myocardial infarction and hypertension (Roberts et al., 2016). Given paracetamol's well-documented ability to deplete hepatic GSH, as seen from our review findings, we posit that some of the evidence on the impact of APAP on cardiovascular impacts may be mediated, in part, through reduced H_2S production consequent to GSH depletion. Considering the valuable role of H_2S in the cardiovascular system (Liang et al., 2017), this hypothesis merits further investigation, as it could provide novel insights into paracetamol's pharmacological profile and potentially inform clinical practice in patients with cardiovascular comorbidities.

It is widely reported that APAP is metabolised in the liver by the glucuronidation (50 - 60 %) and sulfonation (25 - 30 %) pathways, with cytochrome P450 (particularly CYP2E1) oxidation accounting for less than 10% of the total (this generates a trace amount of the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) that is eliminated by GSH conjugation) (Freo et al., 2021). NAPQI directly damages mitochondria and triggers an inflammatory response that can lead to cellular apoptosis (Jaeschke, McGill and Ramachandran, 2012). In the current review, significant reductions in GSH levels occurred among different populations, including healthy subjects and those with fever, headache and alcohol consumption, and post-surgical patients. Clearly, APAP is sensitive to GSH stores and / or SAA intake, given that they are the precursor for GSH synthesis. The reported effect, GSH depletion, can be more pronounced and of prime significance if GSH and / or their nutritional status is depleted, as would be seen in: elderly individuals, as raised previously (Erden-İnal, Sunal and Kanbak, 2002; Kumar et al., 2023); malnourished people with low protein intake (Reid and Jahoor, 2001; Cahyani, Puryatni and Permatasari, 2017); or people following specific dietary patterns (e.g. vegetarian) (Gajski et al., 2018; Manley, 2019; Rowicka et al., 2023), in which increased levels of NAPQI could aggravate APAP toxicity or other adverse reactions. Indeed, these problems have been reported widely in animal studies. Increased susceptibility to APAP toxicity in ageing was reported by Tanimizu et al. (2020), with serum GSH reduced significantly, by approximately 34%, in aged mice (> 80 weeks) compared to young mice (8 - 10 weeks) at 24 hours of intraperitoneal APAP injection (300 mg / kg) (P = 0.012). Secondly, in an animal model that mimicked undernourished patients, wherein rats fed restricted diet of protein and energy were seen to be more vulnerable to hepatic GSH reduction than rats fed *Ad libitum* after 500 mg / kg of APAP (P < 0.01), thus suggesting restricted-fed

rats are more susceptible to APAP-induced hepatotoxicity (Kondo et al., 2012). Moreover, in vegetarian or vegan diets, SAA intake may be reduced, as evidenced by low dietary methionine in a group (Schmidt et al., 2016). Interestingly, rats fed a low-methionine diet for three weeks had liver GSH reduced by nearly 60% within three days and greater APAP-induced liver damage, compared to rats on a regular diet (Price and Jollow, 1989). These animal studies, alongside others (Wendel, Feuerstein and Konz, 1979; Reicks, Calvert and Hathcock, 1988; Taguchi et al., 2015), suggest that SAA intake is critical to prevent risk for GSH reduction. Whether low protein intakes and other specific dietary patterns with low SAAs predispose to undesirable effects in APAP usage in humans needs further research.

In the current review, among aged individuals, long-term APAP administration (14 days) led to an increase in SAA intakes due to increased demand for GSH during APAP treatment. However, for long-term care residents or malnourished or hospitalised elderly patients, increased SAA intakes may not be possible, and this could lead to undesirable effects in users (Pujos-Guillot et al., 2012). Malnutrition is common among the elderly (Dent et al., 2023; Fauzy et al., 2023), perhaps due to multifaceted factors, such as the prevalence of low protein consumption among this age group (Krok-Schoen et al., 2019) and age-related physiological changes and comorbidity (Zanetti et al., 2023). In older malnourished adults, a four-fold increased risk of developing sarcopenia, i.e. age-related muscle loss, has been reported, in comparison to older adults who were well-nourished (Beaudart et al., 2019).The interaction or combination of these two factors, age and nutritional status, needs further investigation in humans using APAP.

Lastly, while there was limited human data from the current review to draw a conclusion regarding plasma Cys, Tau or other TSP metabolites, insignificant

reductions were observed in these biomarkers after APAP usage (Pujos-Guillot et al., 2012). Animal studies showed plasma Cys significantly reduced, by 10%, in APAP-treated rats compared to untreated rats (Mast, et al., 2017). Also, it has been observed that serum sulphates in mice drop significantly two hours after paracetamol is administered (Hazelton Hjelle and Klaassen, 1986). Other TSP-associated proteins and metabolites have been reported to be impacted by APAP, such as the expression of CBS and / or CSE in liver tissues (Elshazly, El-Moselhy and Barakat, 2014; Li et al., 2019), this corresponding with reductions in H_2S in brain tissues (Wiliński et al., 2011) and elevations in serum homocysteine (Nykolaichuk and Kopylchuk, 2022). These changes may contribute to some of the known cardio impacts of APAP usage in humans (Shen et al., 2015; Roberts et al., 2016). Further studies are needed to clarify whether APAP and other therapeutics alter levels of TSP metabolites and metabolic enzymes in humans.

Limitations and future work

Major limitations to this review would be a low number of studies that met the inclusion criteria and the reported short-term duration of APAP treatment. The primarily-male population is also a factor: gender variations in APAP-induced glutathione reduction have been investigated in mice, and it suggested that female mice display lower susceptibility to APAP toxicity, with earlier replenishment of the GSH store following its depletion than for males (Masubuchi, Nakayama and Watanabe, 2011). This may be due to the hormone oestradiol, which causes females to have higher GSH levels than males (Cruikshank, Reed and Nijhout, 2024). Analysis based on gender should be a part of future clinical trials investigating the effects of APAP on TSP metabolites. Future studies should also take into consideration the nutritional status / SAAs dietary intake of participants, as this was overlooked by most of the included studies. We highlighted

animal studies that showed APAP can affect a wider range of TSP metabolites, and it would be interesting to measure these metabolites, such as Tau and H_2S, in humans using APAP and whether or not their reduction contributed to some side effects of APAP. Evidence has also associated APAP use with increased cardiovascular risk. This may potentially be linked to reduced H_2S production resulting from APAP-induced glutathione depletion, an interesting area warranting further investigation. The GSH reduction reported in the current review was among participants whose GSH and nutritional status were most likely far above the threshold; that, in theory, puts other vulnerable populations at greater risk of having APAP-indued GSH depletion leading to increased risk of toxicity, for example those of advanced age, malnourished individuals with low protein intake and people following different dietary patters with SAA insufficiency who use APAP on a chronic basis. There is a need for human studies in order to understand the relationship between paracetamol, the malnourished elderly and sarcopenia. If such were carried out, the logical question to investigate, in order to reduce the pro-sarcopenic effect of long-term APAP treatment, is whether or not a high-protein diet, or foods high in SAA (like meat), can alleviate such effects. This might help reduce the cost to patients and society and reduce strain on the healthcare system.

Other medications that detoxify through the same route as APAP should also be assessed against TSP metabolites in future studies, such as cisplatin (anti-cancer), theophylline (prescribed for asthma) and valproic acid (anti-epilepsy).

Conclusion and clinical implications

APAP is widely prescribed to treat chronic pain but, in recent times, it has been reported than many people in the general population are now self-medicating with this drug. Despite a lack of risk factors for dysregulation of GSH homeostasis, we reported

that significant GSH depletion occurs upon short-term APAP use. This reinforces the idea that more attention should be paid by clinicians when prescribing APAP; and all patients should receive personalised counselling regarding APAP use. Modifying plasma concentrations of GSH may be possible with targeted dietary changes, such as a higher requirement for Cys in food to aid in the synthesis of GSH during APAP treatment. Indeed, dietary Cys was found to be positively correlated with GSH in red blood cells among healthy individuals ($r = 0.765$, $P < 0.001$) (Margaritelis et al., 2021). Supplementing the diet with Cys among elderly subjects led to enhanced GSH synthesis (Sekhar et al., 2011). This could be used in a clinical setting with recommendations to consume foods that may help with glutathione status, such as meat, chicken and fish or supplementations, if needed for people with low food intake, such as the elderly.

Findings from the current review are important since they allow for informed decisions about prescribing pharmaceuticals and suggest potential solutions to circumvent problems like co-administrative approaches to drug use, dietary manipulation to aid SAA intakes and to allow for the creation of safe and efficient prescription strategies for susceptible individuals.

Chapter 4 Impact of common medications on dietary vitamin B_6 intake and plasma pyridoxal 5′-phosphate level in UK adults: National Diet and Nutrition Survey Rolling Programme (NDNS) (2008 - 2017)

4.1 Abstract

Vitamin B_6 in its biologically active form, pyridoxal 5′-phosphate (PLP), is a coenzyme involved in numerous cellular processes including the trans-sulphuration pathway (TSP). Reductions in dietary vitamin B_6 and PLP levels could affect the flux of TSP and are associated with increased relative risk of age-related diseases such as cognitive dysfunction. The aim of this study is to assess dietary intake of vitamin B_6 and plasma PLP concentration among the UK population and to investigate the impact of common medications on vitamin B_6 status. We conducted a study using a cross-sectional analysis of the National Diet and Nutrition Survey Rolling Programme (NDNS) (2008 - 2017), including UK adults aged ≥ 19 years. The total dietary vitamin B_6 and plasma PLP concentrations were analysed by sex and age group, stratified by decade. Plasma PLP concentration of < 20 nmol L^{-1} is considered to be deficiency in vitamin B_6, whereas a concentration of 20 - < 30 nmol L^{-1} indicates a marginal deficiency. Commonly-used medications were grouped by therapeutic usage. Multiple linear regression analyses were used to assess associations between plasma PLP concentration / dietary vitamin B_6 and medications. Data were analysed using IBM SPSS® Statistics software. The Mann-Whitney U-test was used to analyse continuous data when comparing two groups, while with more than two groups, the Kruskal–Wallis H test followed by Bonferroni post-hoc test was used. Analysing categorical variables was performed using the Chi-square test. *P* values ≤ 0.05 were considered statistically significant. Results showed that median (25^{th} - 75^{th} percentile) dietary vitamin B_6 intake of the UK population generally met the reference nutrient intake (RNI) reaching 1.7 (1.4 - 2.3) mg/day^{-1} (the RNI for males is 1.4 mg/day^{-1} and 1.2 mg/day^{-1} for females); and the median (25^{th} - 75^{th} percentile) plasma PLP concentration in the entire population was 42.8 (28.6 - 63.9) nmol L^{-1} and was higher than the threshold for

vitamin B_6 deficiency. However, data showed that both plasma PLP concentrations and dietary vitamin B_6 intake tend to decline with age ($P < 0.001$). 10 % of subjects were found to have vitamin B_6 deficiency and 17 % had marginal deficiency status. The NDNS data set included twelve reported therapeutic drugs. Of these, only antidepressants were associated with low dietary vitamin B_6 ($P = 0.007$, $R^2 = 0.15$). Seven drug classes were associated with plasma PLP concentration reduction, namely lipid-lowering drugs, analgesics, antibacterials, antidiabetics, antidepressants, Ca blockers and prescribed asthma medications ($P = 0{\cdot}01$, $R^2 = 0.06$; $P < 0{\cdot}001$, $R^2 = 0.18$; $P = 0{\cdot}04$, $R^2 = 0.08$; $P = 0{\cdot}002$, $R^2 = 0.08$; $P = 0{\cdot}03$, $R^2 = 0.08$; $P = 0{\cdot}03$, $R^2 = 0.07$ and $P = 0{\cdot}003$, $R^2 = 0.07$, respectively). We have highlighted populations who could develop vitamin B_6 deficiency/marginal deficiency, *viz.* the elderly and medication users. Therefore, it is crucial for clinicians to assess/screen for vitamin B_6 status among these vulnerable groups. If deficiencies occur, foods high in vitamin B_6 should be encouraged, such as meat and dairy products. If necessary, vitamin B_6 supplementation may be taken into consideration to improve vitamin B_6 status. This would assist in lowering potential health-related comorbidities. A causal link between plasma PLP and dietary vitamin B_6 reduction with TSP enzymes and metabolites should be investigated in future studies, particularly among the elderly.

Key words: Trans-sulphuration pathway, Vitamin B_6, PLP, Elderly, Medications.

4.2 Introduction

The B_6 vitamins comprise three non-phosphorylated pyridine derivatives, namely pyridoxal (PL), pyridoxamine (PM) and pyridoxine (PN) (Stover and Field, 2015). A wide range of foods, including meat, dairy products, beans, nuts, potatoes and various fruits and vegetables are the source of these derivatives (Brown, Ameer and Beier, 2017). After being passively absorbed in the intestine, they are phosphorylated in the liver by pyridoxal kinase (EC 2.7.1.35) into their phosphorylated forms: pyridoxal 5'- phosphate (PLP), pyridoxamine 5'-phosphate (PMP) and pyridoxine 5'-phosphate (PNP). PMP and PNP are further converted by pyridoxine 5'-phosphate oxidase (EC 1.4. 3.5) into the active form of vitamin B_6, PLP (Stover and Field, 2015). The reference nutrient intake (RNI) for dietary vitamin B_6 for adults aged ≥ 19 years is 1.4 mg day^{-1} for men and 1.2 mg day^{-1} for women (British Nutrition Foundation, 2021). Since the characterisation of PLP in 1942 (Snell and Pearson, 1942), this molecule has been found to serve as a cofactor for over 140 different enzymes (Wilson et al., 2019) to catalyse crucial metabolic reactions, such as amino acid, carbohydrate and lipid metabolisms, neurotransmitter biosynthesis and immune function (Brown, Ameer and Beier, 2017). Therefore, PLP impacts on several important physiology processes due to its roles in a myriad cellular metabolic pathways, including the trans-sulphuration pathway (TSP) (Sbodio, Snyder and Paul 2019). **Figure 4.1** highlights the role of PLP in TSP in humans. Indeed, PLP is critical for the proper function of two important enzymes in the TSP: cystathionine-β-synthase (CBS, EC 4.2.1.22) and cystathionine- γ-lyase (CSE, EC 4.4.1.1) to convert homocysteine into cystathionin and cystathionine to cysteine, respectively, alongside the production of hydrogen sulphide (H_2S). As such, PLP is important for the flux of TSP. In fact, tissue and plasma PLP concentrations are now known to affect cellular antioxidant biosynthesis, including

glutathione (Itoh et al., 2024); H_2S (DeRatt et al., 2014; Gregory et al., 2016), cysteine (Itoh et al., 2024); and homocysteine elevation (Hou et al., 2012). These studies shed light on the sensitivity of TSP metabolites to vitamin B_6 status.

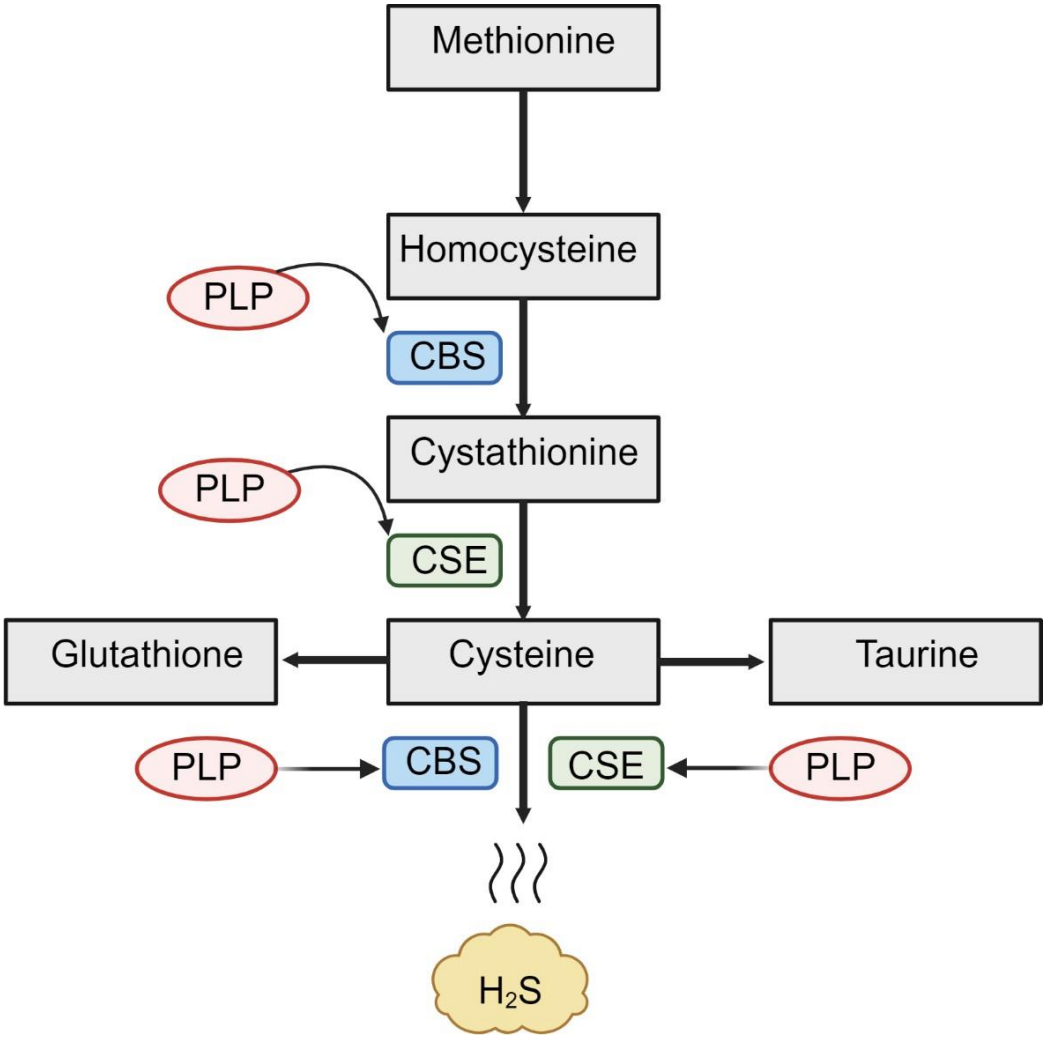


Figure 4.1 Generalised overview of the role of the active form of vitamin B_6, PLP, in the side reactions of trans-sulphuration pathway (TSP) within mammalian tissues. The functions of both enzymes CBS (EC 4.2.1.22) and CSE (EC 4.4.1.1) will be governed by the status of PLP, leading to maintenance of the flux of TSP. **Abbreviations**: **PLP**, pyridoxal 5'-phosphate; **CBS**, cystathionine β synthase; **CSE**, cystathionine-γ-lyase; **H_2S**, hydrogen sulphide (illustration created with BioRender.com).

Aside from metabolic roles, PLP has potential anti-oxidant (Bilski et al., 2000) and anti-inflammatory properties (Yanaka et al., 2005). Indeed, PLP functions in redox

pathways (Mukhopadhyay et al., 2024) and the immune system (Shabbir et al., 2024). Research indicates that reduced serum/plasma PLP levels are strongly associated with an increased risk of cardiovascular diseases (CVD) (Wang et al., 2024), such as myocardial infarction (Page et al., 2009) and venous thrombosis (Cattaneo et al., 2001); cancer(s) (Gylling et al., 2017); diabetes (Rubí, 2012); increased risk of cancer among diabetic patients (Merigliano et al., 2018); oxidative stress (Shen et al., 2010); immune system disruption and C-reactive protein (CRP) level elevations (Friso et al., 2001); and inflammation conditions (Kelly et al., 2004). Additionally, low vitamin B_6 intakes are corelated with age-related diseases such as stroke (Choe et al., 2016); all-cause mortality (Zhao et al., 2019); cancer(s) (Mocellin, Briarava and Pilati, 2017); and depression (Odai et al. 2020). Moreover, in recent years, both vitamin B_6 intake and serum PLP levels have been associated with sarcopenia, i.e. age-related loss of muscle mass (Kato et al., 2024). In fact, low dietary vitamin B_6 or decreases in PLP concentration occur in aging (Porter et al., 2016) but are also known to be influenced by lifestyle factors, including smoking (Ulvik et al., 2010); chronic alcoholism (Lumeng and Li, 1974); vegetarianism (Schüpbach et al., 2017); and vitamin B_6 intake below requirement (Tang, Xu and Shiu-Ming, 2018). In fact, some researchers have proposed that medication usage may also drive vitamin B_6 deficiency (Porter et al., 2019; Rojo-Sebastián et al., 2020). In **chapter 3**, we assessed the impact of medications on the flux of TSP by targeting its metabolites, however, side reactions of this pathway could also affect its flux (**Figure 4.1**). As such, in view of the vulnerability of TSP to changes in vitamin B_6 status, I wanted to investigate the impact of commonly-used medications on vitamin B_6 intake and PLP concentration in humans. We conducted a secondary analysis using the National Diet and Nutrition Survey Rolling Programme (NDNS) (2008 - 2017), including UK adults aged ≥ 19 years. The current

chapter contains the published manuscript of the study: (https://pubmed.ncbi.nlm.nih.gov/36789783/).

Aim

The current work aims to assess vitamin B_6 status (including both dietary intake and PLP concentration) in humans, and to investigate the impact of medications on it, as this could contribute to long-term health impacts in susceptible populations, e.g. the elderly.

It is important to mention that, prior to the impact of COVID-19, the research highlighted in this chapter was intended to be conducted on human participants, to assess the impact of medication usage in the elderly population and its impact on vitamin B_6 status and TSP metabolites, including serum glutathione and H_2S. However, due to the severity of COVID-19 restrictions, we had to mitigate potential COVID-19 transmission and therefore conducted the present secondary analysis.

Hypothesis

Certain commonly-used medications used by adults can negatively impact dietary vitamin B_6 and plasma PLP concentration.

Objective

The current secondary analysis utilised the NDNS dataset (2008 - 2017) to determine dietary vitamin B_6 and plasma PLP concentrations among a UK cohort (≥ 19 years) and to evaluate the effects of a variety of medications on these nutrients in humans.

4.3 Method

4.3.1 The National Diet and Nutrition Survey Rolling Programme (NDNS-RP)

The NDNS-RP is a national cross-sectional survey conducted to evaluate the nutritional status, dietary habits and nutrient intake of people in private households in the UK who are at least 1.5 years old. Data from the NDNS were obtained via the UK Data Service (https://www.ukdataservice.ac.uk). The user guide for UK data and other sources (Venables et al., 2022) provides a detailed methodology; in short, this survey was conducted in all four UK countries - England, Scotland, Wales and Northern Ireland - in order to generate representative data for the UK population. The survey was designed to recruit 1000 participants a year, comprising 500 children aged 1.5 to 18 years and 500 adults aged 19 years and above, plus an additional 600 for a boost sample. Random sampling was performed using the Postcode Address File, which is a database of all addresses in the United Kingdom. To improve cost-effectiveness, these addresses were then grouped into primary sampling units, which are smaller geographic regions based on postcode sectors. A randomly selected list of addresses was taken from each of these units. Trained interviewers contacted the addresses to enrol the participants based on two stages: the interviewer stage (first stage) and the nurse stage (second stage), in order to reach this sample. When we began the work for the current study, 2017 was the most recent timepoint in NDNS. Therefore, the individual and personal level dietary data from all years (2008 - 2017) were combined into an SPSS file format. The current study included both male and female participants who were at least 19 years old.

4.3.2 First stage: dietary data

Every participant underwent a face-to-face computer-assisted personal interview during the first visit by a trained fieldworker. Four days' estimated food diaries, which were explained to the participants, were used to collect dietary data. In the first year of the study, food records were kept for both weekend days and started on Thursday, Friday, or Saturday. In the second year, however, the study was structured to overrepresent weekdays relative to weekends. In year three and onwards, dietary recording was initiated on any day of the week for four days in a row (equally representing all weekdays). In a brief, participants were asked to record all foods and beverages they had during the four days, both inside and outside of their homes, along with the brand name, amount consumed and recipe, if they had cooked it themselves. To estimate portion sizes, weights listed on labels (e.g. 200 mL can of lemonade) or household measurements (e.g. 4 teaspoons of honey) were utilised. Next, a code was assigned to each food or drink item, and dietary assessment was carried out using Diet In Nutrients Out (DINO), a platform developed at the Medical Research Council (MRC) Elsie Widdowson laboratory (MRC EWL) in Cambridge, based on food composition data from Public Health England's NDNS Nutrient Databank. Additionally, the Food Standards Agency reviews this database once a year to update the current food codes or remove foods that are no longer available for sale in the UK.

According to the British Nutrition Foundation (2021), the reference nutrient intake (RNI) for dietary vitamin B_6 for adults 19 years of age and older is 1.4 mg day^{-1} for men and 1.2 mg day^{-1} for women. The current study reported on vitamin B_6 intake solely from diet, excluding supplements. Only those who had completed three or four days of the food diary were invited to take part in blood measurements in the survey's second stage.

4.3.3 Second stage: laboratory measurements and medication use

The second phase of the surveys, which took the form of a nurse visit, wherein trained nurses took blood samples in the homes of participants, was open only to those who had participated in a computer-assisted personal interview and completed at least three dietary records. The nursing stage was carried out 2 - 4 months after the last reviewer visit. After an overnight fast, phlebotomists or nurses with training in venepuncture obtained blood samples, which were then kept in a cool box at about 4°C. Within two hours of collection, samples were sent to a laboratory that was recruited locally for quick analysis. Following processing, the samples were kept at -40°C until they were transported on dry ice and delivered to MRC EWL; there, they were kept frozen at -80°C until additional analysis was performed. The current work evaluated plasma PLP level, as it is one of the most clinically-sensitive indicators for vitamin B_6 status (Lotto, Choi and Friso, 2011). The concentration of PLP in plasma was determined in the NDNS using reverse-phase High Performance Liquid Chromatography (HPLC) with post-column derivatisation and fluorometric detection, as described elsewhere (Rybak and Pfeiffer, 2004).

In this study, a plasma PLP concentration of < 20 nmol L^{-1} is considered to be deficiency in vitamin B_6, whereas a concentration of 20 - < 30 nmol L^{-1} indicates a marginal vitamin B_6 deficiency (Leklem, 1990; Spinneker et al., 2007). Information on the use of medications was also gathered during this visit. Nevertheless, details about the dosages and duration of the drugs taken were not provided.

4.3.4 Ethics

The Declaration of Helsinki's guidelines were followed when conducting the NDNS and the Oxfordshire A Research Ethics Committee provided ethical approval for all procedures involving human subjects for the NDNSD-RP 2008–2013 (Ref. No.

07/H0604/113), while the Cambridge South NRES Committee provided approval for the NDNS–PR 2014–2017 (Ref. No. 13/EE/0016).

The ISRCTN registry has the study listed under the number ISRCTN17261407 (https://www.isrctn.com/ISRCTN17261407). All participants provided written informed consent.

4.3.5 Data analysis

IBM SPSS® Statistics, Version 27, was used for the analysis. Normality of data distribution was evaluated using Shapiro–Wilks, with results indicating that data are not normally distributed ($P < 0.05$). As such, continuous variables were presented as median (25^{th} - 75^{th} percentile), with maximum (*Max*) and minimum (*Min*) values also reported. Categorical variables were reported as frequencies and percentages (%). The total dietary intake of vitamin B_6 in the sample, as well as the plasma PLP concentrations, were analysed by sex and age group, stratified by decades. Mann-Whitney U-test was used for analysing continuous data when comparing two groups, while for more than two groups, Kruskal–Wallis H test followed by Bonferroni post-hoc test was used. Analysing categorical variables was performed using Chi-square test. We examined relationships between dietary vitamin B_6 intakes and plasma PLP concentrations in GenStat 22^{nd} edition using linear regression analysis. In order to test potential associations between plasma PLP concentration/dietary vitamin B_6 and medications, the PLP concentration was $\log_{10}$-transformed for normalisation and then subjected to multiple linear regression analyses. Throughout the investigation, *P* values ≤ 0.05 were regarded as statistically significant.

4.4 Results

4.4.1 Study population selection and characteristics

From the NDNS dataset between the years 2008 and 2017, there were 13,350 participants, of which 6548 were children and consequently excluded from analysis (**Figure 4.2**). In the current study therefore, 6802 participants aged ≥ 19 years old were included. **Table 4.1** presents the participants' demographic characteristics. The population's median (25^{th} and 75^{th} percentiles) age was 49 (36 - 63) years old, with female population predominant over male (59 % and 41 %, respectively). The population's median (25^{th} - 75^{th} percentile) BMI was 27 (24 - 31) kg / m^2, with 29 % of the total being classified as obese (BMI ≥ 30 kg / m^2) and 38 % as overweight (BMI = 25 - 29.9 kg / m^2). Not all individuals, though, had their BMI status reported. The largest proportion of the respondents lived in England (57 %), followed by Scotland (16 %), Wales (14 %) and Northern Ireland (13 %). **Table 4.1** also presents an overview of participant characteristics with regards to medication use. The NDNS dataset included twelve reported therapeutic drugs, namely: analgesic; antibacterial; antidiabetic; antidepressant; four types of antihypertensive drugs (including angiotensin-converting enzyme (ACE) inhibitor, beta blocker, calcium (Ca) blocker and diuretic); antiplatelet, asthma prescribed medication; lipid lowering; and proton pump inhibitor (PPI) (**Table 4.1**). Among those who reported on medication usage, 32 % of the total population used medications, stratified as: lipid lowering medication (19 %); ACE inhibitor users (14 %); Ca^{2+} blocker (9 %); analgesics (9 %); diuretics (9 %); antidepressants (9 %); PPI (8 %); beta blockers (8 %); asthma (5 %); antiplatelet (5 %); antidiabetic (4 %); and antibacterial (1 %). The majority of the reported drugs were used by females rather than males, except for ACE inhibitor, beta blocker, antiplatelet and lipid lowering medications. Nonetheless, there were associations between sex and the use of

analgesic, antidepressant, ACE inhibitor, beta blocker, antiplatelet and lipid lowering medications (P = 0.007, P < 0.001, P < 0.001, P = 0.01, P = 0.03 and P < 0.001, respectively). All medication users in the current study were older than non-users (P < 0.01).

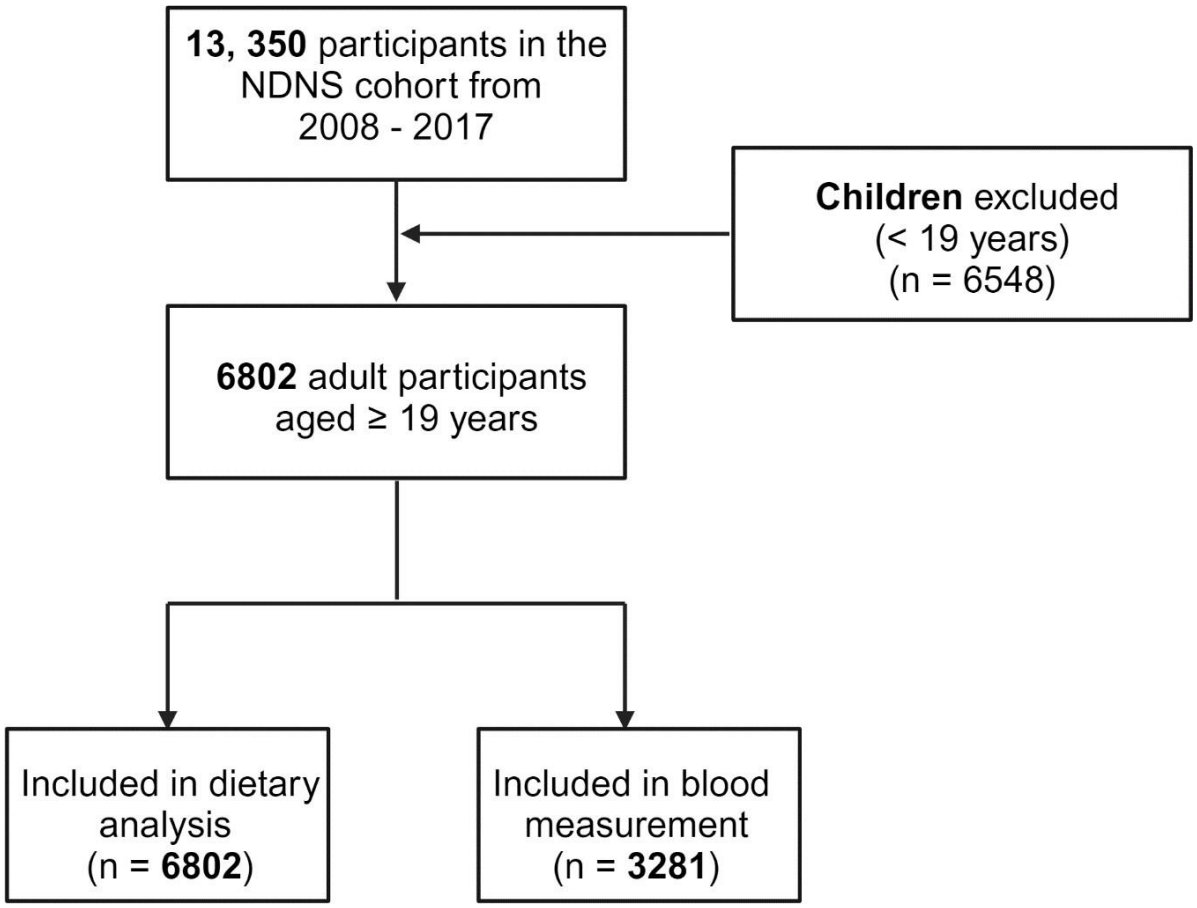


Figure 4.2 Summary flow chart describing the selection of the final population sample included in the current analysis and overall participant numbers derived from the combined NDNS database, 2008 - 2017. **Abbreviations: NDNS**, National Diet and Nutrition Survey.

Table 4.1 Characteristics of total UK adults and medication users / non-users aged ≥ 19 years old from the NDNS, 2008 - 2017.

	Total	Analgesic		Antibacterial		Antidiabetic		Antidepressant		ACE inhibitor		Beta blocker	
		User	Non-user	User	Non-user	User	Non-user	User	Non-user	User	Non-user	User	Non-user
N of participants (%)	**6802** (100%)	**194** (13%)	**1260** (87%)	**26** (2%)	**1428** (98%)	**89** (6%)	**1365** (94%)	**185** (13%)	**1269** (87%)	**309** (12%)	**2149** (88%)	**173** (7%)	**2285** (93%)
Median (25th – 75th percentiles) of age	**49** (36 - 63) ***Min***: 19 ***Max***: 96	**63** (51 - 72) ***Min***: 20 ***Max***: 95	**49** (36 - 63) ***Min***:19 ***Max***: 95	**59** (45 - 70) ***Min***: 19 ***Max***: 89	**51** (38 - 64) ***Min***: 19 ***Max***: 95	**64** (47 - 72) ***Min***: 19 ***Max***: 93	**51** (37 - 64) ***Min***: 19 ***Max***: 95	**54** (44 - 66) ***Min***: 19 ***Max***: 94	**51** (37 - 64) ***Min***: 19 ***Max***: 95	**67** (57 - 74) ***Min***: 26 ***Max***: 93	**48** (36 - 62) ***Min***: 19 ***Max***: 95	**70** (59 - 78) ***Min***: 24 ***Max***: 92	**49** (36 - 63) ***Min***: 19 ***Max***: 95
Median (25th – 75th percentiles) of BMI (kg/m^2)	**27** (24 - 31) ***Min***: 15 ***Max***: 53	29 (25 - 33) ***Min***: 15 ***Max***: 48	27 (24 - 30) ***Min***: 17 ***Max***: 53	28 (25 - 32) ***Min***: 20 ***Max***: 38	27 (24 - 30) ***Min***: 15 ***Max***: 53	31 (27 - 36) ***Min***: 18 ***Max***: 46	27 (24 - 30) ***Min***: 15 ***Max***: 53	28 (25 - 33) ***Min***: 18 ***Max***: 48	27 (24 - 30) ***Min***: 15 ***Max***: 53	29 (26 - 33) ***Min***: 19 ***Max***: 47	27 (24 - 30) ***Min***: 15 ***Max***: 53	29 (27 - 33) ***Min***: 17 ***Max***: 46	27 (24 - 30) ***Min***: 15 ***Max***: 53
BMI (N; %)													
Underweight	(**24**; 1%)	(**1**; 1%)	(**11**; 1%)	(**0**; 0%)	(**12**; 1%)	(**1**; 1%)	(**11**; 1%)	(**1**; 1%)	(**11**; 1%)	(0%)	(12; 1%)	(**1**; 1%)	(**11**; 1%)
Normal	(**590**; 32%)	(**36**; 21%)	(**393**; 33%)	(**3**; 14%)	(**426**;32%)	(**10**; 12%)	(**419**;33%)	(**33**; 20%)	(**396**; 34%)	(**32**; 17%)	(**397**;34%)	(**12**; 13%)	(**417**; 33%)
Overweight	(**706**; 38%)	(**63**; 37%)	(**458**; 39%)	(**10**; 45%)	(**511**;39%)	(**27**; 34%)	(**494**;39%)	(**67**; 41%)	(**454**; 38%)	(**70**; 37%)	(**451**; 39%)	(**36**; 38%)	(**485**; 39%)
Obese	(**539**; 29%)	(**69**; 41%)	(**316**; 27%)	(**9**; 41%)	(**376**; 28%)	(**42**; 53%)	(**343**;27%)	(**63**; 38%)	(**322**;27%)	(**87**; 46%)	(**298**; 26%)	(**45**; 48%)	(**340**; 27%)
Sex													
Number of males (%)	**2810** (41%)	**65** (34%)	**551** (44%)	**8** (31%)	**608** (43%)	**44** (49%)	**572** (42%)	**48** (26%)	**568** (45%)	**155** (50%)	**854** (40%)	**87** (50%)	**922** (40%)
Number of females (%)	**3992** (59%)	**129** (66%)	**709** (56%)	**18** (69%)	**820** (57%)	**45** (51%)	**793** (58%)	**137** (74%)	**701** (55%)	**154** (50%)	**1295** (60%)	**86** (50%)	**1363** (60%)

Table 4.1 (continued) Characteristics of total UK adults and medication users aged ≥ 19 years old from the NDNS, 2008 – 2017

	Ca blocker		Diuretic		Antiplatelet		Asthma prescribed		Lipid lowering		PPI	
	User	Non-user	User	Non-user	User	Non-user	User	Non-user	User	Non-user	User	Non-user
N of participants (%)	**199** (8%)	**2259** (92%)	**188** (8%)	**2270** (92%)	**107** (7%)	**1347** (93%)	**114** (8%)	**1340** (92%)	**405** (16%)	**2053** (84%)	**184** (13%)	**1270** (87%)
Median (25th – 75th percentiles) of age	**67** (59 - 75) ***Min***: 38 ***Max***: 93	**49** (36 - 63) ***Min***: 19 ***Max***: 95	**72** (63 - 79) ***Min***: 39 ***Max***: 94	**48** (36 - 62) ***Min***: 19 ***Max***: 95	**72** (64 - 79) ***Min***: 41 ***Max***: 95	**49** (36 - 63) ***Min***: 19 ***Max***: 95	**57** (44 - 69) ***Min***: 19 ***Max***: 95	**50** (37 - 64) ***Min***: 19 ***Max***: 95	**68** (60 - 75) ***Min***: 36 ***Max***: 95	**46** (35 - 59) ***Min***: 19 ***Max***: 95	**67** (55 - 76) ***Min***: 24 ***Max***: 95	**48** (36 - 62) ***Min***: 19 ***Max***: 95
Median (25th – 75th percentiles) of BMI (kg/m^2)	30 (26 - 33) ***Min***: 18 ***Max***: 48	27 (24 - 30) ***Min***: 15 ***Max***: 53	30 (27 - 34) ***Min***: 15 ***Max***: 48	27 (24 - 30) ***Min***: 17 ***Max***: 53	29 (25 - 32) ***Min***: 17 ***Max***: 47	27 (24 - 30) ***Min***: 15 ***Max***: 53	28 (25 - 31) ***Min***: 15 ***Max***: 49	27 (24 - 30) ***Min***: 17 ***Max***: 53	29 (26 - 33) ***Min***: 17 ***Max***: 46	27 (23 - 30) ***Min***: 15 ***Max***: 53	28 (26 - 31) ***Min***: 15 ***Max***: 47	27 (24 - 30) ***Min***: 17 ***Max***: 53
BMI (N; %)												
Underweight	(**1**; 1%)	(**11**; 1%)	(**1**; 1.1%)	(**11**; 1%)	(**2**; 2%)	(**10**; 1%)	(**1**; 1%)	(**11**; 1%)	(**1**; 1%)	(**11**; 1%)	(**2**; 1%)	(**10**; 1%)
Normal	(**20**; 18%)	(**409**; 33%)	(**11**; 12.2%)	(**418**; 33%)	(**18**; 18%)	(**411**; 33%)	(**24**; 24%)	(**405**;33%)	(**35**; 17%)	(**394**; 35%)	(**30**; 19%)	(**399**; 33%)
Overweight	(**40**; 36%)	(**481**; 39%)	(**31**; 34.4%)	(**490**;39%)	(**33**; 34%)	(**488**;39%)	(**43**; 42%)	(**478**;38%)	(**76**; 36%)	(**445**; 39%)	(**72**; 45%)	(**449**; 38%)
Obese	(**49**; 45%)	(**336**; 27%)	(**47**; 52.2%)	(**338**;27%)	(**45**; 46%)	(**340**; 27%)	(**34**; 33%)	(**351**; 28%)	(**97**; 46%)	(**288**;25%)	(**56**; 35%)	(**329**;28%)
Sex												
Number of males (%)	**93** (47%)	**916** (41%)	**76** (40%)	**933** (41%)	**56** (52%)	**560** (42%)	**47** (41%)	**569** (42%)	**215** (53%)	**794** (39%)	**71** (39%)	**545** (43%)
Number of females (%)	**106** (53%)	**1343** (59%)	**112** (60%)	**1337** (59%)	**51** (48%)	**787** (58%)	**67** (59%)	**771** (58%)	**190** (47%)	**1259** (61%)	**113** (61%)	**725** (57%)

Table 4.1 (continued) Characteristics of total UK adults and medication users aged ≥ 19 years old from the NDNS, 2008 – 2017

	Total	Analgesic		Antibacterial		Antidiabetic		Antidepressant		ACE inhibitor		Beta blocker	
		User	Non-user	User	Non-user	User	Non-user	User	Non-user	User	Non-user	User	Non-user
Region N (%)													
England	**3905** (57%)	**120** (62%)	**858** (68%)	**18** (69%)	**960** (67%)	**56** (63%)	**922** (68%)	**121** (65%)	**857** (68%)	**223** (72%)	**1431** (66%)	**112** (65%)	**1542** (67%)
Scotland	**1073** (16%)	**11** (6%)	**72** (6%)	**1** (4%)	**82** (6%)	**10** (11%)	**73** (5%)	**14** (8%)	**69** (5%)	**10** (3%)	**129** (6%)	**4** (2%)	**135** (6%)
Wales	**952** (14%)	**43** (22%)	**202** (16%)	**5** (19%)	**240** (17%)	**18** (20%)	**227** (17%)	**29** (16%)	**216** (17%)	**46** (15%)	**380** (18%)	**30** (17%)	**396** (17%)
Northern Ireland	**872** (13%)	**20** (10%)	**128** (10%)	**2** (8%)	**146** (10%)	**5** (6%)	**143** (10%)	**21** (11%)	**127** (10%)	**30** (10%)	**209** (10%)	**27** (16%)	**212** (10%)

Table 4.1 (continued) Characteristics of total UK adults and medication users aged ≥ 19 years old from the NDNS, 2008 – 2017

	Ca blocker		Diuretic		Antiplatelet		Asthma prescribed		Lipid lowering		PPI	
	User	Non-user	User	Non-user	User	Non-user	User	Non-user	User	Non-user	User	Non-user
Region N (%)												
England	**143** (72%)	**1511** (67%)	**124** (66%)	**1530** (67%)	**59** (55%)	**919** (68%)	**75** (66%)	**903** (67%)	**249** (61%)	**1405** (68%)	**115** (63%)	**863** (68%)
Scotland	**9** (4%)	**130** (6%)	**12** (6%)	**127** (6%)	**11** (10%)	**72** (5%)	**4** (4%)	**79** (6%)	**31** (8%)	**108** (6%)	**7** (4%)	**76** (6%)
Wales	**31** (16%)	**395** (17%)	**39** (21%)	**387** (17%)	**23** (22%)	**222** (17%)	**22** (19%)	**223** (17%)	**75** (19%)	**351** (17%)	**41** (22%)	**204** (16%)
Northern Ireland	**16** (8%)	**223** (10%)	**13** (7%)	**226** (10%)	**14** (13%)	**134** (10%)	**13** (11%)	**135** (10%)	**50** (12%)	**189** (9%)	**21** (11%)	**127** (10%)

Abbreviations: PPI, proton pump inhibitor; **BMI**, body mass index. Percentages of medication user, BMI classification among user / non-user and sex among user / non-user are sup-group percentages. Not all the participants had their BMI reported.

4.4.2 General patterns of dietary vitamin B_6 and plasma pyridoxal 5'-phosphate (PLP) concentration in the UK population

Assessment of the entire population revealed that the median (25^{th} - 75^{th} percentile) intakes of dietary vitamin B_6 met the RNI, reaching 1.7 (1.4 - 2.3) mg day^{-1} (the RNI for males is 1.4 mg day^{-1} and 1.2 mg day^{-1} for females). In addition, it was found that men consumed higher amounts of dietary vitamin B_6 than women (2.1 (1.6 – 2.8) mg day^{-1} and 1.6 (1.2 - 2) mg day^{-1}, respectively ($P < 0.001$), with both sexes meeting their RNI (**Table 4.2**). Using multiple linear regression analysis, we found a significant association ($P < 0.001$, $R^2 = 0.12$) between dietary intake of vitamin B_6, sex and age group in the total population (**Appendix 4.1**). This confirmed the general trend of age-dependent reductions in dietary vitamin B_6 in each gender group ($P < 0.001$) (**Table 4.2**). While median intakes were above the RNI in each age group for both sexes, among participants aged ≥ 80 years males reached a lowest of 1.6 (1.3 - 2.2) mg day^{-1} ($P < 0.001$) (RNI is 1.4 mg day^{-1}) and females reached a lowest of 1.4 (1.1 - 1.9) mg day^{-1} ($P < 0.001$) (RNI is 1.2 mg day^{-1}) (**Table 4.2**). Among males, the significant reduction in dietary vitamin B_6 was observed in the oldest group (≥ 80 + group) in comparison to all other groups ($P < 0.001$), while among females, this trend was more apparent when compared to the following age groups: 30 - 39 years old, 40 - 49 years old, 50 - 59 years old, and 60 - 69 years old ($P < 0.05$) (**Table 4.2**).

The NDNS dataset also collects plasma PLP levels in participants. The median (25^{th} - 75^{th} percentile) plasma PLP concentrations in the entire population was 42.8 (28.6 – 63.9) nmol L^{-1} and were higher than the threshold for vitamin B_6 deficiency (< 20 nmol L^{-1}) (**Table 4.2**). Additionally, plasma PLP concentrations above the cut-off of less than 20 nmol L^{-1} were present in 90 % of the population as a whole. Nonetheless, 10 % of the population had a vitamin B_6 deficiency (plasma PLP concentration < 20 nmol L^{-1})

and 17 % had a marginal deficiency (plasma PLP concentration 20 - < 30 nmol L^{-1}) (**Appendix 4.2**). We also found that plasma PLP concentrations significantly decreased with age in both sexes, with males reaching a lowest of 28.7 (16.8 - 41.2) nmol L^{-1} ($P < 0.001$) and females reaching a lowest of 31.5 (20.1 - 53.3) nmol L^{-1} ($P < 0.001$) among participants aged ≥ 80 years (**Table 4.2**). These values are still higher than the deficiency cut-off range. Importantly, a statistically-significant reduction was observed in males between the 80 + years group and the following age groups: 60 - 69 years, 50 - 59 years, 40 - 49 years, 30 - 39 years, and 19 - 29 years ($P < 0.001$). Likewise, a significant decrease was seen between females aged 80 years or older and those in the 50 - 59 years group (**Table 4.2**).

As with dietary vitamin B_6, the median (25^{th} - 75^{th} percentile) plasma PLP concentrations in males were higher than in females (47.4 (31.1 - 69.8) nmol L^{-1} **vs** 39.9 (27.1 - 58.8) nmol L^{-1}, respectively; $P < 0.001$); both sexes, however, were above the cut-off of vitamin B_6 deficiency (< 20 nmol L^{-1}). . Moreover, 12 % of female participants were deficient in vitamin B_6 in contrast to 9 % of male participants ($P < 0.05$) (**Appendix 4.2**). Furthermore, the regression analysis showed a significant association ($P < 0.001$, $R^2 = 0.12$) between the age group and plasma PLP concentration in the overall population, but not with gender ($P = 0.2$) (**Appendix 4.1**). We have found a positive significant correlation, but weak ($P < 0.001$, $R^2 = 0.07$), between dietary vitamin B_6 intake and $\log_{10}$ plasma PLP concentration.

Table 4.2 Dietary vitamin B_6 intake and plasma PLP concentration among UK adults aged ≥ 19 years based on sex and age group, 2008-2017.

Marker	N	Median (25^{th} - 75^{th} percentile)	Median (25^{th} - 75^{th} percentile) between sex		*P*	Median (25^{th} - 75^{th} percentile) among age group (year)							
						19 - 29	**30 - 39**	**40 - 49**	**50 - 59**	**60 - 69**	**70 - 79**	**≥ 80**	***P***
Dietary vitamin B_6 intake (mg day^{-1})	6802	1.7 (1.4-2.3) ***Min***: 0.1 ***Max***: 18	M	2.1 (1.6-2.8) ***Min***: 0.2 ***Max***: 15.3 (**n** = 2810)	<0.001	2.2 (1.6-2.9) ***Min***: 0.3 ***Max***: 11.8 (**n** = 363)	2.2 (1.7-2.9) ***Min***: 0.5 ***Max***: 13.1 (**n** = 475)	2.1 (1.7-2.9) ***Min***: 0.6 ***Max***: 15.3 (**n** = 566)	2.1 (1.6-2.8) ***Min***: 0.2 ***Max***: 9.1 (**n** = 519)	2.1 (1.7-2.7) ***Min***: 0.4 ***Max***: 7.4 (**n** = 449)	2.0 (1.5-2.5) ***Min***: 0.5 ***Max***: 5.4 (**n** = 291)	1.6 (1.3-2.2) ***Min***: 0.4 ***Max***: 6.1 (**n** = 147)	<0.001
			F	1.6 (1.2-2) ***Min***: 0.1 ***Max***: 17.9 (**n** = 3992)		1.5 (1.2-2.1) ***Min***: 0.1 ***Max***: 9.6 (**n** = 600)	1.6 (1.3-2) ***Min***: 0.2 ***Max***: 18.1 (**n** = 721)	1.6 (1.3-2.1) ***Min***: 0.1 ***Max***: 10.4 (**n** = 793)	1.6 (1.3-2) ***Min***: 0.1 ***Max***: 6.8 (**n** = 652)	1.6 (1.3-2.1) ***Min***: 0.3 ***Max***: 3.8 (**n** = 579)	1.5 (1.2-2.1) ***Min***: 0.4 ***Max***: 5.1 (**n** = 423)	1.4 (1.1-1.9) ***Min***: 0.4 ***Max***: 4.2 (**n** = 224)	<0.001
Plasma PLP concentration (nmol L^{-1})	3281	42.8 (28.6-63.9) ***Min***: 6 ***Max***: 602	M	47.4 (31.1-69.8) ***Min***: 6 ***Max***: 355 (**n** = 1363)	<0.001	67.3 (46-87.4) ***Min***: 20 ***Max***: 248 (**n** = 139)	54.3 (39-83.4) ***Min***: 10 ***Max***: 317 (**n** = 218)	51.5 (35.5-71.1) ***Min***: 9 ***Max***: 355 (**n** = 285)	48.1 (31.6-65.4) ***Min***: 9 ***Max***: 241 (**n** = 279)	37.5 (27.4-60) ***Min***: 11 ***Max***: 216 (**n** = 255)	32.1 (20.2-53.1) ***Min***: 8 ***Max***: 197 (**n** = 131)	28.7 (16.8-41.2) ***Min***: 6 ***Max***: 104 (**n** = 56)	<0.001
			F	39.9 (27.1-58.8) ***Min***: 7 ***Max***: 602 (**n** = 1918)		40.1 (28.7-60.3) ***Min***: 7 ***Max***: 397 (**n** = 227)	40.3 (28-61.6) ***Min***: 9 ***Max***: 292 (**n** = 312)	37.6 (26.5-54.3) ***Min***: 8 ***Max***: 548 (**n** = 434)	45.7 (30.2-67.4) ***Min*** :8 ***Max***: 602 (**n** = 351)	37.8 (25.4-56.2) ***Min***: 8 ***Max***: 380 (**n** = 319)	42.0 (25.9-59.4) ***Min***: 7 ***Max***: 249 (**n** = 191)	31.5 (20.1-53.3) ***Min***: 8 ***Max***: 278 (**n** = 84)	<0.001

Abbreviations: PLP, Pyridoxal 5' phosphate; ***Min***, minimum value; ***Max***, maximum value; **N**, participant number. Plasma PLP concentration of < 20 nmol L-1 is indicative of vitamin B_6 deficiency, while 20 - < 30 nmol L-1 is indicative of marginal deficiency. RNI for dietary vitamin B_6 intake for adults aged 19 years and above is 1.4 mg day-1 for males and 1.2 mg day-1 for females. Mann–Whitney U-test was used to assess the significant differences between two categorical data, while the Kruskal–Wallis H test followed by Bonferroni post-hoc test was used for more than two groups as described in the method: Data are expressed as median (25th - 75th percentiles). *P* ≤ 5 considered statistically significant.

4.4.3 Dietary vitamin B_6 and plasma pyridoxal 5'-phosphate (PLP) concentration and medication usage

Following analysis of general population characteristics, general pattern of dietary vitamin B_6 and plasma PLP levels, we next assessed the impact of medications on their respective levels. A key aim was to determine the impacts of medication on vitamin levels in view of higher rates of polypharmacy in ageing populations.

Comparison of dietary vitamin B_6 intakes and plasma PLP concentrations between users and non-users is presented in **Table 4.3**. The median (25^{th} - 75^{th} percentile) dietary vitamin B_6 intakes were unaffected by the majority of the reported medications (**Table 4.3**), with the exception of those taking analgesics, antidepressants, calcium blockers, diuretics and proton pump inhibitors. The median (25^{th} - 75^{th} percentile) intake of dietary vitamin B_6 was significantly lower in drug users of these classes of medication when compared to non-users (1.4 (1.1 - 1.8) mg day^{-1} **vs** 1.6 (1.2 - 2.1) mg day^{-1}; 1.4 (1 - 1.9) mg day^{-1} **vs** 1.6 (1.2 - 2.1) mg day^{-1}; 1.5 (1.2 - 2) mg day^{-1} **vs** 1.6 (1.3 - 2.2) mg day^{-1}; 1.5 (1.2 - 1.9) mg day^{-1}**vs** 1.6 (1.3 - 2.2) mg day^{-1}; 1.5 (1.1 - 1.9) mg day^{-1} **vs** 1.6 (1.2 - 2.1) mg day^{-1}, $P < 0.001$, $P < 0.001$, $P = 0.021$, $P = 0.008$, $P = 0.023$, respectively) (**Table 4.3**). Regression analysis, on the other hand, showed that lower dietary vitamin B_6 intake was associated only with antidepressants ($P = 0.007$, $R^2 = 0.15$).

Additionally, when compared to the non-users, nearly all medication users (apart from the antibacterial and ACE inhibitor groups) had significantly lower plasma PLP levels ($P \leq 0.01$: **Table 4.3**). Of the twelve medications that were reported, we found that seven drugs were associated with reduced plasma PLP concentrations — *viz.* lipid-lowering drugs, analgesics, antibacterials, antidiabetics, antidepressants, Ca blockers

and prescribed asthma medication (P = 0·01, R^2 = 0.06; P < 0·001, R^2 = 0.18; P = 0·04, R^2 = 0.08; P = 0·002, R^2 = 0.08; P = 0·03, R^2 = 0.08; P = 0·03, R^2 = 0.07 and P = 0·003, R^2 = 0.07, respectively) (**Appendix 4.1**). These findings confirmed the overall trend of plasma PLP reduction among the medication users. Importantly, all medication users had a higher percentage of participants with vitamin B_6 deficiency than non-users (**Appendix 4.3**). This finding reflected the importance of the current analysis to better understand long-term health among medication users, especially in elderly populations.

Table 4.3 The impact of therapeutic drug use on dietary vitamin B6 and plasma PLP concentration among UK adults aged ≥ 19 years old, 2008 - 2017

Medication	Participant No.	Median (25^{th} – 75^{th} percentiles) of dietary vitamin B_6 (mg day^{-1})	*Min*	*Max*	*P*	Participant No.	Median (25^{th} – 75^{th} percentiles) of PLP concentration (nmol L^{-1})	*Min*	*Max*	*P*
Analgesic (user)	194	1.4 (1.1 - 1.8)	0.2	8.9	< 0.001	112	31.5 (20.2 - 48.4)	8	175	< 0.001
Analgesic (non-user)	1260	1.6 (1.2 - 2.1)	0.4	11.8		847	41.8 (28.5 - 62.1)	8	388	
Antibacterial (user)	26	1.7 (1.3 - 2.2)	1	6.8	0.1	16	32 (21.9 - 43.4)	10	147	0.1
Antibacterial (non-user)	1428	1.5 (1.2 - 2.1)	0.2	11.8		943	41.3 (27.4 - 59.5)	8	388	
Antidiabetic (user)	89	1.5 (1.1 - 1.9)	0.6	3.4	0.07	54	26.2 (19.1 - 42)	9	286	< 0.001
Antidiabetic (non-user)	1365	1.5 (1.2 - 2.1)	0.2	11.8		905	41.7 (28.4 - 61)	8	388	
Antidepressant (user)	185	1.4 (1 - 1.9)	0.2	9	< 0.001	114	33.9 (22.1 - 57)	8	257	0.008
Antidepressant (non-user)	1269	1.6 (1.2 - 2.1)	0.4	11.8		845	41.4 (28.6 - 59.7)	8	388	
ACE inhibitor (user)	309	1.6 (1.3 - 2)	0.4	6.4	0.3	195	38.2 (23.3 - 57.1)	7	257	0.1
ACE inhibitor (non-user)	2149	1.6 (1.3 - 2.2)	0.1	18		1460	41.4 (28 - 60.8)	8	388	
Beta blocker (user)	173	1.5 (1.6 - 2.1)	0.4	3.9	0.1	88	32.8 (22.5 - 53)	8	240	0.013
Beta blocker (non-user)	2285	1.6 (1.3 - 2.2)	0.1	18		1567	41.4 (28.1 - 60.5)	7	388	
Ca^{2+} blocker (user)	199	1.5 (1.2 - 2)	0.6	18	0.021	124	32.5 (21.3 - 50.3)	9	240	< 0.001
Ca^{2+} blocker (non-user)	2259	1.6 (1.3 - 2.2)	0.1	13.2		1531	41.5 (28.4 - 61.6)	7	388	
Diuretic (user)	188	1.5 (1.2 - 1.9)	0.4	4.1	0.008	115	32.2 (20.4 - 55.2)	7	198	< 0.001
Diuretic (non-user)	2270	1.6 (1.3 - 2.2)	0.1	18		1540	41.5 (28.4 - 60.8)	8	388	
Antiplatelet (user)	107	1.6 (1.2 - 2)	0.4	3.1	0.3	64	28.9 (19.6 - 54.8)	8	240	0.004
Antiplatelet (non-user)	1347	1.5 (1.2 - 2.1)	0.2	11.8		895	41.5 (28.4 - 59.8)	8	388	

Table 4.3 (continued) The impact of therapeutic drug use on dietary vitamin B6 and plasma PLP concentration among UK adults aged ≥ 19 years old, 2008 – 2017

Medication	Participant No.	Median (25^{th} – 75^{th} percentiles) of dietary vitamin B6 (mg day^{-1})	*Min*	*Max*	*P*	Participant No.	Median (25^{th} – 75^{th} percentiles) of PLP concentration (nmol L^{-1})	*Min*	*Max*	*P*
Asthma prescribed (user)	114	1.5 (1.2 - 2)	0.4	9.1	0.5	69	31.3 (20.1 - 45.6)	8	261	0.001
Asthma prescribed (non-user)	1340	1.5 (1.2 - 2.1)	0.2	11.8		890	41.4 (28.1 - 60.8)	8	388	
Lipid lowering (user)	405	1.5 (1.3 - 2.1)	0.4	6.4	0.4	245	36.6 (22.1 - 52.5)	7	257	< 0.001
Lipid lowering (non-user)	2053	1.6 (1.3 - 2.1)	0.1	18		1410	41.6 (28.7 - 62.1)	8	388	
Proton pump inhibitor (user)	184	1.5 (1.1 - 1.9)	0.4	6.8	0.023	107	32.8 (23 - 57)	8	240	0.018
Proton pump inhibitor (non-user)	1270	1.6 (1.2 - 2.1)	0.2	11.8		852	41.5 (28.2 - 9.7)	8	388	

Abbreviations: PLP, Pyridoxal 5' phosphate; ***Min***, minimum value; ***Max***, maximum value; **N**, participant number. Plasma PLP concentration of < 20 nmol L-1 is indicative of vitamin B6 deficiency, while 20 - < 30 nmol L-1 is indicative of marginal deficiency. RNI for dietary vitamin B6 intake for adults aged 19 years and above is 1.4 mg day-1 for male and 1.2 mg day-1 for female. The Mann–Whitney U-test was used to assess the significant differences between two categorical data. Data are expressed as median (25th - 75th percentiles). $P \leq 5$ considered statistically significant.

4.5 Discussion

PLP is a critical cofactor for numerous enzymes and is involved in the metabolism of fatty acids, carbohydrates and amino acids (Parra, Stahl and Hellmann, 2018). The involvement of PLP in the TSP is widely known. Finkelstein and Chalmers (1970), for instance, measured the activity of hepatic CBS and CSE in rats that were vitamin B_6-adequate and B_6-deficient, and found reductions by 57 % for CBS and 85 % for CSE. The activity of each was partially restored *in vitro* via the addition of PLP, confirming the correlation with PLP insufficiency and enzymatic function (Finkelstein and Chalmers, 1970). Polymorphisms in CBS and CSE can also drive reductions in these enzymatic functions and are linked to several health conditions, such as neurodegeneration, liver disease and CVD (Beard and Bearden, 2011; Wang and Hegele, 2003). In fact, intracellular PLP availability and its nutritional status ultimately determine CBS and CSE activity and consequently the products and the flux of TSP (Gregory et al., 2016; DeRatt et al., 2014). Perturbation in TSP has been linked to a variety of pathological conditions, such as CVD and diabetes (Wang et al., 2022); neurodegenerative disorders (Smith and Refsum, 2021); and osteoporosis (Berry and Moustafa, 2021). Importantly, the use of medications can lead to vitamin B_6 deficiency (Porter et al., 2019; Rojo-Sebastián et al., 2020), which could impact the flux of this pathway. In England and Wales, between 2004 and 2019, the rate of medication usage increased overall by 42.6 % (Naser et al., 2022). In addition, the trend of polypharmacy, defined as regular use of ≥ 5 drugs, has increased in several countries worldwide: in the UK (Rawle et al., 2018); the USA (Wang et al., 2023); the Netherlands (Oktora et al., 2019); Denmark (Jørring Pallesen et al., 2022); Sweden (Zhang et al., 2020); Canada (Harris et al., 2022); and Australia (Wylie et al., 2020). This trend

towards increased medication usage and polypharmacy can increase the possibility of having a larger population at risk of vitamin B_6 deficiency or marginal deficiency.

We evaluated the effect of drugs on TSP flux in **chapter 3** by focusing on its metabolites; side reactions of this pathway may also have an impact on TSP flux (**Figure 4.1**). As such, in view of the vulnerability of TSP to changes in vitamin B_6 status, the current study sought to use the available NDNS datasets to ascertain plasma PLP concentration and dietary vitamin B_6 intake in free-living adults representative of the UK population (≥ 19 years of age), at the same time assessing the impact of medications on these important nutrients. Overall, the current analysis from the real-world data showed that most participants met the RNI for vitamin B_6 intake and had plasma PLP concentrations above 20 nmol L^{-1}, which indicates that they were above the cut-off for vitamin B_6 deficiency. However, 10 % (n = 344) of the entire population were deficient and 17 % (n = 558) were marginally deficient (20 - < 30 nmol L^{-1}). With increasing age, our findings showed a tendency to reduction in the plasma PLP concentrations and vitamin B_6 intake in both sexes ($P < 0.001$). Most of the reported medications in the NDNS were associated with reduction in plasma PLP concentrations, with all medication users having a higher prevalence of vitamin B_6 deficiency than non-users (**Appendix 4.3**).

While overt vitamin B_6 deficiency is thought not to be commonly found among the general population (Stach, Stach and Augoff, 2021), evidence suggests that marginal deficiency (20 - < 30 nmol L^{-1}) is widespread in various geographical regions; for example, in Canadian populations, marginal deficiency accounted for 11 %, while deficiency was 1.5 % (Ho et al., 2016). However, our findings suggested that both marginal and clear deficiency are commonly observed among the UK population, in line with the findings from populations in Korea (15 % deficiency **vs** 20 % marginal

deficiency) (Kim and Cho, 2014); and the USA (11 % deficiency **vs** 17 % marginal deficiency) (Ye et al., 2010). The discrepancy may be partly explained by the fact that the Canadian study included only young adults with mean ± standard deviation (SD) of 26.7 ± 4.2 years with higher qualification levels (71 %) – nearly three times higher than the current study (25 %). In fact, low educational status has been linked to low plasma PLP concentration (Minović et al., 2020). Nevertheless, marginal vitamin B_6 deficiency has been correlated with high risk of inflammation-related diseases (Lotto, Choi and Friso, 2011; Kumrungsee et al., 2022). This can be a critical issue among the elderly, as systemic inflammation during the aging process is associated with both mortality and morbidity (Chung et al., 2019).

The susceptibility to the reduction in dietary vitamin B_6 and plasma PLP concentrations tracked in our study with age in both sexes. Our findings are in parallel with previous observations (Rose et al., 1976; Morris et al., 2008; Kjeldby et al., 2013). In fact, in recent years, among the elderly, dietary vitamin B_6 and/or serum PLP levels were found to be negatively associated with sarcopenia (Kato et al., 2024); frailty (Balboa-Castillo et al., 2018); accelerated aging (Root and Longenecker, 1983); all-cause mortality (Wang et al., 2024); cognitive decline (Hughes et al., 2017; Palacios et al., 2019); high risk of disability in activities of daily living (Bartali et al., 2006); hip fracture incidents (Dai et al., 2013); and depression (Moore et al., 2019). Our findings raise a concern here among the elderly population as this could further drive age-related diseases (Porter et al., 2016) or unwanted side-effects attributed to the use of polypharmacy, such as malnutrition (Zanetti et al., 2023); falls and functional impairment (Wastesson et al., 2018); and overall decline in quality of life (Cantlay, Glyn and Barton, 2016). In our cohort, the prevalence of polypharmacy, ≥ 5 drugs, showed an increase with age (**Appendix 4.4**), which was in parallel with previous studies

(Linjakumpu et al., 2002; Zhang et al., 2016). This further emphasises the significance of our findings in this vulnerable population. The observed higher rates of polypharmacy among older adults are reflective of having multiple chronic conditions that require multiple medications.

Although vitamin B_6 status is still above the cut-off of deficiency in the elderly groups in the current analysis, the NDNS participants are people living in their own homes in the UK; however, it is possible that the observed reductions in dietary vitamin B_6 intake and plasma PLP concentration are more notable among care-home residents. Making efforts to prevent this critical issue is significant, not just from the standpoint of the healthcare system, but also from the socioeconomic perspective: it is imperative to better understand therapeutic strategies targeting an aging society. Using vitamin B_6 as an example, it is widely reported that adequate vitamin B_6 status in the elderly is associated with improved cognitive performance (Xu et al., 2022); and physical performance (Grootswagers et al., 2021); lower risk of mobility impairment (OR = 0.66) (Struijk et al., 2018); fragility fractures (Yazdanpanah et al., 2007); and risk of CVD (Jayedi and Zargar, 2019). Monitoring dietary vitamin B_6 should therefore be considered among the elderly in the clinical practice.

Another finding derived from the current research is a weak positive correlation between vitamin B_6 intake and plasma PLP; it was, however, statistically significant ($P < 0.001$, $R^2 = 0.07$). This was in parallel with Ho et al.'s (2016) study, but not with that of Morris et al. (2008), who found a stronger correlation ($r = 0.32$, $P < 0.001$). The fact that our data reflect dietary B_6 from food alone, rather than supplementary B_6, may help to explain the weaker correlation between plasma PLP and dietary B_6 intake in our study and that of Ho et al., when compared to the Morris et al. (2008) data. Moreover, we found that males consumed more dietary vitamin B_6 than females, a

similar observation having previously been reported with a mean ± SD of 2.17 ± 0.67 mg day^{-1} and 1.84 ± 0.60 mg day^{-1}, male vs female (P < 0.001) (Kim and Cho, 2014). This may be due to the fact that males usually consume a higher calorie intake and amount of meat, a rich vitamin B_6 source, compared to females (Lombardo et al., 2020).

One of the key findings in the current work was that use of various therapeutic drugs classes are common among the participants (32 %). Across the twelve drug groups reported in the NDNS (**Table 4.1**), lipid lowering mediation was the most-used drug in UK adults. Analysis from the National Health and Nutrition Examination Survey (NHANES), a nationally-representative survey of the US population, showed that lipid lowering medication was also the most commonly used medication in the US population aged ≥ 60 years in 2015 - 2016 (Martin et al., 2019). This could be indicative of high rates of cardiovascular diseases in these two nations. All the medication users in the current cohort were older than the non-users, confirming once again the prevalence of using medication in older individuals, with females using more of the reported medications than males (n = 8). This result was in line with previous studies (Roe, McNamara and Motheral, 2002; Fernández-Liz et al., 2008; Glaeske et al., 2012) and could be explained by the likelihood that females see/consult their doctors more frequently than males (Wang et al., 2013), or the fact that females have longer life expectancy than males (Baum et al., 2021).

Interestingly, we found that only antidepressants were associated with reduction in dietary vitamin B_6 intake. Although more studies are needed to explore potential mechanisms, antidepressants – particularly selective serotonin reuptake inhibitors (SSRIs) – may reduce dietary vitamin B_6 levels by increasing the body's need for them. Indeed, these drugs elevate brain serotonin, a neurotransmitter that requires B_6 for

synthesis (Stover and Field, 2015). Further research is needed to evaluate B_6 metabolism among antidepressant-using individuals; and into whether or not they need higher vitamin B_6 requirements during treatment periods. Additionally, the present analysis showed seven drugs were linked to reduced plasma PLP concentrations: lipid-lowering drugs, analgesics, antibacterials, antidiabetics, antidepressants, Ca^{2+} blockers and prescription asthma medication. Although lower plasma PLP concentrations among medication users are above the threshold of vitamin B_6 deficiency, these drug groups may still have an effect on people at risk of suboptimal vitamin B_6 to deficiency, such as the elderly. Indeed, we found that the majority of medication usage in our study increased with older individuals (**Appendix 4.5 (A)** and **(B)**). This pattern was also found among the populations of the US (Kantor et al., 2015), Finland (Linjakumpu et al., 2002) and Denmark (Christensen et al., 2019).

In fact, all medication users had a higher prevalence of vitamin B_6 deficiency than non-users (**Appendix 4.3**). While additional research is required to examine the mechanisms through which the use of these medications may result in lowering PLP concentrations, possible mechanisms for reduction are reduced food intake appetite (Litchford, 2020); limited bioavailability (Mason, 2010); or impaired vitamin B_6 utilisation such as in theophylline, asthma prescription drug (Ubbink et al., 1990) by inhibition of pyridoxal kinase, the enzyme that converts pyridoxal to PLP (Walters, 1988). Regardless, the present study indicates a negative impact of commonly-prescribed drugs on vitamin B_6 status, and that adults who receive these medications on a long-term basis, especially the elderly, should be closely monitored. Important connections between vitamin B_6 intake, plasma PLP concentrations and medication use were discovered in the current study.

Dietary supply of vitamin B_6 remains the primary source of PLP in humans. However, in deficiency or suboptimal cases, supplementation could be considered among individuals with low food intake, such as the elderly or malnourished. Human intervention studies have evaluated the effects of vitamin B_6 supplementation, either alone (Cheng et al., 2016) or in combination with other B-vitamins (Smith et al, 2010), on health. These studies reported that vitamin B_6 supplementation reduced homocysteine levels in plasma leading to medicate medication of antioxidant capacity (Cheng et al., 2016) and lowering cognitive decline (Li et al., 2021; Smith et al, 2010). Improvement in burn wound healing (Stach, Stach and Augoff, 2021); enhanced immune system (Kumrungsee et al., 2020); and memory performance (Deijen et al., 1992); were also documented in the literature upon vitamin B_6 supplementation. These studies therefore demonstrate the positive effects of optimal vitamin B_6 intakes on health.

Limitations and future work

The current study as a whole emphasises the usefulness of the NDNS dataset for the evaluation of health- and nutrient-related interactions, using as it does a large representative sample of the UK population. However, limitations of the current work should be acknowledged. Firstly, since plasma PLP concentration is currently the only recognised biomarker with cut-offs to define B_6 deficiency (National Academies Press, 2000), we used it as our single indicator to evaluate B_6 status. Although PLP concentration is a clinically-useful biomarker (Lotto, Choi and Friso, 2011), it does not take into account the metabolic turnover of vitamin B_6 in the relevant population; rather, it represents the circulating concentration of vitamin B_6. Numerous factors can influence it, including alkaline phosphatase concentrations, serum albumin and inflammation (Ueland et al., 2015). Functional biomarkers that are sensitive, like

plasma cystathionine (Lamers, 2011), are essential for determining intracellular vitamin B_6 status. Secondly, a significant limitation in the NDNS dataset is reporting the included medications only by category, without any data as to dosage and duration. As such, we were not able to provide accurate correlations between exact drug name and dietary vitamin B_6/plasma PLP concentration. Thirdly, the participants in the current study are free-living people in the UK; this might limit the generalisability of findings. Lastly, although we highlighted that several commonly-used medications used by the general population can impact on vitamin B_6 status, other non-reported drugs, such as antiepileptic treatments, can also cause vitamin B_6 deficiency (Mintzer, Skidmore and Sperling, 2012). As such, attention should be drawn to the possibility that a wider range of medications could affect vitamin B_6 status. Further research is required.

Although COVID-19 restrictions prevented us from conducting a human-based study to confirm a causal link between PLP reduction and TSP enzymes and metabolites, this approach should be investigated in future studies. However, our findings align with the possibility that restoration of vitamin B_6 homeostasis among medication users may positively influence the disruption in TSP induced by these medications and overall health. Again, further investigations are needed. It would also be interesting to assess vitamin B_6 status among a wider range of drugs and assess possible mechanisms. This would help healthcare professionals targeting at-risk populations to prevent vitamin B_6 deficiency/marginal deficiency.

Conclusion and clinical implications

In conclusion, in this study, dietary vitamin B_6 intake of the UK population generally met the RNI; and plasma PLP concentration was above the cut-off of deficiency. However, 10 % of subjects had vitamin B_6 deficiency and 17 % had marginal deficiency

status. This study revealed a tendency for both vitamin B_6 intake and PLP concentrations to decrease with age. Significantly, human plasma PLP levels were consistently found to be negatively impacted by medication use. We have highlighted populations who could be at risk of vitamin B_6 deficiency or marginal deficiency *viz.* medication users and the elderly (especially those on medications). Therefore, it is crucial for clinicians to assess and/or screen the vitamin B_6 status among these vulnerable groups. If deficiency occurs, foods high in vitamin B_6 should be encouraged, such as meat and dairy products. If necessary, vitamin B_6 supplementation may be taken into consideration to improve vitamin B_6 status. Dietitians should take into considerations the use of medications and age when assessing the nutritional status of these populations. This would assist in lowering potential health-related comorbidities.

Chapter 5 Validation of a human hepatoma HepG2 cell model of oxidative stress and apoptosis to aid in medication assessment; using paracetamol and sulphasalazine

5.1 Abstract

Oxidative stress and apoptosis may accelerate the development or progression of long-term diseases such as neurodegenerative disorders and cardiovascular diseases. Several pharmacological drugs are implicated in inducing oxidative stress, which can be a potential mechanism behind certain adverse drug effects and toxicities. The aim of this study is to validate and develop a human hepatoma liver cell model (HepG2) to aid in the assessment of commonly used-drugs, namely paracetamol (APAP) and sulphasalazine (SSZ) (0 – 4 mM), and to further understand the molecular mechanisms of drug-induced apoptosis in HepG2 cells. We conducted a series of *in vitro* studies to assess cytotoxicity and early and late oxidative markers in HepG2 cells. Results showed that both APAP and SSZ reduce cell viability, with SSZ exhibiting a concentration-dependent effect ($P = 0.002$), while APAP requires higher concentrations than SSZ to induce significant cytotoxicity in our cell line ($P < 0.01$). Our mechanistic studies demonstrated that both drugs induce the formation of reactive oxygen species (ROS), lipid peroxidation and mitochondrial depolarisation in a time-dependent manner ($P < 0.01$, for both drugs), suggesting a possible mechanism involved in drug cytotoxicity. Both drugs induced GSH depletion in a concentration-dependent manner ($P < 0.001$ for both drugs). Low APAP concentrations (≤ 1 mM) were found to significantly decrease GSH levels in cells ($P < 0.001$) without immediately affecting their survival. However, this GSH depletion appeared to increase the cells' vulnerability to further oxidative stress, i.e. DNA damage, as observed in this study; indicating that GSH depletion is likely one of the key mechanisms in APAP-induced cytotoxicity.

APAP-mediated apoptosis in HepG2 cells is partially caspase-dependent, while caspase-3 plays a more prominent role in the apoptotic cascade induced by SSZ compared to other pan-caspases. The incomplete protection observed upon caspase inhibition suggests the involvement of caspase-independent mechanisms, possibly including necrosis, especially at higher concentrations. Significant cleaved PARP was observed at the highest concentrations of both APAP and SSZ (P = 0.002 and P = 0.003, respectively). For a wider picture, this study highlights significant concerns for vulnerable populations, such as the elderly, who often have reduced GSH levels. This susceptibility can be further compounded by the common practice of polypharmacy in this population.

Key words: Medication, Paracetamol, Sulphasalazine, Apoptosis, Oxidative stress.

5.2 Introduction

Under both physiological and pathological states, metabolism produces reactive oxygen species (ROS) (Jomova et al., 2023). Superoxide anions (O_2^-), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) are generally collectively referred to as ROS (Krishnamurthy et al., 2024). ROS carry out important physiological roles including controlling intracellular transcription factor activation, gene expression and differentiation, as well as cell proliferation (Filip and Albu, 2018). However, abnormally high productions of ROS results in oxidative stress. This excess in turn reacts with other molecules leading to lipid peroxidation (Su et al., 2019), protein and enzyme degradation and nucleic acid damage (Juan et al., 2021), and eventually leads to apoptosis, cell death. These events are often associated with the onset or development of several chronic and age-related diseases (Liguori et al., 2018) such as diabetes (Oguntibeju, 2019); cancer (Reuter et al., 2010); neurological disorders (Singh et al., 2019); and cardiovascular diseases (Dubois-Deruy et al., 2020), including atherosclerosis (Kattoor et al., 2017).

While ROS react with a wide range of biomolecules, such as deoxyribonucleic acid (DNA) and lipids, intracellular thiols (- SH) are one of their main targets (Baba and Bhatnagar, 2018). Owing to their high nucleophilicity, thiols found in proteins and peptides are especially susceptible to direct oxidation by ROS, which can change the structure and function of proteins. However, thiols also provide a strong defence against oxidative stress-induced biochemical disruptions (He et al., 2017; Kükürt et al., 2021). Glutathione (GSH), a trans-sulphuration pathway (TSP) metabolite, is one of the key intracellular thiol antioxidant species found in mammalian cells. It is crucial for preserving the redox homeostasis of various organelles, for cell signalling and for protecting cells from oxidative stress (Franco and Cidlowski, 2009; Ribeiro, 2023).

Therefore, thiol, including GSH, is one component of many others to mitigate the effects of ROS overproduction and achieve a redox equilibrium. Other mechanisms include enzymatic (e.g. superoxide dismutase (SOD) and catalase) and non-enzymatic systems (e.g. carotenoids, vitamin E and C) (Ighodaro and Akinloye, 2018). In fact, there are some exogenous factors triggering ROS formation and contributing to oxidative stress and apoptosis, such as cigarette smoke, radiation, alcohol abuse and air pollution (Sharifi-Rad et al., 2020). Importantly, medication use has also been reported to induce ROS and oxidative stress (Deavall et al., 2012; Li et al., 2020; Salimi et al., 2020; Ahmed et al., 2023; Umemura, 2023). Oxidative stress significantly impacts human health, warranting extensive research attention. Considering this and the widespread use of pharmaceutical drugs (Zhang et al., 2016), we wanted to validate and develop human hepatoma (HepG2) cells as a model for assessing oxidative stress and apoptosis in our laboratory. I have selected two medications: paracetamol (APAP), a widely known analgesic used to treat mild to moderate pain, and sulphasalazine (SSZ), an anti-inflammatory drug used to treat ulcerative colitis and other inflammatory bowel diseases (Prescott, 2000; Linares, Alonso and Domingo, 2011). These drugs were chosen as representatives in the current study because of their well-known impact on thiols status, specifically on the TSP metabolite GSH, which is a critical component of the body's antioxidant defence systems (Tirmenstein and Nelson, 1990; Jannuzzi, Kara and Alpertunga, 2018; Sendo et al., 2022). The ability of APAP and SSZ to disrupt thiol balance can induce oxidative stress and potentially trigger apoptosis, making them valuable tools for this investigation. Furthermore, the liver serves as the primary detoxifying organ and a critical site of the TSP. Given this important metabolic role, human liver cancer HepG2 cells have been extensively utilised for drug and toxicity testing, which is an advantage of this cell

model. Indeed, HepG2 cells are widely used for *in vitro* toxicological models (Ramirez et al., 2018; Lőrincz et al., 2021). They have been used to study a range of differing biological and clinical processes, such as immunotherapy (Li et al., 2016), drug resistance (Fujita et al., 2019) and tumorigenesis signalling (Ji et al., 2010).

In **chapters 3** and **4**, we assessed the impact of commonly-used medications on TSP metabolites and their cofactors (vitamin B_6) in humans. In the current chapter, we employ an *in vitro* approach to further understand the molecular mechanisms of drug-induced apoptosis in HepG2 cells. This will help validate our cell line for medication assessment in the subsequent chapter, where we will evaluate the effects of drugs on TSP metabolites, specifically H_2S and its key enzyme production.

Hypothesis

The hypothesis was that commonly-used medications can induce oxidative markers in our HepG2 cell line and downregulate the TSP metabolites, GSH.

Aim

The aim of this study was to validate a cellular model of oxidative stress and apoptosis to aid in the assessment of drugs that impact on thiol status in a hepatoma HepG2 model and, additionally, to clarify and describe the molecular mechanisms underlying drug-induced apoptosis in HepG2 cells.

Objective

This study sought to address the following key research objectives:

1) To assess cytotoxicity of APAP and SSZ in HepG2 cells.

2) To determine the impact of individual drugs on early markers for the apoptotic cascade, namely: reactive oxygen species (ROS) formation, lipid peroxidation and mitochondrial membrane potential.
3) To examine later-stage events of apoptosis including Lactate dehydrogenase (LDH), deoxyribonucleic acid (DNA) damages, caspase involvement and poly-ADP ribose polymerase (PARP) cleavage.

In addition, I also assessed the status of GSH, a TSP metabolite, upon drug treatment. These investigations will assist us in examining and characterising the molecular pathways underlying drug-induced apoptosis in HepG2 cells.

5.3 Materials and methods

A detailed description of all the techniques used in this chapter is presented in the Materials and Methods chapter (**chapter 2**).

5.3.1 Chemicals

Paracetamol (APAP) and Hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich (St Louis, USA) and Sulphasalazine (SSZ) from Across Organics.

5.3.2 Cell culture

HepG2 cells were cultured in DMEM, supplemented with 100 ml / L FBS, 10 000 units / ml penicillin and 10 mg / ml streptomycin, as detailed in **chapter 2** (**2.2.1.1 - 2.2.1.3**). Seeding density of the included experiments in the current chapter is presented in **Table 5.1**.

Table 5.1 HepG2 cell seeding densities for various assays used in the current study

Plate seeding number	Assay
10,000 cells / well in clear 96 well plate	• Cell viability (crystal violet) • Caspase inhibitors
20,000 cells / well in clear 96 well plates	• Lactate dehydrogenase assay (LDH)
10,000 cells / well in black plate (with clear bottoms)	• DCFDA (ROS production) • JC-1 (mitochondria depolarisation)
400,000 cells / well in clear 6 well plates	• GSH measurement • Malondialdehyde (MDA) level • Cleaved PARP • Fluorescent imaging (DAPI, CellROX® Deep Red, MitoSOX™ Red and Image-iT®) (DNA damage, ROS and lipid peroxidation)
Petri dish 2.2 million cells	• Western blot

5.3.3 Cell viability testing

Cell viability was determined using the crystal violet assay, as previously described (Feoktistova, Geserick and Leverkus, 2016) and detailed in **section 2.2.3.1.** Viability assays were performed to assess the effects of APAP and SSZ on HepG2 cells at various concentrations and time points. Previous *in vitro* studies showed that both APAP and SSZ induced cytotoxicity at 4 mM (González et al. 2017; Yin et al., 2023). In line with these studies, we selected a concentration range of 0 - 4 mM, testing eight different concentrations (0, 0.06, 0.12, 0.25, 0.5, 1, 2 and 4 mM), to assess the concentration-dependent effects of these drugs over a 24-hour treatment period. Replicates were performed independently (n = 18 for APAP and n = 24 for SSZ) on separate days with drugs prepared fresh for each individual experiment. Plates were incubated at the indicated time point, then crystal violet assay was performed, as described in **section 2.2.3.1.** Results are presented as % of cell viability in comparison to control.

5.3.4 Measurement of reactive oxygen species (ROS) and lipid peroxidation (LPO)

Intracellular ROS production was quantified by DCFDA and visualised using two fluorogenic probes; CellROX® Deep Red and MitoSOX™ Red. In all ROS experiments, 4 mM concentration of drugs was used with a range of time points: 0, 30, 60 and 120 minutes, in line with previous literature (Park, 2016).

DCFDA assay was performed as described in **section 2.2.3.2.** The dye was added to the cells and incubated for one hour at 37°C with 5% CO_2, before cells treated with APAP (4 mM), SSZ (4 mM), hydrogen peroxide (H_2O_2) at 1 mM (used as positive controls (Basu and Sur, 2018) or control (untreated cells) diluted in HBSS. In cell-free

wells, 100 µl of HBSS was added; this was known as background. The fluorescence intensity was measured using the FluoStar Omega fluorescent plate reader and recorded at 485 / 520 nm. From each fluorescence measurement, background fluorescence was subtracted. Each drug was replicated independently (n = 18 for each treatment) and results are expressed in relative fluorescence units (RFU). Cytoplasmic ROS formation was visualised using CellROX®, while mitochondrial ROS (superoxide [$O_2^{\bullet-}$]) was imaged using MitoSOX™, as described in **section 2.2.3.5**. Cells were treated with 4 mM APAP, 4 mM SSZ, 1 mM H_2O_S or control (untreated cells) diluted in HBSS. APAP and SSZ were imaged with EVOS microscope at 30, 60 and 120 minutes, while untreated cells and positive control were imaged at the 120 minutes endpoint.

Lipid peroxidation was visualised by using Image-iT® Lipid Peroxidation Kit according to the manufacturer's recommendations, as detailed in **section 2.2.3.5**. The probe labels reduced and oxidised cell membranes. Upon lipid oxidation, peak emission shifts from red to green (~590nm to ~510nm, respectively). HepG2 cells were treated with cumene hydroperoxide (at 100 µM diluted in PBS [2 mL / well]) as a positive control (provided with the kit), control (untreated cells), 4 mM APAP and 4 mM SSZ. APAP and SSZ were imaged with EVOS microscope at 30, 60 and 120 minutes, while untreated cells and positive control were imaged at the 120 minutes endpoint. Malondialdehyde (MDA) was determined using Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich) following the supplier instructions, as described in **2.2.3.3**. MDA levels were measured by the colorimetric change using a plate reader at 532 nm. The protein content of the samples was determined by using the Lowry assay (**section 2.2.3.10**) to standardise the data, results being presented as nmol MDA / mg protein (n = 3).

5.3.5 Mitochondrial membrane potential assessment using the fluorometric probe, JC-1

The mitochondrial membrane potential (Δ Ψ m) was assessed with JC-1, as detailed in **section 2.2.3.4**. Briefly, cells were loaded with 2 µM of JC-1 in each well of a 96- well plate (black plate with clear bottom) for 30 minutes, then treated with 4 mM APAP, 4 mM SSZ, 1 mM H_2O_2 or control (untreated cells) diluted in HBSS for 0, 30, 60 and 120 minutes. In cell-free wells, 100 µl of HBSS was added; this was known as background. From each fluorescence measurement, background fluorescence was subtracted. After drug treatment, fluorescence intensity was recorded with a fluorescence plate reader. JC-1 exists as a monomer and fluoresces green in mitochondria with low membrane potential. It exists as an aggregate and fluoresces red in mitochondria with a high membrane potential. Δ Ψ m was determined by the ratio of fluorescence intensity between 590 and 530 nm (red / green). Depolarised mitochondria are indicated by decreased Δ Ψ m. Results are presented as ratios of red to green fluorescence (n = 6).

5.3.6 Intracellular glutathione (GSH) levels

Intracellular GSH levels were estimated using a quantification kit according to the supplier's instructions, as described in **section 2.2.3.6**. HepG2 cells were treated with various concentrations of APAP (0, 0.06, 0.12, 0.25, 0.5, 1, 2 and 4 mM) and SSZ (0, 0.06, 0.12, 0.25, 0.5, 1, 2 and 4 mM) for 24 hours before scraping and lysis of the cells. The absorbance was read at 415 nm using a BMG Fluostar plate reader. Then GSH contents of the samples were detected and calculated, based on the standard curves by using the y = mx + c equation. Protein content of the samples was determined by

using the Lowry assay (**section 2.2.3.10**) to standardise the data. Results are presented as nmol GSH / mg protein (n = 3 each for drug).

5.3.7 DNA damage assessment: 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) staining

DNA damage was assessed using DAPI dye as described elsewhere with slight modifications (Chazotte, 2011) and detailed in **section 2.2.3.6.** HepG2 cells were treated with differing concentrations (0, 0.06, 0.12, 0.25, 0.5, 1, 2 and 4 mM) of APAP and SSZ, control (untreated cells) and positive control (1 mM H_2O_2) diluted in EBSS for 24 hours. Images were obtained using an EVOS microscope. We looked at nucleus condensation and nuclear bubbling as a feature of apoptosis (Thornberry and Lazebnik, 1998).

5.3.8 Assessment of membrane leakage as an indicator of apoptosis: lactate dehydrogenase (LDH) leakage

Activity of LDH in the medium was measured following the supplier instructions for the CyQUANT™ LDH Cytotoxicity Assay Kit. Cells were treated with 0, 0.06, 0.12, 0.25, 0.5, 1, 2 and 4 mM of APAP and SSZ for 24 hours. For maximum LDH activity, cells were treated with 100 µl of FBS free media, as per manufacturer's instructions. The total LDH activity was measured as detailed in **section 2.2.3.7.** Results are presented as % LDH leakage (n = 9 for each drug):

$$\frac{\text{LDH in medium}}{\text{Total LDH activity}} * 100$$

5.3.9 Assessment of caspase and poly-ADP ribose polymerase (PARP) involvements

To determine whether caspase plays a part in the cytotoxic response of cells to SSZ and APAP, HepG2 cells were pre-treated with caspase inhibitors, namely Z- DEVD-FMK (caspase 3 inhibitor) and Z- VAD-FMK (broad-range caspase inhibitor) one hour prior to drug treatment, as described elsewhere (Rose et al., 2003). Next, cells were treated with control (untreated cells), 4 mM APAP and 4 mM SSZ for 24 hours; cells were treated with the drugs without caspase inhibition for the purpose of comparison. Cell viability was assessed using crystal violet as described in **section 2.2.3.1**. Results are presented as % of cell viability in comparison to control (n = 12 for each drug).

The level of cleaved PARP was estimated following the manufacturer's instructions for the PARP Cleavage [214 / 215] Human ELISA Kit (Thermo Fisher Scientific). Prior to cell harvesting, HepG2 cells were treated with various concentrations of the drugs (0, 0.06, 0.12, 0.25, 0.5, 1, 2 and 4 mM) for 24 hours. ELISA cell extraction and PARP measurement are detailed in **section 2.2.3.9**. Protein content of the samples was determined by using the Lowry assay (**section 2.2.3.10**) to standardise the data. Results are expressed as cleaved PARP / mg protein (n = 2 for APAP and n = 3 for SSZ).

5.3.10 Western blot

For western blot analysis of caspases, HepG2 cells were treated with 0, 0.6, 0.12, 0.25, 0.5, 1, 2 and 4 mM of APAP and 0, 0.6, 0.12, 0.25, 0.5, 1, 2 and 4 mM of SSZ, separately for 24 hours before cells were scrapped to extract protein. The protocols outlined in **section 2.2.3.10** were followed for protein extraction, normalisation and western blotting. Then results were analysed using ImagJ (Stael et al., 2022). Results are presented as blots and their relative densitometry analyses (n = 3 for each drug).

5.3.11 Data analysis

Data are presented as mean ± standard error of mean (SEM), unless otherwise stated. Data were analysed using one-way ANOVA when comparing between three or more groups, followed by a post-hoc Tukey test, while independent t-tests were performed when comparing between two groups. **(*)** indicates $P < 0.05$ and **(**)** indicates $P < 0.01$ throughout the chapter.

5.4 Results

It should be acknowledged that some concentration- / time-dependent experiments in this chapter could not be performed, owing to the consequences of COVID-19 restrictions (e.g. lab access [two-hour working slot]).

5.4.1 APAP and SSZ promote cell death in HepG2

Initial experiments were carried out to separately determine the cytotoxicity impacts of both drugs on the HepG2 cell model using crystal violet assay.

As shown in **Figure 5.1**, both APAP and SSZ (0 – 4 mM) induced HepG2 cell viability reduction at 24 hours' treatment.

APAP induced significant cell viability reductions at 2 and 4 mM APAP for 24 hours in comparison to control, with viability reaching 68 ± 2.2 % and 25 ± 2.4 % (% Ctrl ± standard error of mean [SEM]) ($P < 0.01$), respectively. At lower concentrations (0.06 mM to 0.5 mM), APAP shows no toxicity. In fact, there is a slight proliferation detectable at these low concentrations, potentially due to a compensatory mechanism. On the other hand, SSZ showed concentration-dependent losses in HepG2 cell viability at 24 hours' treatment ($P = 0.002$). While the viability of cells treated with SSZ was significantly pronounced at ≥ 0.12 mM for 24 hours compared to control (untreated cells), viability reached 78 ± 1.9 %, 69 ± 2.4 %, 59 ± 1.7 %, 55 ± 1.5 %, 41 ± 1.0 % and 25 ± 0.43 % when treated with 0.12, 0.25, 0.5, 1, 2 and 4 mM SSZ (% Ctrl ± SEM) ($P < 0.01$) respectively. These data, considered together, showed that both APAP and SSZ can induce HepG2 cell death in our cell line. However, APAP requires higher concentrations than SSZ to induce significant cytotoxicity.

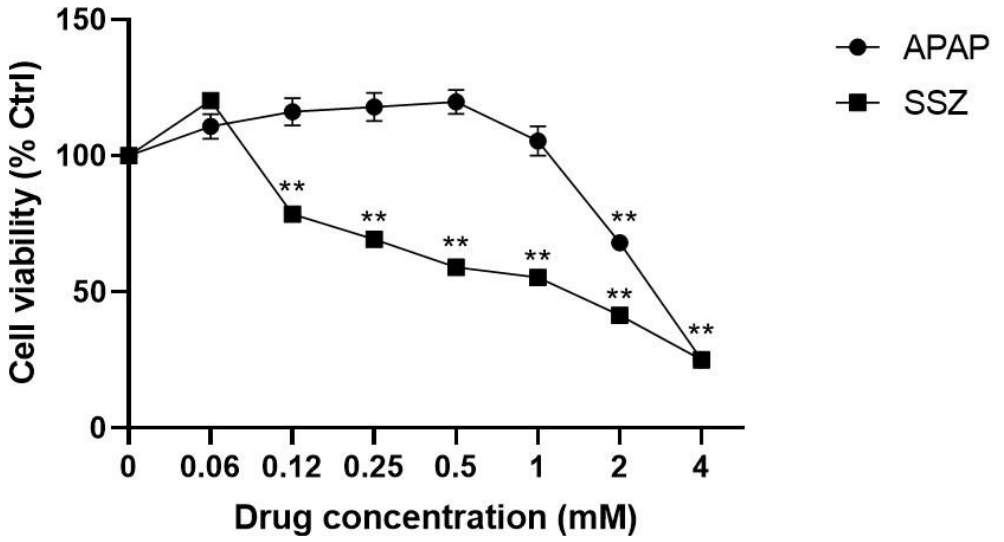


Figure 5.1. Concentration-dependent loss in HepG2 cell viability induced by APAP and SSZ, as determined by the crystal violet assay. Cells were treated with 0 (control < 0.1% DMSO) and the drugs at the indicated concentrations for 24 hours (n = 18 for APAP and n = 24 for SSZ). These replicates were performed independently on separate days using freshly prepared reagents. Data are expressed as the mean ± SEM. **(**)** *P* < 0.01.

5.4.2 Intracellular ROS production and lipid peroxidation

One key hallmark of apoptosis in mammalian cells is an early increase in intracellular ROS production. ROS formation is widely recognised as an early event in the apoptotic cascades induced by a range of cellular stressors, including drugs. To determine APAP- and SSZ-induced cell death *via* ROS generation, we used a range of methodologies, including DCFDA analysis, cell-ROS detection probes and lipid peroxidation. All of these studies were conducted for short-term exposure of the drugs (0, 30, 60 and 120 minutes) at 4 mM concentration. First, I conducted a pilot study to validate the DCFDA probe in our cell line by using 1 mM H_2O_2, a positive control. Significant elevation in ROS production was observed in a time-dependent manner (*P* < 0.001) **Figure 5.2 (A)**. This indicated that ROS generation occurred and can be

detected with the DCFDA probe. Following the validation step, HepG2 cells were treated with 4 mM of APAP and SSZ. Results show that both APAP and SSZ cause time-dependent ROS generation ($P = 0.023$ and $P = 0.034$, respectively). At each time point, treatment with 4 mM of either drug significantly elevated ROS production, compared to untreated control cells ($P < 0.01$).To confirm these findings, two additional ROS markers, CellROX® and MitoSOX™, were used to visualise free radical species generation. **Figure 5.2 (B**) shows cytoplasmic and mitochondrial ROS formation at 120 minutes post-drug treatment at 4 mM concentrations. The CellROX® probe indicated increased cytoplasmic ROS generation, as evidenced by greater red fluorescence in cells treated with 4 mM APAP or SSZ, compared to untreated cells. This effect was comparable to the positive control (1 mM H_2O_2). Similarly, the mitochondria-specific ROS detection marker MitoSOX™, which detects superoxide anion (·O2) production, showed higher mitochondrial superoxide levels in APAP- and SSZ-treated cells, compared to untreated cells at 120 minutes, as indicated by increased red fluorescence. Both cytoplasmic and mitochondrial ROS were detected in a time-dependent manner, correlating with the earlier DCFDA findings. Fluorescent images at 120 minutes are presented here; images for earlier time points (30 and 60 minutes) for both drugs can be found in **Appendix 5.1** (CellROX®) and **Appendix 5.2** (MitoSOX™). Together, these findings from this set of experiments suggest that the rapid increase in ROS can be a possible mechanism in APAP- and SSZ-induced death of HepG2 cells.

Moreover, ROS production often occurs in parallel with the occurrence of lipid peroxidation (LPO) to cell membranes, an event often occurring during apoptosis. As such, LPO was also assessed using the Image-iT® lipid peroxidation probe. Upon lipid oxidation, the peak emission shifts from red to green. Indeed, fluorescent images in

Figure 5.2 (B) showed that both APAP and SSZ (4 mM) increased LPO at 120 minutes of treatment, when compared to untreated cells as detected by increased green fluorescence. This fluorescence was comparable to the positive control, cumene hydroperoxide (100 µM). In fact, APAP and SSZ triggered LPO in a time-dependent manner that occurred in parallel with ROS production, *viz.* 30, 60 and 120 minutes (the earlier timepoints of LPO are presented in **Appendix 5.3**). Additionally, assessment of LPO was confirmed using quantitative methods by measuring malondialdehyde (MDA). MDA levels increase following lipid peroxidation and are a product of oxidised lipid degradation **(Figure 5.2 (C).** our results showed that both 4 mM APAP and SSZ significantly elevated MDA levels in a time-dependent manner ($P = 0.005$ and $P = 0.01$, respectively). For APAP treatment, MDA reached a maximum level at 120 minutes (6.8 ± 0.02 nmol / mg protein **vs** 1.2 ± 0.01 nmol / mg protein [mean ± SEM] for APAP **vs** control, respectively; $P = 0.003$). Similarly, when HepG2 cells were treated with 4 mM SSZ, MDA levels reached 5.2 ± 0.01 nmol / mg protein at 120 minutes compared to 1.2 ± 0.01 nmol / mg protein in control cells ([mean ± SEM]; P = 0.006). The pattern of LPO and MDA increase correlates with the ROS formation seen in the previous experiment, supporting the proposition that oxidative stress can be a potential mechanism of APAP toxicity.

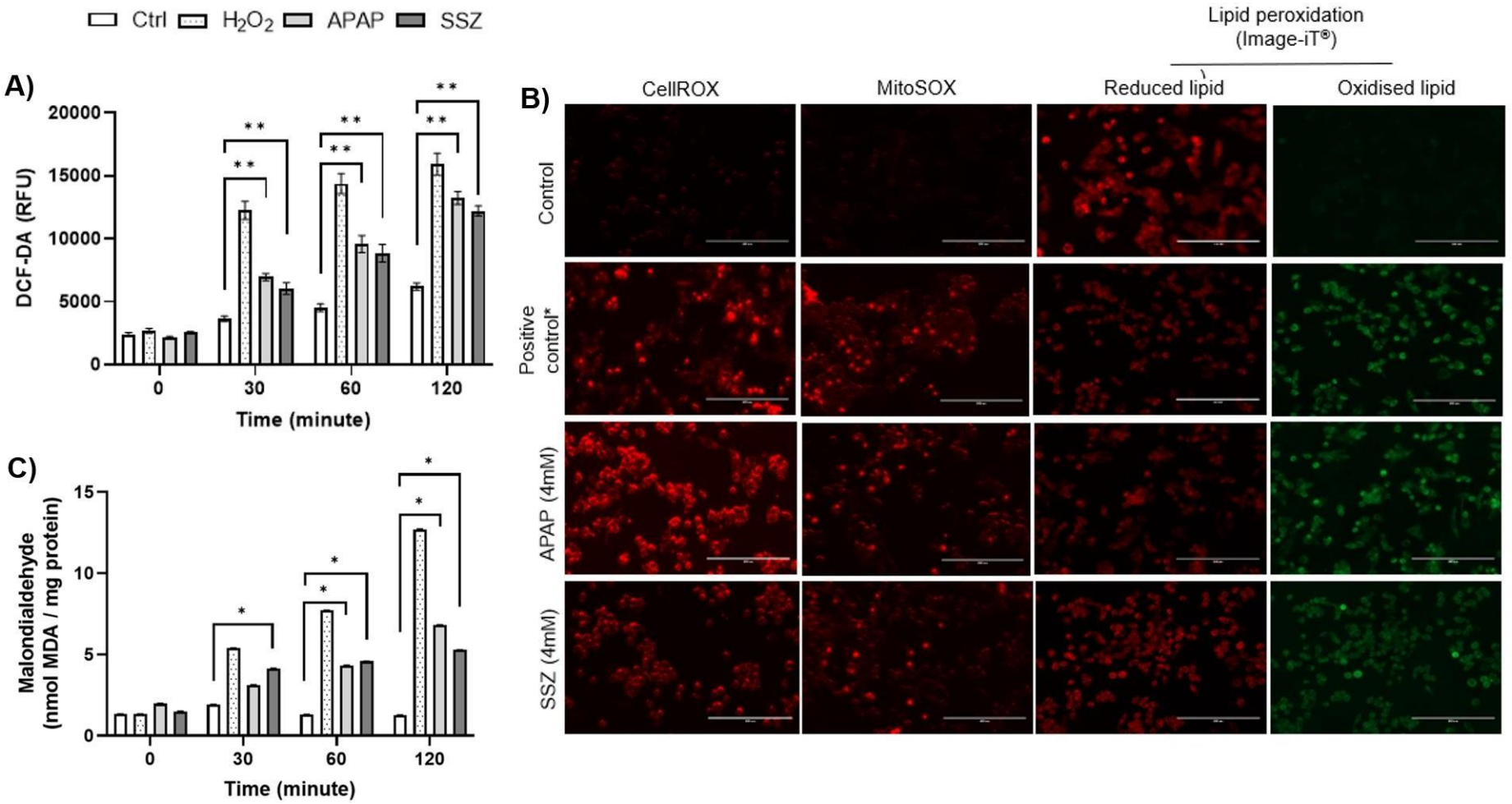


Figure 5.2 (A) ROS levels were measured at 0, 30, 60 and 120 minutes after exposure to 4 mM concentrations of the drugs by DCFDA staining. **(B)** Visualisation of cytoplasmic (CellROX), mitochondrial (MitoSOX™) ROS formation and lipid peroxidation (Image-iT®) in HepG2 cells at 120 minutes of treatment. The positive control for the CellROX and MitoSOX™ is 1 mM H_2O_2, while 100 µM of cumene hydroperoxide is the positive control for the lipid peroxidation experiment. **(C)** MDA levels were assessed at 0, 30, 60 and 120 minutes after exposure to 4 mM concentrations of the drugs using the Lipid Peroxidation (MDA) Assay Kit. **(*)** $P < 0.05$ and **(**)** $P < 0.01$ versus untreated cells. Data represent means ± SEM of independent experiments (n = 18 for DCFDA and n = 3 for MDA).

5.4.3 Mitochondrial membrane potential (Δ Ψ m)

Δ Ψ m is critical to the regulation of ATP production, mitochondrial function and overall mitochondrial health. Excessive ROS can damage the electron transport chain (ETC) components in the inner mitochondrial membrane. This damage, in turn, leads to a reduction in Δ Ψ m, significantly impacting cellular health. As such, we also assessed Δ Ψ m using the mitochondrial specific dye, JC-1 (**Figure 5.3**). A decrease in the red / green fluorescence intensity ratio of this fluorescent probe is indicative of mitochondrial depolarisation. This was confirmed when HepG2 cells were treated with 1 mM H_2O_2, a positive control, for 0, 30, 60, 120 minutes, and results showed time- dependent reduction in Δ Ψ m ($P < 0.001$).

Following this validation pilot study, we assessed the impact of APAP and SSZ at 4 mM on Δ Ψ m in HepG2 cells for various time points (0, 30, 60 and 120 minutes) (**Figure 5.3)**. Results showed that both drugs enhanced loss of mitochondrial membrane potential in a time-dependent manner ($P < 0.01$). At each time point, there are significant losses, compared to the respective controls ($P < 0.05$ for 30 minutes time point and $P < 0.01$ for 60 and 120 minutes), suggesting mitochondrial depolarisation is a critical process in cell death. These results are correlated with the other oxidative markers measured earlier, *viz.* ROS and LPO.

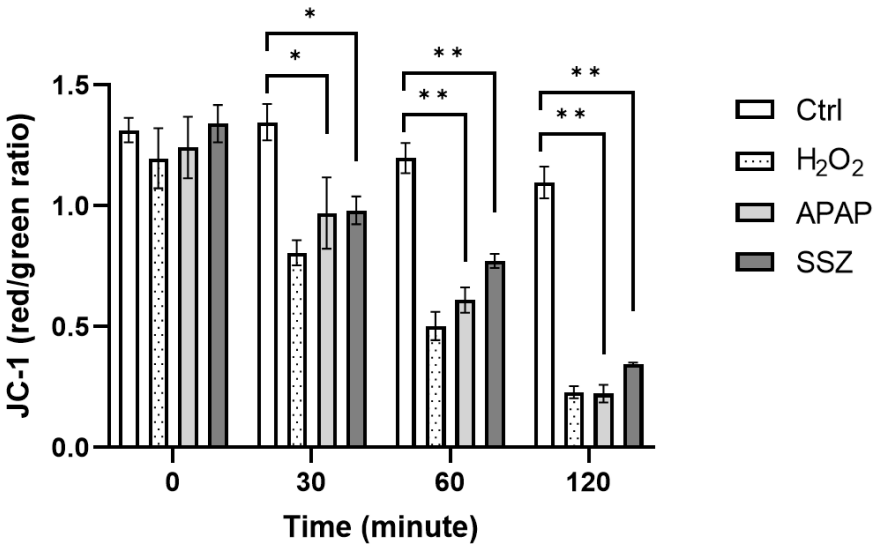


Figure 5.3 Mitochondrial membrane potential was assessed at 0, 30, 60 and 120 minutes after exposure to 4 mM concentrations of the drugs by JC-1 staining. 1 mM H_2O_2 represents a positive control. **(*)** $P < 0.05$ and **(**)** $P < 0.01$ versus untreated cells. Data represent means ± SEM of independent experiments (n = 6).

5.4.4 Glutathione levels

After examining the initial indicators of oxidative stress, I proceeded to evaluate the levels of intracellular glutathione using a commercially-available kit (**Figure 5.4**). This assessment was conducted to help elucidate the molecular mechanisms through which APAP and SSZ were inducing cell death.

Results showed that intracellular GSH levels decreased significantly in a concentration-dependent manner at 24 hours when cells were treated with differing concentration ranges of APAP and SSZ treatment ($P < 0.001$). For both drugs, all the indicated concentrations (**Figure 5.4**) significantly reduced intracellular GSH compared to controls (untreated cells) ($P \leq 0.001$), even at the lowest concentration (0.06 mM). GSH levels reached the lowest at 2 mM APAP concentration in comparison

to control (11.05 ± 0.05 nmol GSH / mg protein **vs** 33.19 ± 0.09 nmol GSH / mg protein (mean ± SEM) APAP **vs** control, respectively, $P < 0.001$); while GSH levels reached the lowest at 1 mM SSZ in contrast to control (16.81 ± 0.41 nmol GSH / mg protein **vs** 35.47 ± 3. 49 nmol GSH / mg protein (mean ± SEM) SSZ **vs** control, respectively, $P < 0.001$). Notably, when cells were seeded according to the manufacturer's recommendation (**section 2.2.3.6)** and treated with 4 mM APAP or 2 mM and 4 mM SSZ, there was insufficient protein for analysis, potentially due to the severe cell death at these concentrations at 24 hours' treatment (≈ ≤ 40 % cell viability). Nevertheless, GSH depletion over 24 hours may contribute to the observed cell viability decrease induced by SSZ. The significant decrease in cell viability at higher APAP concentrations (2 and 4 mM) correlates with the severe GSH depletion at these concentrations. However, APAP at ≤ 1 mM concentrations failed to induce cell viability reduction, despite causing significant GSH depletion. This observation suggests that APAP toxicity induces GSH depletion even at low concentrations, indicating that GSH reduction is likely one of the key mechanisms in APAP-induced cytotoxicity, however not the only one; this aligns with the established understanding of APAP metabolism, where GSH detoxifies the reactive metabolite NAPQI.

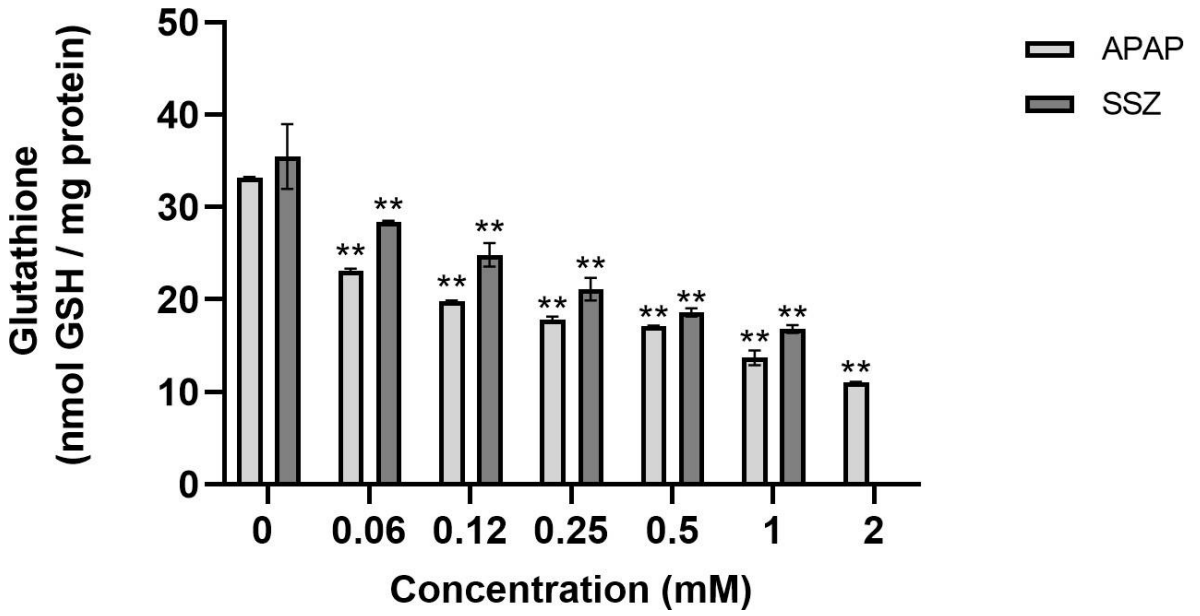


Figure 5.4 Intracellular GSH levels measured in HepG2 cells treated with the indicative concentrations of the drugs for 24 hours using a colorimetric assay. **(**)** *P* < 0.01 versus respective controls (zero concentrations). As for SSZ at 2 mM, not enough protein was detected at this concentration. Data represent means ± SEM of three independent experiments (n = 3).

5.4.5 APAP and SSZ induced apoptosis in HepG2 cells

In this study, apoptosis was assessed using LDH leakage, DNA damages using DAPI nuclear staining, caspase inhibitor, PARP cleavage and protein expression of caspases using western blot analysis.

5.4.5.1 Lactate dehydrogenase (LDH) leakage and DAPI assessment

LDH is located in the cytoplasm and, on plasma membrane damage, LDH is released into the extracellular medium. LDH is widely used as a marker of cell viability and membrane integrity. In this part of the study the LDH assay was performed to determine whether individual drugs impacted on cell membrane integrity. As shown in **Figure 5.5,** LDH leakage at 24 hours was determined with various drugs concentrations (0 - 4 mM). Results showed significantly-induced extracellular LDH at 4 mM APAP, compared to the control (untreated cells), 27.3 ± 0.95 % **vs** 6.6 ± 0.42 %,

respectively (% of leakage ± SEM) ($P < 0.002$). Lower concentrations of APAP failed to induce LDH release at 24 hours' treatment. This LDH leakage data correlates with the cell viability results, showing significant effects primarily at higher APAP concentrations, indicative of later-stage apoptosis. At lower APAP concentrations (≤ 2 mM), cells appear to maintain membrane integrity, despite experiencing some level of stress, as evidenced by GSH depletion. Moreover, a concentration-dependent increase in LDH release was noted for SSZ at the same time point, 24 hours ($P < 0.01$). Indeed, SSZ treatment significantly elevated LDH leakage at concentrations of 0.25, 0.5, 1, 2 and 4 mM (11.6 ± 0.64 %, 18.2 ± 0.39 %, 27.1 ± 0.56 %, 34.4 ± 0.1.4 %, 43.2 ± 1.3 %, ($P < 0.01$, respectively). LDH leakage becomes significant at higher concentrations than GSH depletion, suggesting GSH depletion precedes membrane damage.

We then looked at DNA damage and nucleus condensation as another feature of apoptosis using DAPI staining. **Figure 5.5** showed that H_2O_2 (positive control) increased the proportion of condensed nuclei, as evident from increased blue fluorescence. Following this validation, HepG2 cells treated with 4 mM APAP and SSZ for 24 hours and fluorescent images showed bright nuclei. This suggests that DNA damage is another important mechanism, alongside GSH depletion and membrane damage, in the overall toxicity profile of APAP and SSZ in these cells, which can lead to apoptosis. Additionally, the overall fluorescence in the nuclei of APAP- and SSZ treated-cells was noticed to increase in a concentration-dependent manner at 24 hours (**Appendix 5.4**).

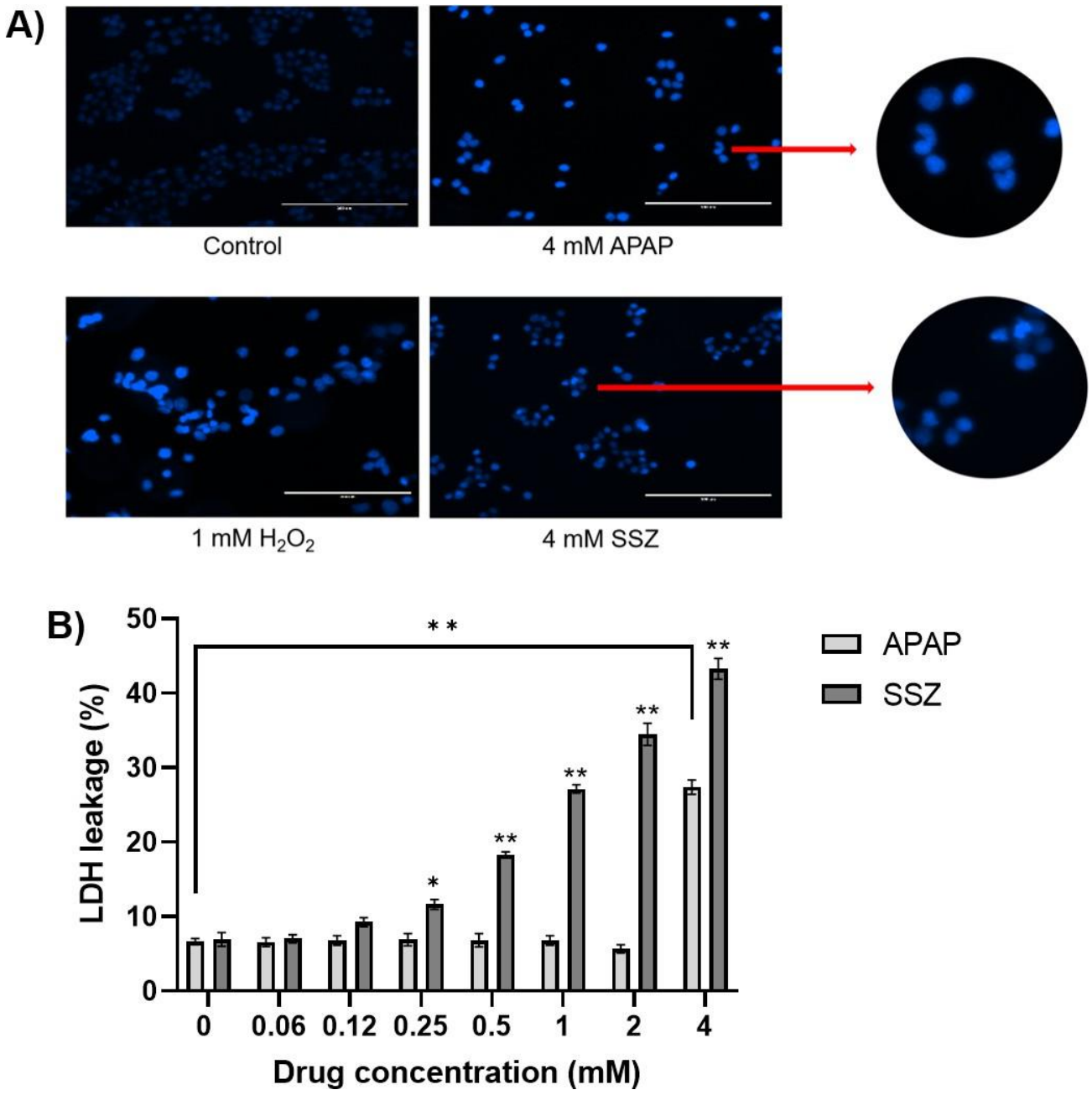


Figure 5.5 (A) DNA damage and nuclear changes stained by DAPI in HepG2 cells at 24 hours' treatment with the drugs at the indicative concentrations. Arrows indicate apoptotic bodies of nuclear condensation observed under EVOS microscope **(B)** LDH leakage (%) at 24 hours of drug treatment at the indicated concentrations. Data represent as % of means ± SEM of independent experiments (n = 9). **(*)** $P < 0.05$ and **(**)** $P < 0.01$ versus respective zero concentrations.

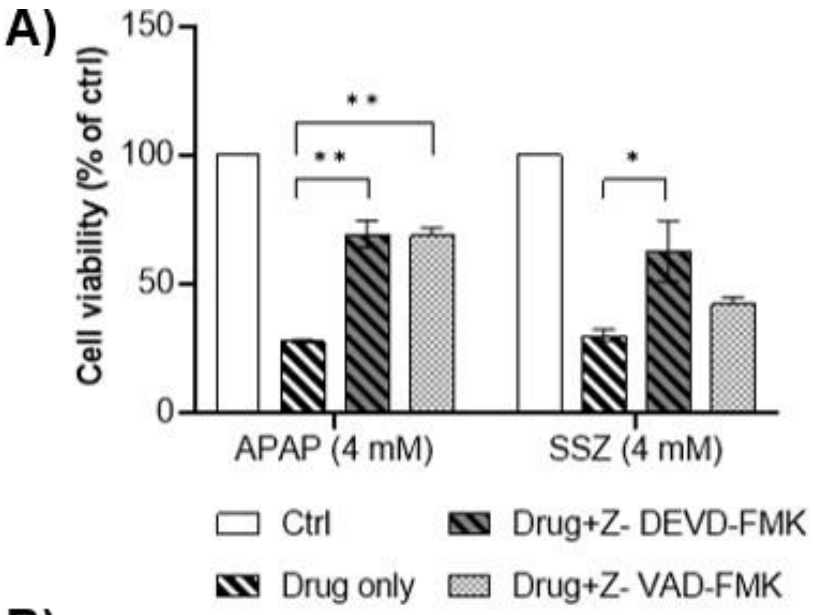
5.4.5.2 Involvement of caspases in APAP- and SSZ- mediated apoptosis

To explore the role of caspases in cell death following exposure to APAP and SSZ treatment, we assessed cell viability after caspase inhibition (**Figure 5.6 (A)**). Two inhibitors were used in this study: Z-DEVD-FMK (a caspase-3 inhibitor) and Z-VAD-FMK (a broad-range caspase inhibitor), applied one hour prior to treatment with 4 mM APAP and SSZ for 24 hours. Inhibition of caspases with either Z-DEVD-FMK or Z-VAD-FMK resulted in an approximately 41% increase in cell viability after APAP treatment ($P < 0.01$), compared to the APAP-only group. Additionally, Z-DEVD-FMK significantly rescued cells treated with 4 mM SSZ more effectively than did Z-VAD-FMK, with increases in cell viability of 33 % and 12 % respectively ($P < 0.006$ and $P = 0.3$), compared to SSZ-only groups. These data suggest that caspases are involved in the apoptotic cascade of the drugs and potentially more for caspase-3 regarding SSZ. Given that caspase inhibition could not completely protect the cells, this suggests that caspases participated in cell death but not as the only mechanism. It would be interesting to assess the impact of caspase inhibitions on concentration ranges of drugs; however, we were not able to assess that because of time constraints.

We then assessed poly-ADP ribose polymerase (PARP) cleavage, a signature of apoptosis, using an ELISA kit. **Figure 5.6 (B)** shows the level of PARP cleavage in HepG2 cells treated with different concentrations of the drugs for 24 hours. We have not detected cleaved PARP at low concentrations of the drugs (< 1 mM). However, the elevation in cleaved PARP level was noted as significant when HepG2 treated with 2 mM APAP and 1 mM SSZ accounted for 7.65 cleaved PARP / mg protein and 6.85 cleaved PARP/ mg protein, compared to 0.44 cleaved PARP/ mg protein of untreated cells ($P = 0.002$ and $P = 0.003$, respectively); this result being reflective of the potential role of PARP cleavage in the apoptotic cascade.

Moreover, to confirm caspase involvement in drug-induced apoptosis in our HepG2 cell line, we conducted a western blot analysis to assess the pan-caspase proteins for caspase 3 and 8. HepG2 cells were treated with differing concentrations of the drugs for 24 hours. Results showed that, although there is a detectable trend of reduction in caspase-3 for both drugs, it is not, however, significant ($P > 0.05$). These negative results might indicate that a full-time course analysis of cells treated with the drugs is required. Unfortunately, time restrictions precluded this series of experiments.

Figure 5.9 illustrates the potential mechanisms of oxidative stress markers and cell death induced by APAP and SSZ, based on our findings.



B)

	Mean ± SEM of cleaved PARP / mg protein	
Concentration (mM)	**APAP**	**SSZ**
0	0.44 ± 0.08	0.44 ± 0.02
0.06	0.45 ± 0.02	0.49 ± 0.03
0.12	0.50 ± 0.13	0.51 ± 0.07
0.25	0.55 ± 0.05	0.78 ± 0.15
0.5	0.71 ± 0.16	0.79 ± 0.06
1	0.80 ± 0.06	6.85 ± 0.23**
2	7.65 ± 0.19**	N/A
n	**2**	**3**

Figure 5.6 (A) HepG2 cells were partially rescued by caspase inhibitors; Z- DEVD-FMK and Z- VAD-FMK, as determined by the crystal violet assay. **(*)** $P < 0.05$ **and (**)** $P < 0.01$ versus drug only group. Data represent means ± SEM of independent experiments (n = 12) **(B)** PARP cleavage was detected at 24 hours of different concentration of the drugs. Data represent means ± SEM **(**)** $P < 0.01$ versus respective zero concentrations. **(N/A)** not enough protein was detected at this concentration

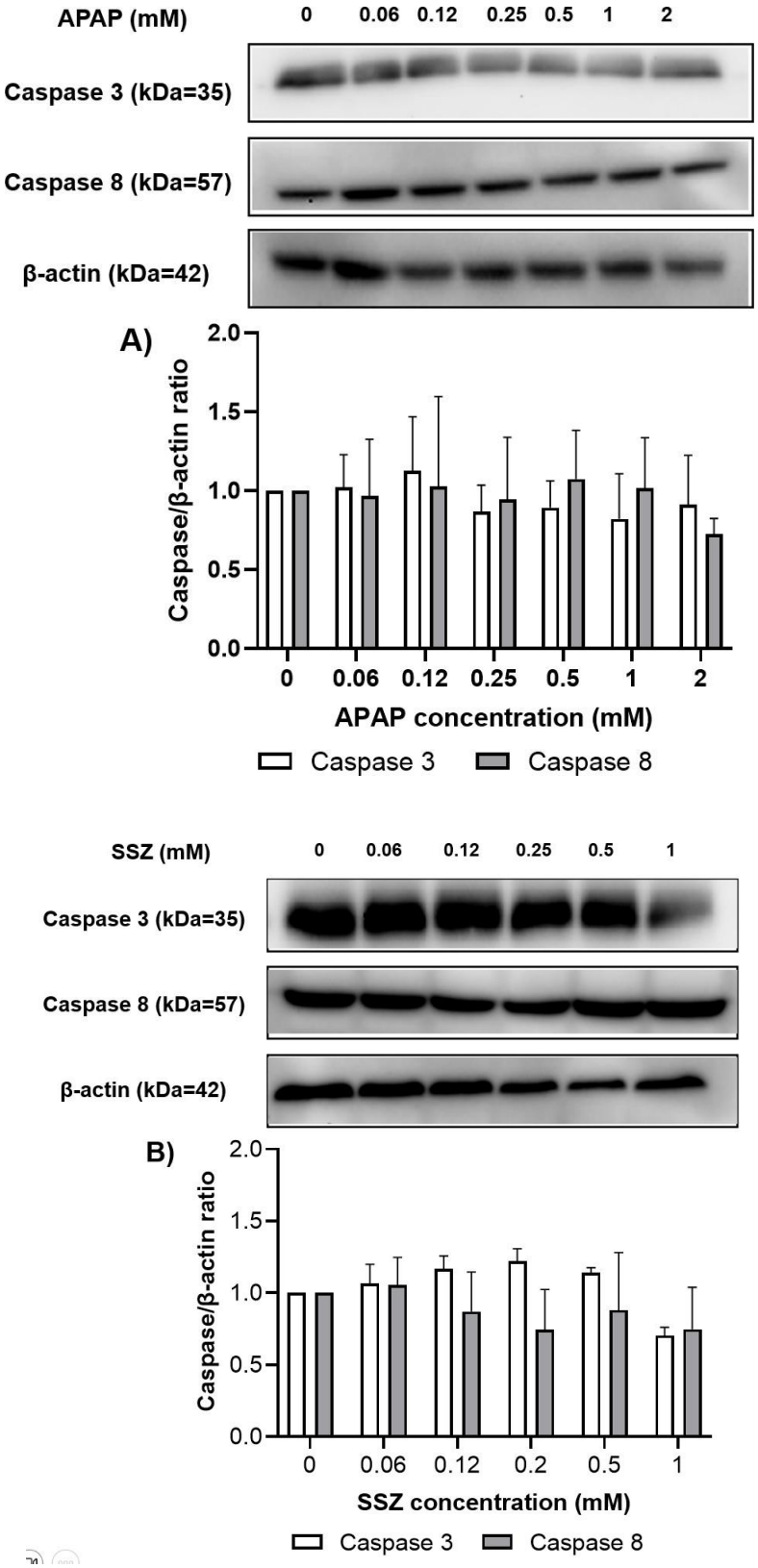


Figure 5.7 Western blot analysis and its densitometry of caspase 3 and caspase 8 expression of in HepG2 cells treated with the indicative concentrations of **(A)** APAP and **(B)** SZZ for 24 hours. Western blots were normalised to actin. Data expressed as mean ± standard deviation (n = 3).

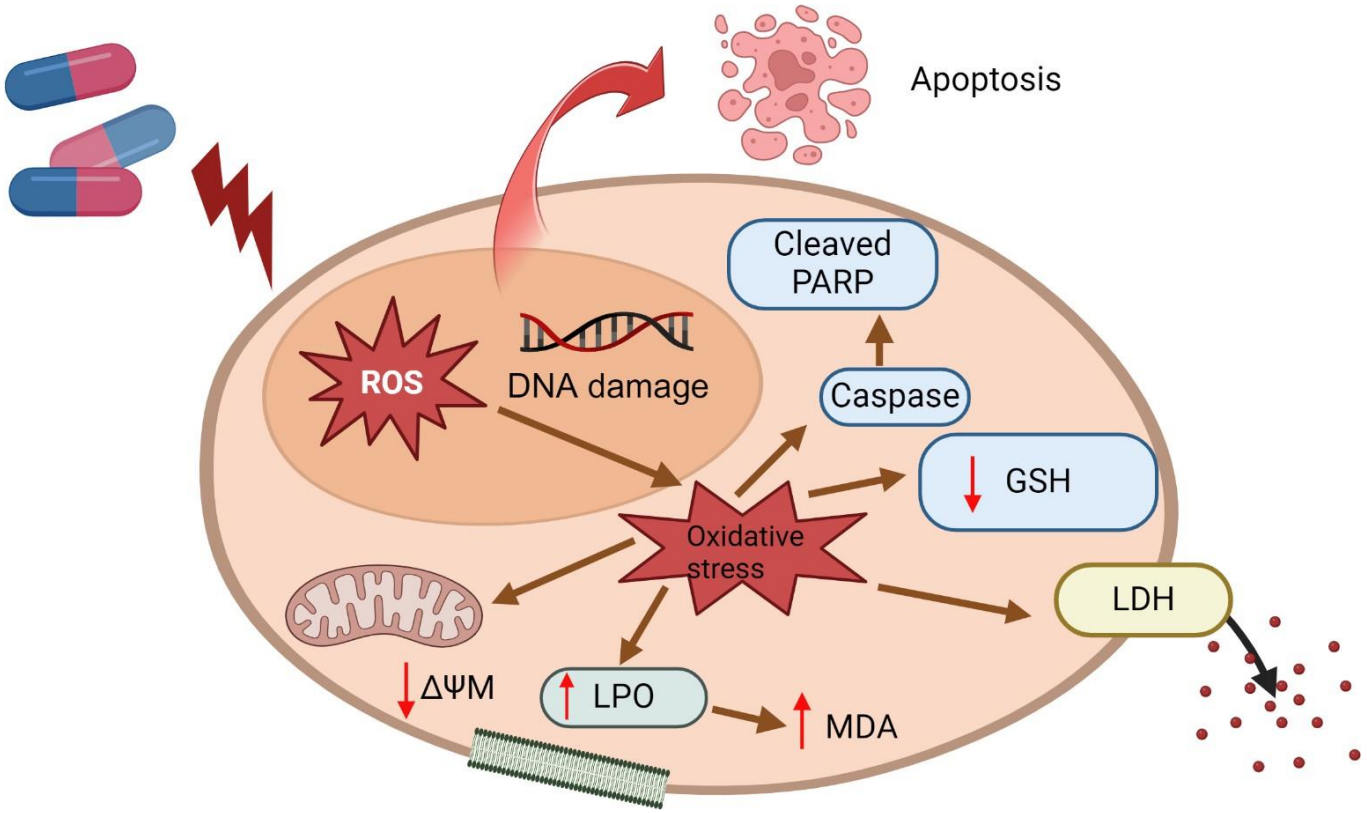


Figure 5.8 Model for APAP- and SSZ-induced oxidative stress and apoptosis in HepG2 cell line. Based on our results, this figure shows the increase in oxidative stress which causes ROS formation, LPO, mitochondrial dysfunction, LDH leakage, GSH reduction, partial caspase involvement, PARP cleavage and eventually cell death. **Abbreviations: ROS**, reactive oxygen species; **GSH**, glutathione; **LPO**, lipid peroxidation, **MDA**, malondialdehyde, **Δ Ψ m**, mitochondrial membrane potential , **LDH**, Lactate dehydrogenase, **PARP**, poly-ADP ribose polymerase (illustration created with BioRender.com).

5.5 Discussion

The liver functions as the main organ for detoxification and is a vital site for the TSP. In this study, we validated and developed a human hepatoma HepG2 cellular model in our laboratory to assess oxidative stress and apoptosis of two commonly-used medications, namely paracetamol (APAP) and sulphasalazine (SSZ). HepG2 cells derived from human hepatocellular carcinoma are now an important tool for pharmaceutical research and drug development (Blidisel et al., 2021). In fact, their use in pharmacological and toxicological studies has been documented since the 1970s (Deng et al., 2019). These cells offer a reliable *in vitro* model for assessing medication toxicity, effectiveness and metabolism (Qiu et al., 2015). APAP is a widely-known analgesic used for alleviating pain, while SSZ is an anti-inflammatory drug used in the management of inflammatory bowel diseases. Both drugs affect thiol status related to the TSP. APAP alters glutathione levels (Jannuzzi, Kara and Alpertunga, 2018), while SSZ influences the cystine transporter (X_c^-) (Shukla et al., 2011). These effects on cellular redox balance make these drugs valuable tools for the current investigation.

Chronic oxidative stress may accelerate the development or progression of long-term diseases like neurodegenerative disorders (Solleiro-Villavicencio and Rivas-Arancibia, 2018); diabetes (Asmat, Abad and Ismail, 2016); cardiovascular diseases (Dubois-Deruy et al., 2020); and ageing-related diseases (Liguori et al., 2018). Thiols, particularly GSH, are major cellular antioxidants; dysregulation in thiol homeostasis plays a role in the pathology of several diseases, such as chronic inflammatory disease (Sandikci et al., 2019); cancer(s) (Hizal et al., 2018; Eryilmaz et al., 2019); and sleep aponia syndrome (Dikis et al., 2019). Several pharmacological drugs are implicated in inducing oxidative stress (Hickey et al., 2001; Sawyer et al., 2010; Deavall et al., 2012; Guillouzo and Guguen-Guillouzo, 2020; Li et al., 2020; Salimi et

al., 2020; Ahmed et al., 2023; Umemura, 2023), and this can be important mechanism behind certain drugs' adverse effects and toxicities (Deavall et al., 2012).

In **chapter 3** and **4**, we have shown the impact of commonly-used medications on some of the TSP metabolites and their cofactor, vitamin B_6, in humans. In this chapter, we aim to gain a more detailed understanding of the impact of medications (using APAP and SSZ as models) at the cellular level, using an *in vitro* approach. This study will help us, firstly, validate the HepG2 cell line and, secondly, explore and characterise the molecular mechanisms of drug-induced apoptosis in our cell line. Additionally, it will aid in evaluating the impact of medications on some TSP metabolites / enzymes, using this model, in the next chapter.

As hypothesised, we have shown that APAP and SSZ induce cytotoxicity in HepG2 via the induction of oxidative stress and apoptosis. We confirm the likelihood that ROS formation, LPO, mitochondria depolarisation, GSH depletion, LDH leakage, DNA damage, caspase-3 and / or other caspase involvement and PARP cleavage are involved in the response of HepG2 cells to these drugs. Both APAP and SSZ induced cell death in HepG2 cells, with APAP requiring higher concentrations than SSZ to achieve significant cytotoxicity. Our findings are consistent with previous studies on the effects of APAP and SSZ on HepG2 cells. Specifically, SSZ at 250 µM reduced HepG2 cell viability to approximately 40% (Kim, Abdullah and Lee, 2019), while treatment with 20 mM APAP resulted in cell viability of 30% (Badr et al., 2016) and 55% at 8 mM APAP (González et al., 2017). These findings underscore the higher cytotoxic potency of SSZ compared to APAP in HepG2 cells. Low concentrations of APAP caused a slight proliferation in our cell line. This observation is interesting and in line with Behrends et al. (2019), who found that, while APAP induces cytotoxicity in HepG2 cells, a slight proliferation can be detected at low concentrations (< 2 mM).

The researchers suggest that APAP at these low concentrations may enhance glycolysis, leading to increased energy production that could support cell growth. Interestingly, a notable discrepancy exists between our findings and those of González et al. (2017). Using the same 4 mM APAP concentration and 24-hour treatment period, they reported 75 % HepG2 cell viability, while we observed only 25 % viability. This substantial difference likely stems from the different assay methods employed: González et al. (2017) used the MTT assay, which measures metabolic activity, while our study utilised the crystal violet assay, which quantifies cell adherence.

In our work, cytotoxicity and cell death were associated with several early markers of oxidative stress, including ROS formation, lipid peroxidation and mitochondrial depolarisation. Numerous biological components are directly attacked and oxidised by ROS, which can cause cellular damage. A critical part of the intricate relationship between oxidative stress and various diseases is overproduction of ROS (Chatterjee, 2016). Our findings demonstrate that both APAP and SSZ induce significant ROS production in HepG2 cells. These results align with previous research. Parikh, Pandita and Khanna (2015) reported a 30-fold increase in DCFDA fluorescence, indicating ROS formation, in HepG2 cells treated with 20 mM APAP, compared to untreated cells. Sendo et al. (2022) observed that 400 µM SSZ increased DCFDA fluorescence by ≈ 2.7- fold, compared to untreated cells in a uterine serous carcinoma cell line, mediating cell death. In our study, the highest DCFDA increases detected were 2.1-fold and 1.9-fold when HepG2 cells were treated with 4 mM APAP and SSZ, respectively. These results are comparable to Sendo et al. (2022). The differences in DCFDA increases across studies can potentially be explained by variations in drug concentrations and cell lines used. Moreover, in line with our observation, Wang et al. (2021) observed that 2 mM SSZ elevated in MDA levels in HepG2 cell line, a LPO product. Our study

found higher LPO as detected by MDA levels (≈ 6 nmol / mg protein) with 4 mM APAP at 120 minutes, versus Zhou et al.'s (2023) lower levels (≈ 4 nmol / mg protein) with 10 mM APAP at 24 hours. This difference likely reflects peak oxidative stress in our shorter time point (Katikaneni et al., 2020), while their longer exposure may show cell death of the most damaged cells. Wu et al. (2022) and Liptay et al. (2002) observed mitochondria depolarisation in HepG2 cells treated with 20 mM APAP and in Jurkat T-lymphocytes with 2 Mm SSZ, respectively. Mechanistic insights from the studies above, together with our work, suggest that ROS, LPO and mitochondria depolarisation can be contributors to cell death. These findings highlight the critical role of understanding drug cytotoxicity, which is a key contributor to drug-induced liver injury.

Glutathione (GSH), a metabolite of the TSP, is a key cellular thiol that serves as a primary antioxidant in mammalian cells. It plays a crucial role in maintaining redox balance across various organelles and is integral to cellular signalling processes (Franco and Cidlowski 2009). Both APAP and SSZ were seen to significantly reduce intracellular GSH levels, even at the lowest concentration used in the study. While the extent of GSH depletion observed in APAP-treated cells at low concentrations did not lead to a reduction in cell viability, it can make the cells more susceptible to additional oxidative stress (Dimova et al., 2005), including DNA damage, consistent with previous literature (Palabiyik et al. 2016). In fact, this phenomenon mirrored a human-based study where GSH deficiency intensified other oxidative stress (i.e. inflammation) among patients with cystic fibrosis (Dickerhof et al., 2017). Our observation suggests that APAP toxicity induces GSH depletion even at low concentrations in our cell line, indicating that GSH reduction is likely one of the key mechanisms in APAP-induced cytotoxicity, alongside ROS, LPO and mitochondria depolarisation. Significant

reductions in GSH induced by APAP were in line with previous work in HepG2 cells (Bai and Cederbaum, 2004; Palabiyik et al. 2016; Jannuzzi, Kara and Alpertunga, 2018; Zhou et al. 2023), primary rats' hepatocytes (Zhou et al., 2015) and rat embryonic liver cells (RLC-18) (Bader et al., 2011). Our finding also highlights significant concerns for vulnerable populations. Elderly people often have reduced GSH levels (Lang et al., 1992) and are at increased risk of adverse effects. This susceptibility is further compounded by the common practice of polypharmacy in this population. For example, recent evidence from *in vitro* study showed that SSZ in combination with cisplatin, an anti-cancer drug, was seen to aggravate cell viability reduction, ROS formation and PARP leakage in uterine serous carcinoma (USC) cells, compared to either SSZ or cisplatin alone (Sendo et al., 2022).

Moreover, HepG2 cells express the xCT cystine / glutamate antiporter system (Liu, Xia and Huang, 2020). Our observation confirmed that SSZ reduced GSH in a concentration-dependent manner in HepG2 cells, aligning with the well-known SSZ mechanism that can deplete GSH through its effect on cellular transport systems. SSZ blocks the cystine / glutamate transporter (system x_c^-). Normally, this transporter takes up cystine and simultaneously pumps glutamate out of the cells (Bridges, Natale and Patel, 2012.). Inside the cell, cystine is broken down into cysteine, its reduced form. By blocking this transporter, SSZ leads to cysteine starvation in the cell, ultimately resulting in GSH depletion (Gout et al., 2001). Our finding is in line with previous work that showed significant reduction in GSH upon SSZ treatment; at 400 µM SSZ (Sendo et al., 2022) in a uterine serous carcinoma cell line; at 0.5 mM in breast cancer cells (Narang, et al., 2007); and at 1.5 mM in TFK-1 cells (Zheng et al., 2021).

Later-stage apoptosis, characterised by LDH leakage, DNA damage and cleaved PARP, along with the partial involvement of caspase-3, was observed in the apoptotic

cascade induced by APAP and sulphasalazine SSZ in our cell line. These later events mirror previous experiments using APAP or SSZ in HepG2 cells (Palabiyik et al., 2016; Choi et al., 2017; Kim, Choi, and Nam, 2020); human thyroid cancer cell lines (Zou et al., 2019); and myocytes (Sykes et al., 2015). We and others have reported that APAP cytotoxicity in human hepatoma cells is partially caspase-dependent (Macanas-Pirard et al., 2005). The incomplete protection observed suggests the involvement of caspase-independent mechanisms, possibly including necrosis, especially at higher concentrations. This finding warrants further investigation. Pre-treatment with the Z-VAD-FMK inhibitor did not significantly rescue SSZ-induced cytotoxicity in our cells, consistent with the literature (Shamaa, 2021; Kim, Abdullah and Lee, 2019). Notably, our results indicate that caspase-3 plays a more prominent role in the apoptotic cascade induced by SSZ compared to other pan-caspases, consistent with Liptay et al. (2002) and supporting the notion that specific caspases may have distinct functions (Green, 2022). Caspase-mediated apoptosis involves the cleavage of crucial proteins for cell function, with PARP being a key substrate. PARP plays a crucial role in DNA repair and cellular stress responses (Chaitanya, Alexander and Babu, 2010). Numerous neurological conditions, including cerebral ischemia (Chaitanya and Babu, 2008) and Parkinson's disease (Kanthasamy et al., 2006), have been linked to the cleavage of PARP by caspase-3. Our results showed that the cytotoxicity of both APAP and SSZ was partially alleviated with caspase-3 inhibition, while significant cleaved PARP was observed at the highest concentrations. This suggests that while caspase-3 is involved in the apoptotic process, other mechanisms may also contribute to the overall cytotoxic effects of these drugs, as explained earlier. Additional research into the specific caspase-mediated pathways activated by various hepatotoxic drugs is necessary. Understanding these mechanisms could offer significant insights into the

molecular foundations of drug-induced liver injury and may aid in the development of targeted therapeutic interventions.

We have demonstrated that APAP and SSZ induce HepG2 cell cytotoxicity through dysregulations in cell cycle and various cellular processes, including ROS generation, lipid peroxidation, mitochondrial depolarisation, GSH depletion, LDH leakage, DNA damage, caspase-3 and/or other caspase involvement, and PARP cleavage. These findings are particularly concerning for long-term medication users, such as the elderly, who often manage multiple chronic conditions through polypharmacy. Screening for GSH among medication users could impact the progression of certain conditions at the earliest stages of disease development, potentially having a substantial impact on population-wide health and longevity. Our data not only expand the current understanding of APAP and SSZ-induced cytotoxicity, but could potentially be extended to the context of vitamin B_6 inadequacy, as explored in **chapter 4**, where we found that analgesics and many other medications were associated with low plasma pyridoxal 5'-phosphate (PLP), the active form of vitamin B_6. This connection is particularly significant given that vitamin B_6, in the form of PLP, serves as a crucial cofactor for enzymes in the TSP. The reduction in both GSH and the cofactor of TSP (PLP) can lead to a compounded effect on the TSP flux overall, potentially leading to increased risk of chronic diseases.

Conclusion

This study showed the susceptibility from APPA and SSZ of a HepG2 cell line to cell death and apoptosis. We confirm the possibility that the response of HepG2 cells to these drugs includes early oxidative markers which are generative of ROS, lipid peroxidation and mitochondrial depolarisation, ultimately leading to reduced cell viability. Further mechanistic investigations revealed concentration-dependent

glutathione depletion for both drugs. Notably, APAP at ≤ 1 mM concentrations failed to reduce cell viability despite causing significant GSH depletion. This GSH reduction can make the cells more susceptible to additional oxidative stress and DNA damage, indicating that GSH depletion is likely one of the key mechanisms in APAP-induced cytotoxicity. Later-stage markers of oxidative damage, including LDH leakage, DNA damage, caspase-3 and/or other caspase involvement, and PARP cleavage, contributed to the mechanisms of APAP- and SSZ-induced cell death. However, our study suggests that HepG2 cell death is not solely dependent on these mechanisms, and other caspase-independent pathways may also be involved. It is evident that drug-induced oxidative stress is a significant problem that might impede the optimal use of several important medications. In the next chapter, we will explore whether APAP and SSZ affect specific TSP metabolites and enzymes, further elucidating the complex mechanisms of drug-induced liver injury and their potential systemic effects.

Chapter 6 Hydrogen sulphide and polysulphide supplementations protect against paracetamol- and sulphasalazine-induced oxidative stress damages in HepG2 cells

6.1 Abstract

Within mammalian systems, hydrogen sulphide (H_2S) serves as a signalling molecule, regulating numerous physiological processes. Recent data suggest that polysulphides (H_2S_n, n > 2) may be responsible for many effects previously attributed to H_2S. Both H_2S and H_2S_n are involved in cellular redox regulation and are implicated in the function of multiple organ systems, such as the cardiovascular and gastrointestinal systems. Their levels can be influenced by various factors, including genetic modifications and diet. However, little is known about the impact of commonly used medications on H_2S and H_2S_n levels. Therefore, we assessed the impact of two commonly used medications namely paracetamol (APAP) and sulphasalazine (SSZ) on H_2S and H_2S_n levels in HepG2 cells using 7-azido-4-methylcoumarin (AzMC) and Sulfane Sulfur Probe 4 (SSP4) probes, respectively. We also evaluated the impact of H_2S donors (NaHS) and H_2S_n donor (Na_2S_2) against oxidative stress markers induced by APAP and SSZ in HepG2 cells. Results showed that APAP can indirectly affect H_2S production in HepG2 cells by significantly reduce the protein expressions of CBS ($P < 0.01$) and CSE ($P = 0.023$), however, no significant direct reduction in H_2S was noticed upon short time APAP exposure (up to 4 hours). SSZ showed interesting findings as CSE expression was significantly elevated when cells were treated for 24 hours with low concentrations (0.12 mM and 0.25 mM; ($P = 0.034$, $P = 0.049$), respectively). At higher concentrations, CSE expression started to decrease, though not statistically significantly. A concentration-dependent reduction in CBS expression, however, not statistically significant, was noticed when cells treated with SSZ. Both NaHS and Na_2S_2 had protection effects as shown by reduction in ROS formation, lipid peroxidation, mitochondria depolarisation, LDH leakage, DNA damage, cleaved PARP

as well as preserving GSH levels. But also, both donors can act as direct scavengers as both NaHS and Na_2S_2 exhibit antioxidant properties, detected by the ABTS and copper reduction assays. In conclusion, APAP and SSZ could indirectly impact H_2S levels in HepG2 cells by reducing the expressions of CBS and / or CSE enzymes. NaHS and Na_2S_2 could be employed as a tool to alleviate drugs induced oxidative stress. It offers a means to manipulate cell protective systems to prevent cellular damage. More research may be necessary to determine whether H_2S and H_2S_n could be a promising therapeutic treatment for the drug toxicity in clinical trials. In the future, studies are needed to explore if H_2S and H_2S_n affecting the drugs anti-inflammatory (SSZ) and analgesic (APAP) activities.

Key words: Hydrogen sulphide, Polysulphide, Antioxidant, Paracetamol, Sulphasalazine

6.2 Introduction

Hydrogen sulphide (H_2S) is the third gaseous mediator discovered in mammals, after nitric oxide (NO) and carbon monoxide (CO) (Rose, Moore and Zhu, 2017). This molecule serves as a signalling molecule and is produced by a variety of cells and tissues (Li, Rose and Moore, 2011). In our body, H_2S is predominantly synthesised through enzymatic reactions *via* the trans-sulphuration pathway (TSP) by two key enzymes, namely cystathionine β-synthase (CBS) (EC 4.2.1.22) and cystathionine γ-lyase (CSE) (EC 4.4.1.1). Recently, another enzymatic pathway *via* cysteine-tRNA synthetase (CARS) can generate persulphides which in turn can be converted into H_2S, however further studies are needed to confirm this phenomena (Zainol Abidin et al., 2023). Moreover, H_2S can also be produced non-enzymatically, for example through glutathione and thiosulphate (Kolluru et al., 2013).

Within mammalian systems, H_2S regulates a number of physiological processes, such as metabolism, vasorelaxation, autophagy, oxidative stress and cell cycle (Cirino, Szabo and Papapetropoulos, 2023). H_2S can induce S- sulphhydration of many different cellular proteins, by adding sulphur, derived from H_2S, to the thiol groups of cysteine residues. This process offers a potential mechanism by which H_2S modifies the function of a broad variety of cellular proteins and enzymes (Kimura, 2021). Several age related diseases (Piragine et al., 2023) are associated with its abnormal levels such as diabetes mellitus (Qian et al., 2018), hypertension (Meng et al., 2015), respiratory (Tian et al., 2012) and neurological diseases (Sharif et al., 2023). The decline in H_2S levels is associated with the development of renal (van den Born et al., 2016) and CVDs as well as overall mortality (van den Born et al., 2019).

Polysulphide (H_2S_n, $n > 2$), on the other hand, is a family of sulphane sulphur compounds that has received a lot of interest lately due to its connection with H_2S.

H_2S_n is produced, partially, by the oxidation of H_2S or its rection with NO (Kimura, 2021; Miyamoto et al., 2017). H_2S_n can also induce S-sulfhydration, highlighting its role in regulating redox signalling (Kasamatsu and Ihara, 2021). Lately, H_2S_n have been identified as potent antioxidants and their functions as mediators in a variety of physiological processes have come to light. They have been reported as a protectant in different tissues of mammalians such as kidney (Sun et al., 2020), heart (Xiong et al., 2023) and central nerve system (Kanemaru et al., 2023). These protection effects maybe mediated by various mechanisms such as anti-inflammatory (Zhang et al., 2019), antioxidant (Sawa et al., 2020), cytoprotecting (Koike et al., 2013) and post-translational modifications of proteins (Braunstein et al., 2020). However, while polysulphides have antioxidant properties, these molecules are complex with potential oxidant properties that depends on the pH and pKa (Switzer, 2023).

Many factors can affect H_2S levels and may potentially subsequently affect its metabolite, H_2S_n. These factors including genetic modification (Myszkowska et al., 2021), smoking and alcohol (Read, Zhu and Yang, 2021) and diet (Rose et al., 2021). It is interesting to note that in a number of model systems, some pharmacological medications that are frequently prescribed have been shown to change the levels of H_2S (Alsaeedi et al., 2023). However, studies on whether H_2S and H_2S_n levels are altered by commonly used medications is still limited. In **chapter 5**, we have shown that paracetamol (APAP) (analgesics) and sulphasalazine (SSZ) (anti-inflammatory) triggered oxidative stress in HepG2 cells, inducing apoptosis. Since both H_2S and H_2S_n involved in cellular redox regulation (Kimura, 2017), we have raised a question to whether or not APAP and SSZ can also affect H_2S and H_2S_n status. To the best of our knowledge whether APAP or SSZ altered H_2S and H_2S_n levels in HepG2 cell line have never been explored.

Additionally, as H_2S and H_2S_n have cytoprotectant properties (Kimura, 2015; Koike et al., 2013), we speculate that these endogenous sulphur compounds, H_2S and H_2S_n, are likely to alleviate the oxidative stress caused by APAP and SSZ, observed in our cell line (as shown in **chapter 5**). Sodium polysulphides (Na_2S_n) are frequently used to investigate the biological activity of polysulphide (Cao et al., 2019). As such, in this work we used Na_2S_n as polysulphide donors namely sodium disulphide [Na_2S_2]. While we used sodium hydrosulphide (NaHS) as H_2S donor which is commonly employed as a pharmacological tool for evaluating the effects of H_2S in biological experiments (Chen and Liu, 2016; Sitdikova, Weiger and Hermann, 2010).

Hypothesis

Our hypotheses point to the possibility that: **(i)** APAP and SSZ can alter H_2S or H_2S_n levels in HepG2 cell line by virtue of impacting of biosynthetic routes of these molecules and, **(ii)** H_2S and H_2S_n have cytoprotective effects against APAP- and SSZ-in hepatoma HepG2 cells by mitigating oxidative stress and cellular damage.

Aim

The aim of this study was to assess the impact of APAP and SSZ on H_2S and H_2S_n levels in HepG2 cells. And to evaluate if H_2S and H_2S_n donors protect oxidative damages induced by APAP and SSZ in HepG2 cells. Additionally, to elucidate a possible mechanism of action, donor compounds were screened for antioxidant activity using antioxidant assays, as detailed below in the method section

Objective

This study sought to address the following key research objectives:

1. To examine the impact of APAP and SSZ on H_2S and polysulphides status in HepG2 cells.

2. To investigate the effects of NaHS and Na_2S_2 on the IC_{50} of APAP and SSZ induced oxidative stress markers in HepG2 cells. This include the same markers identified in **chapter 5** which are ROS, lipid peroxidation, mitochondrial membrane potential, and late apoptotic markers that include GSH level, LDH leakage, DNA damage and PARP cleavage.

3. If protection observed, to explore a possible metabolism underlying the protective effects of NaHS and Na_2S_2 *via* two antioxidant assays.

6.3 Material and method

6.3.1 Chemicals

7-azido-4-methylcoumarin (AzMC) fluorogenic probe, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) [ABTS], antioxidant assay kit (MAK334), reduced glutathione (GSH), homocysteine (Hcy), cysteine (Cys), pyridoxal 5′-phosphate (PLP), Trolox, Sodium hydrosulfide (NaHS) and DL-Propargylglycine (PAG) was all purchased from Sigma-Aldrich (St Louis, USA). Sulfane Sulfur Probe 4 (SSP4) and sodium disulfide (Na_2S_2) were purchased from Dojindo Molecular Technologies. Lead acetate strips were purchased from Johnson Test Papers (Oldbury, UK).

6.3.2 Cell culture

HepG2 cells were cultured in DMEM, supplemented with 100 ml / L FBS, 10 000 units / ml penicillin and 10 mg / ml streptomycin as detailed in **chapter 2** (**2.2.1.1 – 2.2.1.3**). For the cell viability tests, cells were seeded in clear 96-well plates (Costar Flat Bottom with Lid) at a density of 10,000 cells per well. For the lactate dehydrogenase test, cells were seeded at 20,000 cells per well in transparent 96-well plates. For the DCFDA, JC-1, AzMC, and SSP4 assays, cells were seeded at 10,000 cells per well in black (with clear bottoms plates) (µCLEAR®, BLACK CellStar® F-Bottom). Clear six-well plates were used to seed cells at a density of 400,000 cells per well in order to detect cleaved PARP using ELISA, H_2S (Lead acetate strips), GSH and MDA levels. Cells were seeded at a density of 2.2 x 10^6 / petri dish in 100mm x 20mm petri dishes for western blot analysis.

6.3.3 Cell culture treatment

APAP and SSZ were dissolved in DMSO, a final concentration of 10 mmol / L of the drug was reached and then concentrations were adjusted as needed by dilution in

DMEM FBS-free media with the DMSO concentration remaining below 0.1 %. NaHS was dissolved first in ddH_2O, and concentrations needed reached by dissolving in FBS-free media. As manufacturer's recommendations, Na_2S_2 was dissolved in ddH_2O purged with nitrogen gas and then diluted in FBS-free DMEM to reach the desired concentrations as indicated below. All reagents were freshly prepared for each individual experiment.

6.3.4 Measurement of H_2S and H_2S_n levels

Lead acetate strip test (LAST) was conducted as detailed previously (Anishchenko et al., 2019). Briefly, in order to the cells be directly exposed to H_2S release during treatments, lead acetate paper was cut into small, equal-sized squares and fitted into the inner size of the lid of six well plates. Four different sets of conditions were applied to the cells: **i)** control conditions (untreated cells), **ii)** 5 mM Cys + 5 mM PLP, **iii)** 5 mM Cys + 5 mM PLP + 4 mM APAP and **iv)** 5 mM Cys + 5 mM PLP + 4 mM SSZ and plates were covered with tape and incubated for 24 hours (5 % CO_2 at 37 °C) (Anishchenko et al., 2019). This method based on lead acetate strips detect H_2S by forming a visible black precipitate upon contact, which is accounted for the detected H_2S levels. The strips then were photographed and then analysed using ImagJ software. Data presented in arbitrary units (a.u.) of three independent repeat (n = 3).

In parallel with this, fluorescent probes namely AzMC was used to estimate H_2S levels and SSP4 was used to detected H_2S_n levels in HepG2 cells as described in **section 2.2.3.11**. Briefly, HepG2 cells were seeded in 96-well plates (black plates with clear bottom) at a cell density of 1 × 10^4 cell / well for 24 hours. HepG2 cells were loaded with 25 µM AzMC or 10 µM SSP4 in the dark for 30 minutes, washed with PBS twice and then treated with the drugs different concentration (0, 0.06, 0.12, 0.25, 0.5, 1, 2, 4

mM) of APAP and SSZ, separately. Tape was used to cover the plates to reduce volatilisation. Fluorescence measurement was performed at 4 timepoints of treatment incubation (1, 2, 3 and 4 hours). The excitation / emission of AzMC and SSP4 fluorescence was recording at 365 / 450 nm and 482 / 515 nm per manufacturer's recommendations, respectively. Background fluorescence was subtracted from all fluorescence measurements (n = 3).

6.3.5 Oxidative stress markers

6.3.5.1 Cell viability testing – crystal violet assay

Cells were treated for 24 hours with FBS-free media (control, untreated cells), IC_{50} concentrations of APAP (2.5 mM), SSZ (1.3 mM), with or without 20, 40 and 80 µM of NaHS and Na_2S_2, separately, as used in previous experiments (Cao et al., 2018). Cell viability was then determined using the crystal violet assay as described in **section 2.2.3.1** and replicates were performed independently (n = 9).

6.3.5.2 Measurement of reactive oxygen species (ROS) and lipid peroxidation (LPO)

DCFDA was used to measure intracellular ROS production. The dye was then added to the cells and incubated for one hour at 37 °C with 5 % CO_2, as stated in **section 2.2.3.2**. APAP (2.5 mM) or SSZ (1.3 mM) ± 80 µM NaHS and Na_2S_2, H_2O_2 at 1 mM (positive control) or control (untreated cells) diluted in HBSS were applied to the cells for a duration of 120 minutes. Replicates were performed independently (n = 9). Fluorescence was measured with the FluoStar Omega fluorescent plate reader (485 / 520nm). All fluorescence measurements were adjusted for background fluorescence and the results are given in relative fluorescence units (RFU).

Two other probes were employed: MitoSOX™ Red indicator was used to visualise ROS generation in the mitochondria (superoxide [$O_2^{\bullet-}$]) and CellROX® Deep Red reagent was used to visualise ROS in the cytoplasm, both detailed in **section 2.2.3.5**. Cells were exposed to APAP (2.5 mM) or SSZ (1.3 mM) ± 80 µM NaHS and Na_2S_2 or H_2O_2 at 1 mM (positive control) diluted in HBSS. Cells were treated for 120 minutes and images were taken on the EVOS fluorescent microscope. The fluorescence intensity of these probes increases proportionally with increasing ROS levels.

LPO was assessed using the Image-iT® Lipid Peroxidation Kit (C10455-Invitrogen) according to the manufacturer's protocol, as described in **section 2.2.3.5**. Cells were exposed to APAP (2.5 mM) or SSZ (1.3 mM) ± 80 µM NaHS and Na_2S_2 or 100 µM cumene hydroperoxide as a positive control, provided with the kit for 120 minutes. EVOS fluorescent microscope was used to obtain images. Additionally, Malondialdehyde (MDA) measurement was assessed using a commercial Lipid Peroxidation Assay Kit as described in **section 2.2.3.3.** Applying same concentration and time point as for the Image-iT® Lipid Peroxidation Kit above. Replicates were performed independently (n = 3).

6.3.5.3 Mitochondrial membrane potential assessment using the fluorometric probe, JC-1

Mitochondrial membrane potential was assessed using JC-1 dye. Cells were loaded with 2 µM of JC-1 in each well of a 96-well plate (black plate with clear bottom) for 30 minutes, then cells were treated with APAP (2.5 mM) or SSZ (1.3 mM) with or without 80 µM NaHS and Na_2S_2 for 120 minutes as detailed in **section 2.2.3.4**. Replicates were performed independently (n = 6).

6.3.5.4 Intracellular glutathione levels

Intracellular GSH levels were assessed in HepG2 cells treated with control (FBS free media), APAP (2.5 mM) or SSZ (1.3 mM) with or without 80 µM NaHS and Na_2S_2 24 hours using a commercially available kit (Sigma-Aldrich, 38185) following the kit protocol as described in **section 2.2.3.6**. Replicates were performed independently (n = 3).

6.3.5.5 DNA damage assessment: 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) staining

DAPI staining was used, as described in **section 2.2.3.5**, to identify changes in chromatin structure that were indicative of apoptotic cell death. Cells were exposed to APAP (2.5 mM) or SSZ (1.3 mM) ± 80 µM NaHS and Na_2S_2 for 24 hours. H_2O_2 at 1 mM diluted in EBSS served as the positive control. Images were obtained using the EVOS fluorescent microscope. The condensation and fragmentation of their nuclei were assessed to identify apoptotic cells.

6.3.5.6 Assessment of membrane leakage as an indicator of apoptosis: lactate dehydrogenase (LDH) leakage

LDH release in the medium was measured after 24-hour treatment with APAP (2.5 mM) or SSZ (1.3 mM), ± 80 µM NaHS or Na_2S_2. The CyQUANT™ LDH cytotoxicity assay kit (Invitrogen) was used as described in **section 2.2.3.7**, with absorbance read at 415 nm and 655 nm using Biorad microplate reader (Model 680 XR). Replicates were performed independently (n = 6).

6.3.5.7 Assessment of poly-ADP ribose polymerase (PARP) involvements

Cleaved PARP levels were measured in cells treated with APAP (2.5 mM) or SSZ (1.3 mM) ± 80 µM NaHS and Na_2S_2 for 24 hours. A commercial ELISA kit (Thermo Fisher) was used as per **section 2.2.3.9**. Results were normalised to total protein content

determined by Lowry assay (**section 2.2.3.10**). Replicates were performed independently (n = 3).

6.3.6 Western blot

For western blot analysis of CBS and CSE enzymes, HepG2 cells were treated with different concentrations of APAP and SSZ separately for 24 hours before cells were scrapped to extract protein. The protocol outlined in section **2.2.3.10** were followed for protein extraction, normalisation and western blotting. Then results were analysed using ImagJ (Stael et al., 2022). Results presented as blots and their relative densitometry analyses (n = 3 for each drug).

6.3.7 Antioxidant assays

H_2S (NaHS) and H_2S_n (Na_2S_2) samples were prepared as described in **section 6.3.3**. We used concentrations of 20, 40, and 80 µM, matching those used in our cell culture experiments (Cao et al., 2018). Total antioxidant capacity (TAC) of these samples were then evaluated using two different assays: Firstly, TAC of H_2S and H_2S_n was assessed by using Antioxidant Assay Kit (MAK334, Sigma-Aldrich) (copper reduction assay). In this assay, antioxidants in the sample reduce Cu^{2+} to Cu^{+}. The reduced copper (Cu^{+}) then forms a coloured complex with a dye reagent. The intensity of this colour (pink / red), measured at 570 nm, is directly proportional to the sample's TAC. Following the manufacturer's instructions, Trolox standards, provided with the kit, was diluted in ultrapure water to construct a standard curve; with concentration range 0 – 1000 µM. Then, 20 µL of each tested samples and Trolox standards were transferred in a clear flat-bottom 96-well plate. Following this, 100 µL of reaction mix was added to all assay wells and the plate was tapped to ensure even distribution and allowed to incubate for 10 minutes at room temperature and absorbance was measured at 570 nm.

Absorbance values were compared to the standard curve prepared with 0 - 1000 µM Trolox solutions (n = 3).

Secondly, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was also used in the current assay to further assess TAC. In the ABTS assay, ABTS is oxidised to produce its radical cation, ABTS•+, which has a blue-green colour. The sample's antioxidants neutralise this radical, which results in a loss of colour. The degree of colour loss is proportional to TAC allowing for quantitative assessment. The ABTS assay in the current study was conducted according to Lee et al. (2014) and Šola et al. (2020). Briefly, the stock solutions included 7.4 mM ABTS and 2.6 mM potassium persulfate were mixed and allowed to stand overnight at 4°C in the dark. Then, ABTS radical solution was adjusted with phosphate buffered saline (pH 7.4) to an absorbance of 0.7 - 0.8 at 734 nm. For this assay, Trolox was dissolved in methanol to prepare a stock solution, which was then used to construct a standard curve. The concentration range of the Trolox standards was 0 - 1 mM. 190 µL of ABTS radical solution was mixed with 10 µL of each test sample solution and standards in a clear flat-bottom 96-well plate as previously described (Lee et al., 2014). After 6 minutes (Šola et al., 2020), the absorbance was measured at 734 nm using a microplate reader (FLUOstar Omega - BMG Labtech) (n = 3). Total antioxidant capacity was quantified using the Trolox standard curve and results are expressed as follows:

$$\textbf{\% ABTS inhibition} = [(A0 - A1) / A0] \times 100$$

Where **A0** = Absorbance of the control and **A1** = Absorbance of the test sample.

6.3.8 Data analysis

Data is presented as mean ± standard error of mean (SEM), unless otherwise stated. Data was analysed using one-way ANOVA when comparing between three or more

groups followed by post-hoc Tukey test. While independent t-tests were performed when comparing between two groups. **(*)** indicates $P < 0.05$ and **(**)** indicates $P < 0.01$ throughout the chapter.

6.4 Results

6.4.1 Hydrogen sulphide (H_2S) and polysulphide (H_2S_n) measurement

6.4.1.1 Lead acetate strip test (LAST) assay – detection of hydrogen sulphide (H_2S) in HepG2 cells

We used the LAST assay to validate H_2S production in our cell line. This assay's principle is based on the reaction of H_2S with lead ions on acetate paper, forming lead sulphide and resulting in a visible black precipitate (**H_2S + Pb(CH3COO)2 → PbS + 2CH3COOH**). In this pilot study, we aimed to maximise H_2S production by treating HepG2 cells with 5 mM cysteine (Cys) and 5 mM pyridoxal 5′-phosphate (PLP), the active form of vitamin B_6, for 24 hours, in line with previous literature (Anishchenko et al., 2019). Cys is a substrate needed for H_2S biosynthesis, while PLP is required as a cofactor for H_2S -producing enzymes, i.e., CBS and CSE. When HepG2 cells were exposed to cysteine and PLP, H_2S generation was observed compared to the non-treated cells, as evidenced by the visible brown colour formation ($P < 0.01$) (**Figure 6.1**). These results confirm that our HepG2 cells have the capacity to generate H_2S and therefore have the potential to be a useful model for subsequent studies in the current chapter. Following this, when cells were treated with 4 mM APAP or SSZ in the presence of Cys and PLP, we observed significant downregulations in H_2S production at 24 hours by 1.5 and 2 folds with respect to the Cys and PLP treated cells ($P = 0.016$ and $P < 0.01$), respectively (**Figure 6.1 (B)**). Our results suggest that APAP and SSZ can negatively affect H_2S levels in HepG2 cells. This will be further confirmed using a fluorescence probe (AzMC) as described in the next experiment.

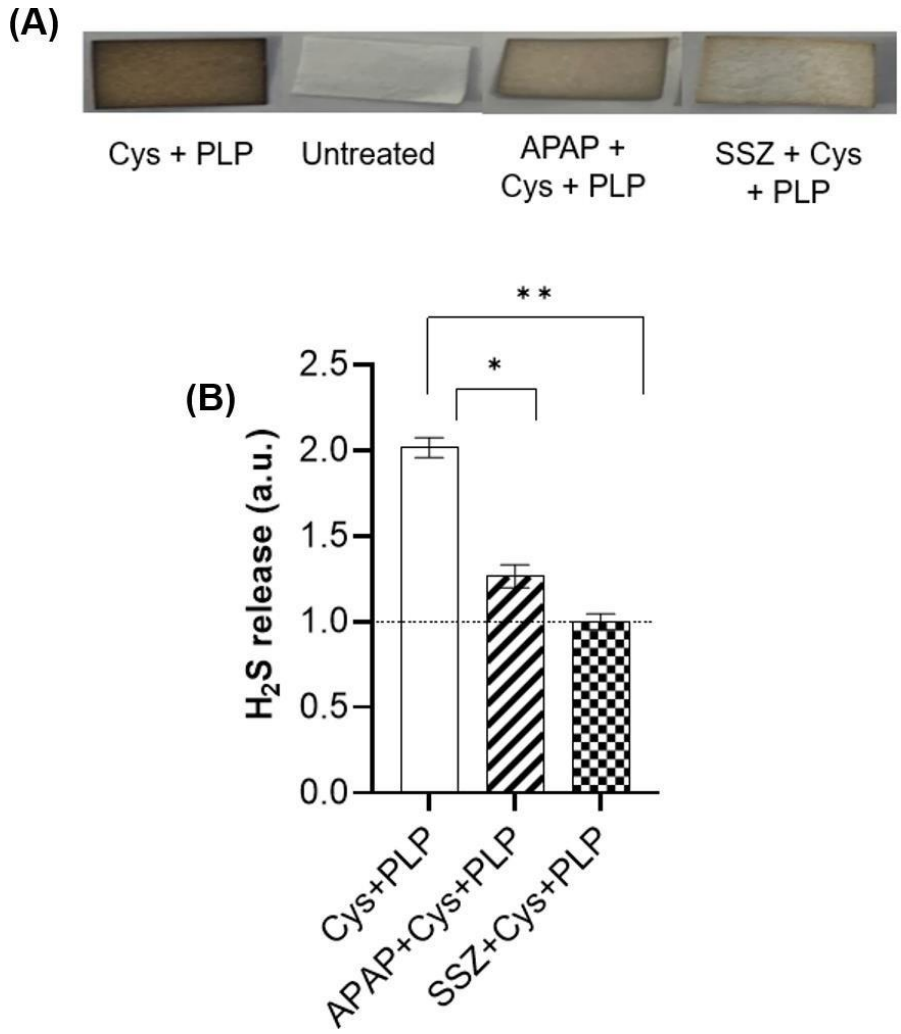


Figure 6.1 (A) Representative photograph of lead acetate strip showing the difference in the degree of black / brown colour formation when HepG2 cells treated for 24 hours with 5 mM Cys + 5 mM PLP, untreated, 5 mM Cys + 5 mM PLP + 4 mM APAP and 5 mM Cys + 5 mM PLP + 4 mM SSZ **(B)** ImagJ analysis of three independent replicates (n = 3). Data recorded in arbitrary units (a.u.) untreated control indicated in dotted line. **(**)** $P < 0.01$ and **(*)** $P < 0.05$. Data represent means ± SEM.

6.4.1.2 Verification of AzMC and SSP4 specificity

To detect H_2S and H_2S_n, we employed two distinct fluorophores. AzMC, with excitation / emission wavelengths of 365 / 450 nm, was used for H_2S detection, while SSP4, with excitation / emission wavelengths of 482 / 515 nm, was utilised to identify H_2S_n. Each fluorophore exhibits specificity for its respective sulphur species. Probes specificity was validated in previous work (Olson and Gao, 2019). To confirm the probes'

specificity under our laboratory conditions, we further tested their reactivity with potential interfering molecules, including sulphur compounds such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH). We examined these molecules, along with NaHS and Na_2S_2, in PBS buffer at concentrations of 0, 100, 300, and 1000 µM (Olson and Gao, 2019).

In PBS buffer, NaHS (H_2S donor) and Na_2S_2 (H_2S_n donor) increased AzMC and SSP4 fluorescence, respectively, in a concentration-dependent manner ($P < 0.01$). Cys, Hcy and GSH had no appreciable effects on AzMC or SSP4 fluorescence, even at the highest concentrations, compared to NaHS and Na_2S_2, respectively (**Figure 6.2 (A) and (B)**). These results confirmed the specificity of AzMC to H_2S and SSP4 to H_2S_n. We further validated the detection of H_2S and H_2S_n in our HepG2 cell line by treating cells with 0 – 1 mM NaHS or Na_2S_2 for 24 hours. Results showed that NaHS and Na_2S_2 increased AzMC and SSP4 fluorescence, respectively, in a concentration-dependent manner ($P < 0.01$) (**Figure 6.2 (C) and (D)**). Additionally, DL-Propargylglycine (PAG, 1 mM) (Cirino et al., 2023), an inhibitor of H_2S synthesis, followed by different concentrations of NaHS (0 – 1 mM) (**Figure 6.2 (C))** caused a reduction in AzMC fluorescence in cells in a concentration dependent manner ($P < 0.01$) (**Figure 6.2 (C)**). However, while PAG is known to inhibit intracellular H_2S, it should be noted that the addition of NaHS represents extracellular H_2S levels. The unexpected observed flat response of AzMC suggested a potential interaction between PAG and AzMC. If time allowed, we could verify the interaction between PAG and AzMC in buffer first and then include additional controls (PAG only) to further validate the inhibitory effect of PAG on H_2S levels. Nevertheless, collectively, these data demonstrate that AzMC and SSP4 have sufficient specificity to be effectively employed for their respective analyses of H_2S and H_2S_n in subsequent experiments.

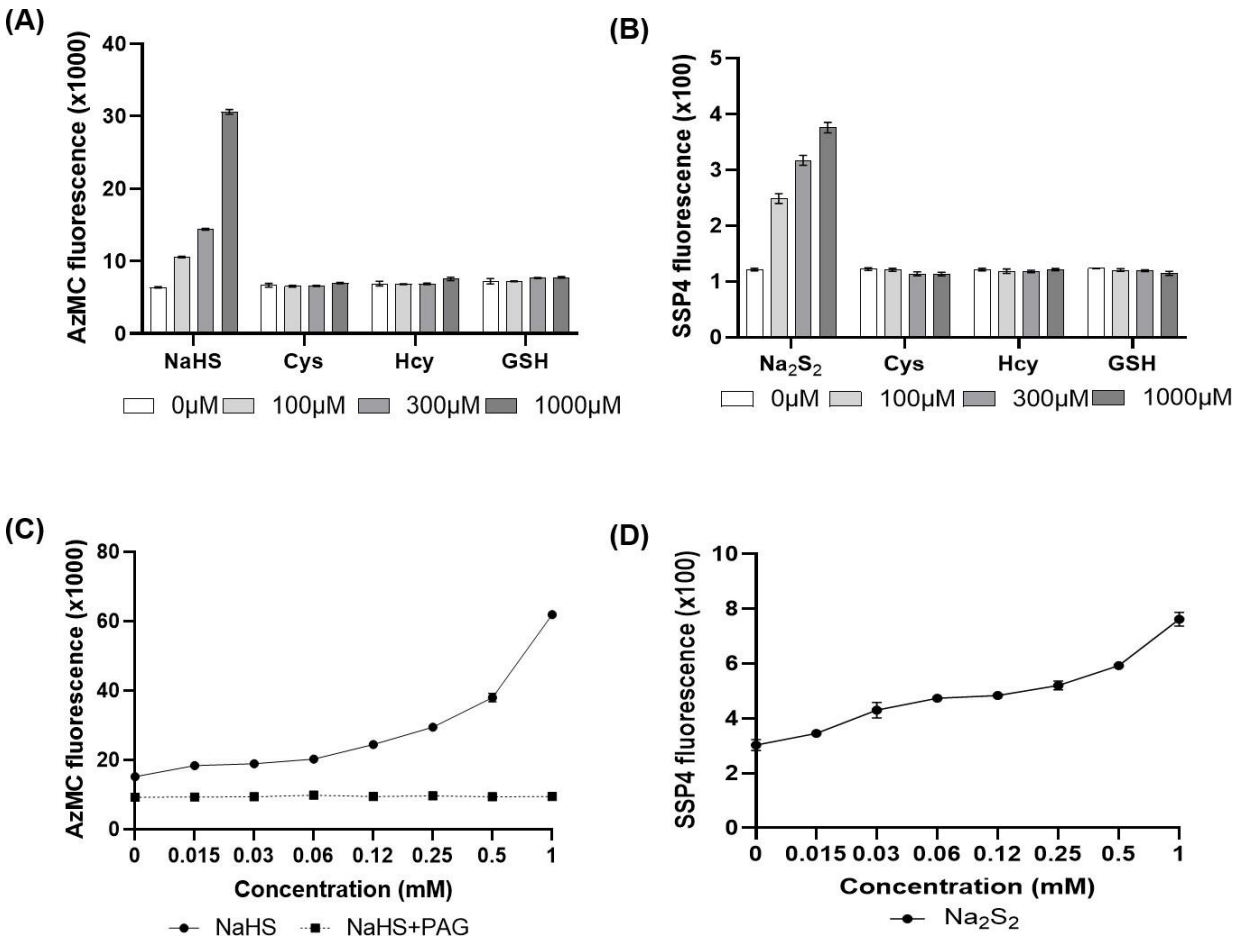


Figure 6.2 Effects of potential interfering molecules on AzMC and SSP4 fluorescence in buffer (**A** and **B**) compared to H_2S and H_2S_n, respectively: Cysteine (Cys) and homocysteine (Hcy), glutathione (GSH) at the indicated concentrations. Mean ± SEM, n = 9 wells. Effects of exogenous NaHS and Na_2S_2 in HeG2 cells at the indicated concentrations on AzMC fluorescence **(C)** and on SSP4 fluorescence **(D)** at 24 hours. An inhibitor of H_2S biosynthesis, D,L-Propargylglycine (PAG) decrease AzMC fluorescence in HepG2 cells at 24 hours. Mean ± SEM n = 9 wells; **(*)** *P < 0.05 and* ***(**)*** *P < 0.01.*

6.4.1.3 Impacts of APAP and SSZ on H_2S and H_2S_n levels

Following the validation experiments, we then assessed the impact of different concentrations of APAP and SSZ (0 - 4 mM) on H_2S and H_2S_n levels in HepG2 cells for four timepoints (0 - 4 hours). As shown in **Figure 6.3**, at each timepoint, there was a tendency of H_2S reduction but this was not statistically significant ($P > 0.05$).

No significant changes were observed in H_2S and H_2S_n levels when HepG2 cells were treated with different concentrations of APAP (0 - 4 mM) for short-term exposure (up to 4 hours). However, we observed non- significant reduction of H_2S at 4 hours. These negative results may be due to the short-term APAP exposure or low number of replicates (n = 3). Long-time treatment would help get a better understanding of APAP on H_2S and H_2S_n levels. Sadly, due to time constrains we could conduct these experiments. In contrast, SSZ showed a concentration and time- dependent reduction in H_2S levels in HepG2 cells ($P < 0.01$). We also observed no significant reduction in H_2S_n with SSZ.

To investigate whether these results could be due to interference of the drugs with the fluorescent probes, we tested the direct interaction of APAP and SSZ with AzMC and SSP4 by adding the probes to the drugs in PBS buffer. Results (**Appendix 6.1**) showed no interference between APAP and either AzMC or SSP4, suggesting that the observed effect with APAP reflects true biological responses. However, there was clear direct interference between SSZ and both probes, indicating that the apparent reduction in H_2S_n levels with SSZ treatment may be, at least partly, due to this interference rather than solely biological effects. The impact of SSZ remain to be clarified.

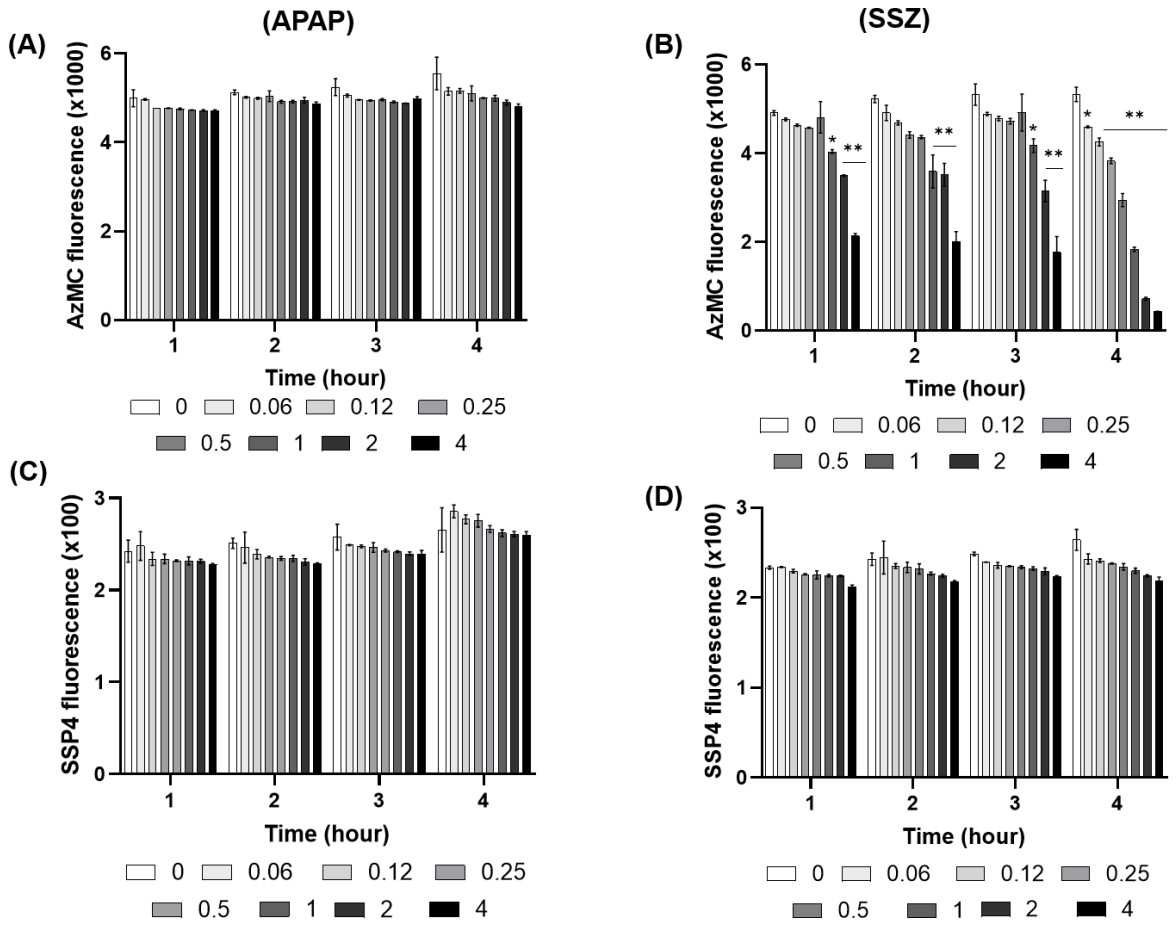


Figure 6.3 Effects of different concentrations of APAP on AzMC **(A)** and SSP4 **(C)** fluorescence in HepG2 cells for the indicated time points. The effects of SSZ on AzMC **(B)** and SSP4 **(D)** fluorescence in HepG2 cells for the indicated time points. Data represent means ± SEM of three independent experiments (n = 3).

6.4.1.4 Impact of APAP and SSZ on CBS and CSE expression in HepG2 cells

We then investigated whether APAP and SSZ indirectly affect H_2S production by altering the expression of critical enzymes involved in H_2S synthesis, namely CBS and CSE enzymes. This will allow us to obtain a bigger picture of the impact of the drugs on H_2S production. To assess this, we used western blot analysis to determine the

expression levels of CBS and CSE in HepG2 cells treated with different concentrations with APAP and SSZ for 24 hours (**Figure 6.4**).

APAP significantly reduced the expression of both CBS ($P < 0.01$) and CSE ($P = 0.023$) in HepG2 cells in a concentration-dependent manner at 24 hours treatment (**Figure 5 A**). Compared to control (untreated cells), CBS expression was significantly reduced starting at 0.12 mM APAP ($P = 0.05$) and at higher concentrations. CSE expression was significantly reduced only at 2 mM APAP ($P = 0.05$). Regarding SSZ, we observed a reduction in CBS expression across different concentrations, although this was not statistically significant ($P = 0.132$) (**Figure 5 B**). Interestingly, CSE expression was significantly elevated when HepG2 cells were treated with 0.12 mM ($P = 0.034$) and 0.25 mM ($P = 0.049$) SSZ for 24 hours, which may be explained as a compensatory mechanism. This increase in CSE expression could be the cell's attempt to maintain H_2S levels in response to H_2S depletion, although our study could not definitively confirm this mechanism due to the limitations in H_2S detection with SSZ. At higher concentrations, CSE expression started to decrease, though not statistically significantly.

These western blot results, combined with our AzMC and SSP4 findings, suggest that APAP treatment significantly reduces the expression of both CBS and CSE, despite showing no significant impact on H_2S or H_2S_2 levels in the short term (up to 4 hours). However, these findings suggest the likelihood of APAP-induced H_2S and H_2S_2 reduction if cells were treated for longer periods (beyond 4 hours). For SSZ, the interpretation would be more complex due to the probe interference issues identified earlier. Given the direct interference of SSZ with the H_2S / H_2S_2 detection probes, it's challenging to definitively link these enzyme expression changes to actual H_2S levels.

However, future studies using alternative H_2S detection methods are needed to clarify the relationship between SSZ-induced changes in enzyme expression and H_2S production in these cells.

It would have been preferable to do more repeats and include a positive control (NaHS), but time constraints prevented this from happening. To better understand these drugs, future research should look into both the expression levels and enzymatic activity of CBS and CSE in HepG2 cells over time.

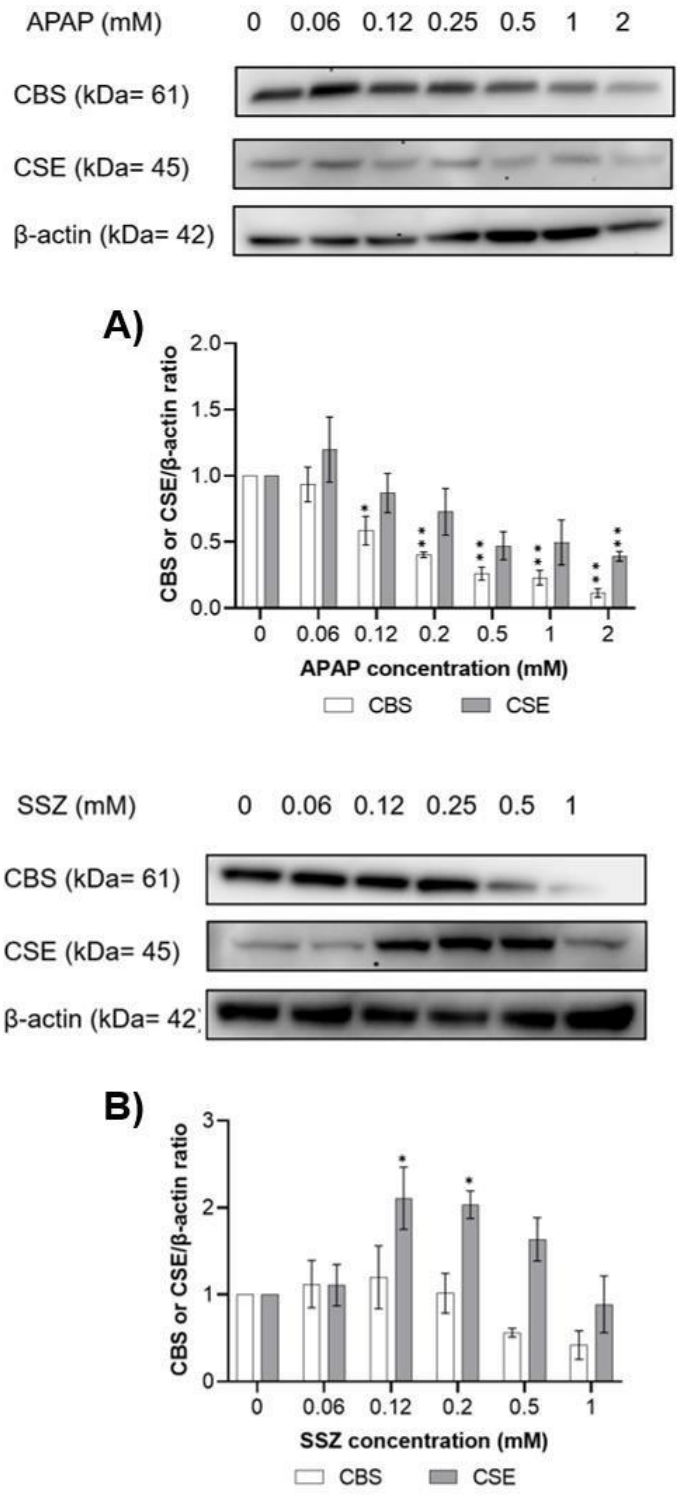


Figure 6.4 Changes in CBS and CSE expressions in HepG2 cells treated with the indicative concentrations of APAP **(A)** and SSZ **(B)** for 24 hours.**(*)** $p < 0.05$ versus untreated cells Data represent means ± SEM of independent experiments (n = 3).

6.4.2 Oxidative stress

In **chapter 5**, we indicted that both APAP and SSZ induced oxidative stress and apoptosis in HepG2 cells. This let us to consider assess whether H_2S or H_2S_n preserve cell viability and reduce oxidative stress in drugs treated cells by using NaHS (H_2S donor) and Na_2S_2 (H_2S_n donor). Notably, due to time constraints, we were not able to test the time and / or concentration effects of these endogenous sulphur compounds for some of the experiments.

6.4.2.1 NaHS and Na_2S_2 inhibit drugs induced cell death in HepG2 cells

The protective effects of H_2S or H_2S_n against drug-induced cytotoxicity were first tested on HepG2 cell viability using crystal violet assay (**Figure 6.5**). We first observed that HepG2 cells treated with 80 µM of either NaHS or Na_2S_2 alone for 24 hours had similar cell viability compared to control (untreated cells) (97 ± 3.1 % **vs** 99 ± 4.8 % **vs** 100 % for NaHS, Na_2S_2, and control, respectively; $P > 0.05$). This result suggests that these H_2S and H_2S_n compounds did not induce cytotoxicity in our cell line under our experimental conditions at the used concentration. As expected, when cells were treated with the IC_{50} of each drug, 2.5 mM APAP and 1.3 mM SSZ, significant reductions in HepG2 viability were observed compared to control (untreated cells) at 24 hours treatment ($P < 0.01$), reaching approximately 50 % (**Figure 6.5**). However, co-treatment with either NaHS or Na_2S_2 significantly elevated cell viability in cells treated with APAP or SSZ at 24 hours in concentration-dependent manners ($P < 0.01$). For APAP-treated cells, HepG2 cell viability increased from 50 ± 3.5 % to 86 ± 1.5 % when co-treated with 80 µM NaHS ($P = 0.007$) (**Figure 6.5 (A)**), and from 52 ± 5.1 % to 93 ± 1.7% when co-treated with 80 µM Na_2S_2 ($P = 0.005$) (**Figure 6.5 (B)**). Similarly, for SSZ-treated cells, viability increased from 47 ± 2.4 % to 80 ± 1.2 % with NaHS co-

treatment (P = 0.008), and from 44 ± 3.0 % to 91 ± 0.3 % with Na_2S_2 co-treatment (P = 0.004) (**Figure 6.5 (A) and (B)**, respectively). These results from crystal violet assay highlight the protective effects of H_2S and H_2S_n in APAP- and SSZ-induced HepG2 viability reduction occurred in a concentration-dependent manner. Based on these results and considering time constraints, we selected the 80 µM concentration of both NaHS and Na_2S_2 for the subsequent experiments, as this concentration exhibited the most pronounced protective effect.

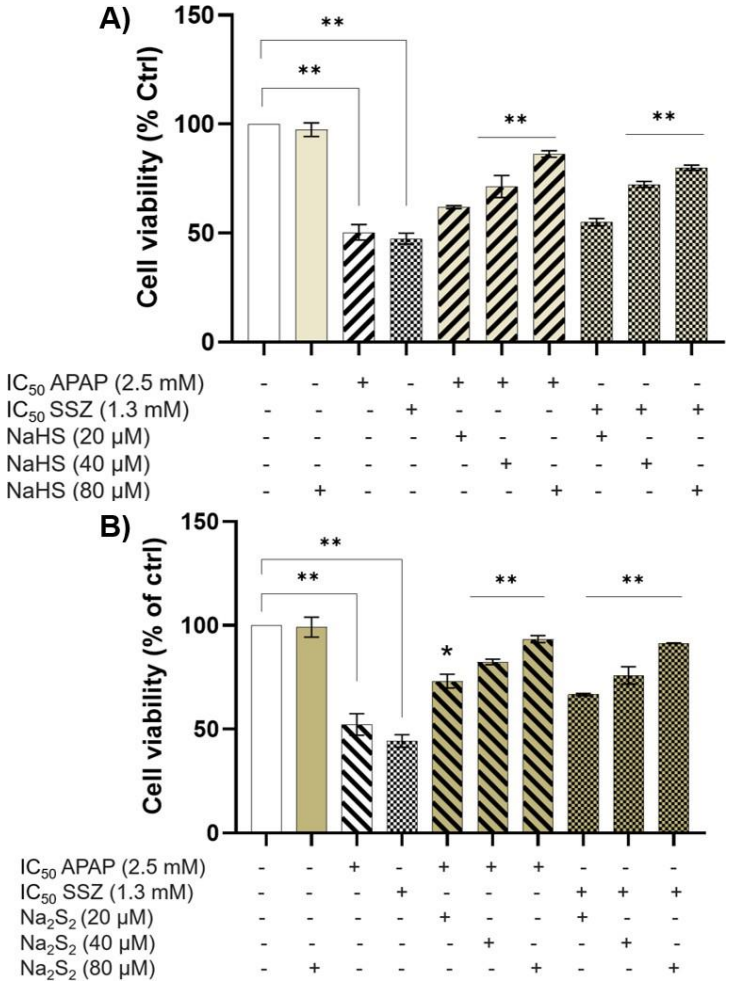


Figure 6.5 Concentration-dependent changes in HepG2 cell viability at 24 hours when APAP (2.5 mM) and SSZ (1.3 mM) co-treated with the indicated concentrations of **(A)** NaHS (H_2S donor) and **(B)** Na_2S_2 (H_2S_n donor**)** as determined by the crystal violet assay. Control represents untreated cells (FBS-free media) (< 0.1% DMSO) (n = 9) Data are expressed as the mean ± SEM.

6.4.2.2 NaHS and Na_2S_2 inhibit drugs induced ROS production and lipid peroxidation in HepG2

It is generally acknowledged that the production of ROS is an early event to the apoptotic cascades that are triggered by various cellular stressors, such as medications. As reported in **chapter 5**, APAP and SSZ induced ROS formation in HepG2 cells in a time-dependent manner. Therefore, it is of great significant to assess if H_2S or H_2S_n donors can protect our cell line from this early oxidative stress marker to better understand the protection mechanism. To explore this, we quantitatively measured ROS levels using the DCFDA assay, while visualising ROS formation with CellROX® and MitoSOX™ probes. This strategy provides both quantitative data and visual evidence of the potential protective effects of NaHS and Na_2S_2 against drug-induced ROS formation in HepG2 cells.

Figure 6.6 demonstrates that both 80 µM of NaHS and Na_2S_2 alone showed DCFDA fluorescence similar to untreated cells at 120 minutes ($P > 0.05$). In contrast, the IC_{50} of APAP (2.5 mM) and SSZ (1.3 mM) significantly increased ROS generation compared to control (untreated cells), as indicated by elevated DCFDA fluorescence ($P < 0.01$). We then investigated the impact of H_2S and H_2S_n donors on drug-induced ROS production. Co-treatment with 80 µM NaHS or Na_2S_2 reduced DCFDA fluorescence at 120 minutes compared to drug-only groups. For APAP, fluorescence reductions of ≈ 41 % (NaHS) and ≈ 35 % (Na_2S_2) were observed ($P < 0.001$, respectively). Similarly, for SSZ, fluorescence reductions of ≈ 44 % (NaHS) and ≈ 42 % (Na_2S_2) were noted ($P < 0.001$, respectively). These results indicate that both tested sulphur-containing compounds significantly mitigated ROS formation induced by APAP and SSZ, possibly explain their cytoprotective observed in the cell viability assay.

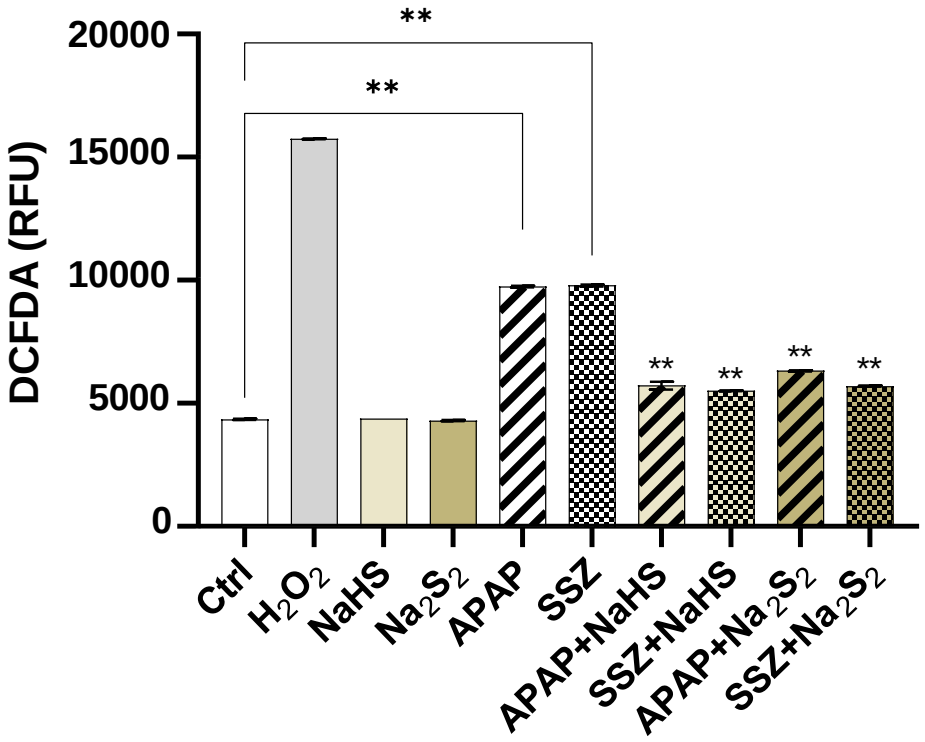


Figure 6.6 ROS levels in HepG2 cells measured by DCFDA assay at 120 minutes. Cells were exposed to control conditions (Ctrl, untreated cells), H_2O_2 (positive control, 1mM), IC_{50} of drugs (APAP 2.5 mM or SSZ 1.3 mM) alone, H_2S donor alone (80 µM NaHS), H_2S_n donor alone (80 µM Na_2S_2) or co-treatments of drugs with the donors. **(**)** $P < 0.01$ compared to control (untreated cells) (indicated by lines in figure); **(**)** $P < 0.01$ for co-treatments compared to their respective drug-only treatments (not indicated by lines). Data represent means ± SEM of independent experiments (n = 9).

We then visualised ROS formation at 120 minutes of treatment to further confirm the DCFDA results. Two fluorescent probes were used: CellROX® Deep Red reagent, which exhibits bright red fluorescence upon ROS formation in the cytoplasm (excitation / emission maxima at 640 / 665 nm) and MitoSOX™, which detects mitochondrial superoxide ($O_2^{\bullet-}$) with excitation / emission maxima of approximately 510 / 580 nm. The red fluorescence intensity of these probes increases proportionally with increasing ROS levels. As shown in **Figure 6.7** and **Figure 6.8**, fluorescence

microscopy observations revealed that compared to drug-only groups, the 80 µM NaHS or Na_2S_2 co-treatment groups exhibited less cytoplasmic ROS content and mitochondrial superoxide production, evidenced by reduced CellROX® and MitoSOX™ red fluorescence, respectively. These findings correlate with the DCFDA results, suggesting that the ROS-suppressing effects H_2S and H_2S_n against APAP and SSZ could be involved in the mechanism of the observed H_2S and H_2S_n-mediated protection of cell viability in the HepG2 cell line. It would be valuable to investigate whether the H_2S and H_2S_n-induced reduction of ROS in HepG2 cells treated with APAP and SSZ occurs in a time-dependent manner. Unfortunately, time constraints prevented us from conducting this set of experiments.

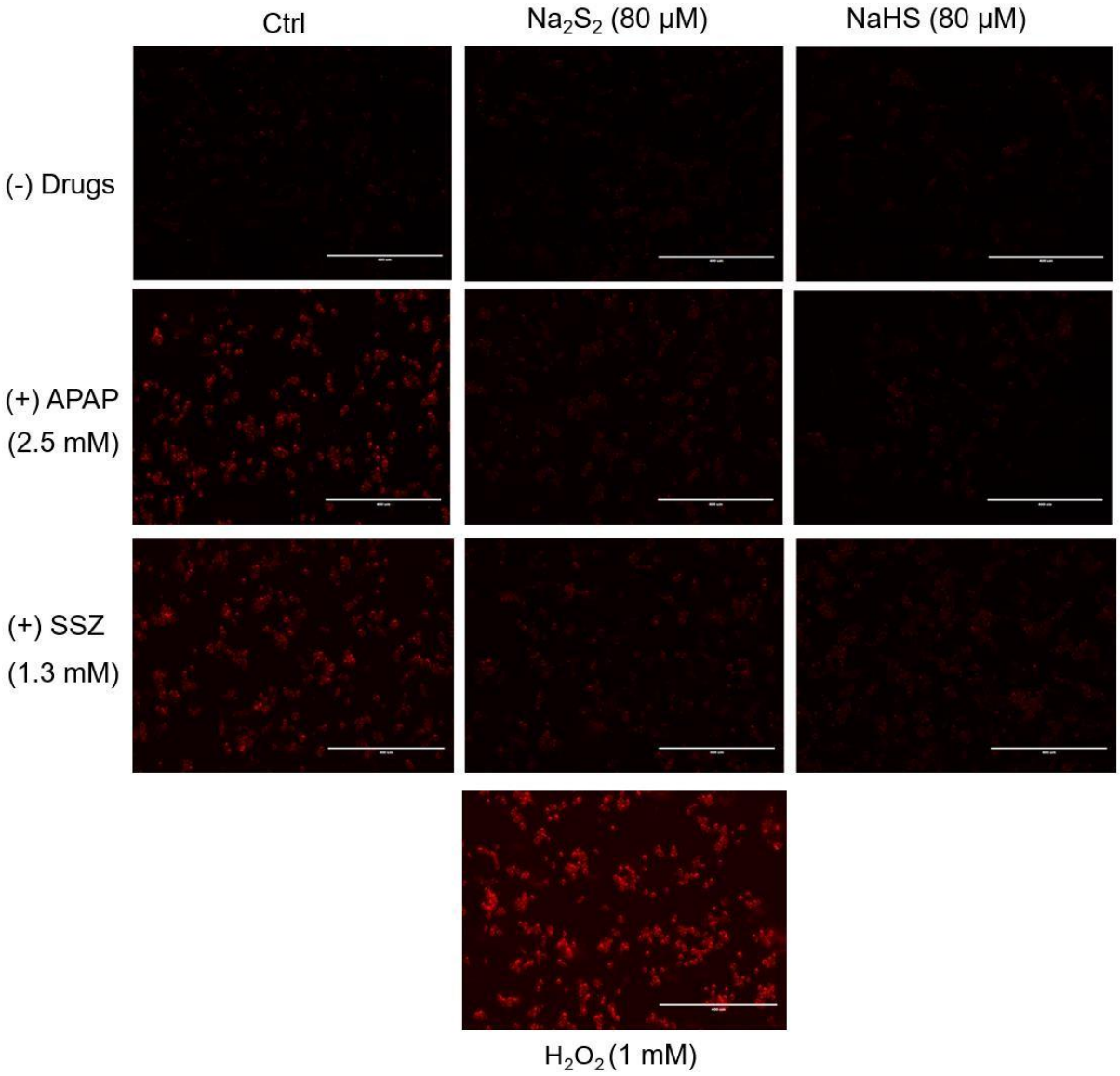


Figure 6. 7 Visualisation of cytoplasmic (using CellROX® probe) ROS formation in HepG2 cells exposed to control conditions (Ctrl, untreated cells), IC_{50} of drugs (APAP 2.5 mM or SSZ 1.3 mM) alone, H_2S donor alone (80 µM NaHS), H_2S_n donor alone (80 µM Na_2S_2), co-treatments of drugs with the donors or H_2O_2 (positive control, 1mM) for 120 minutes. Scale bar represents 400 µm.

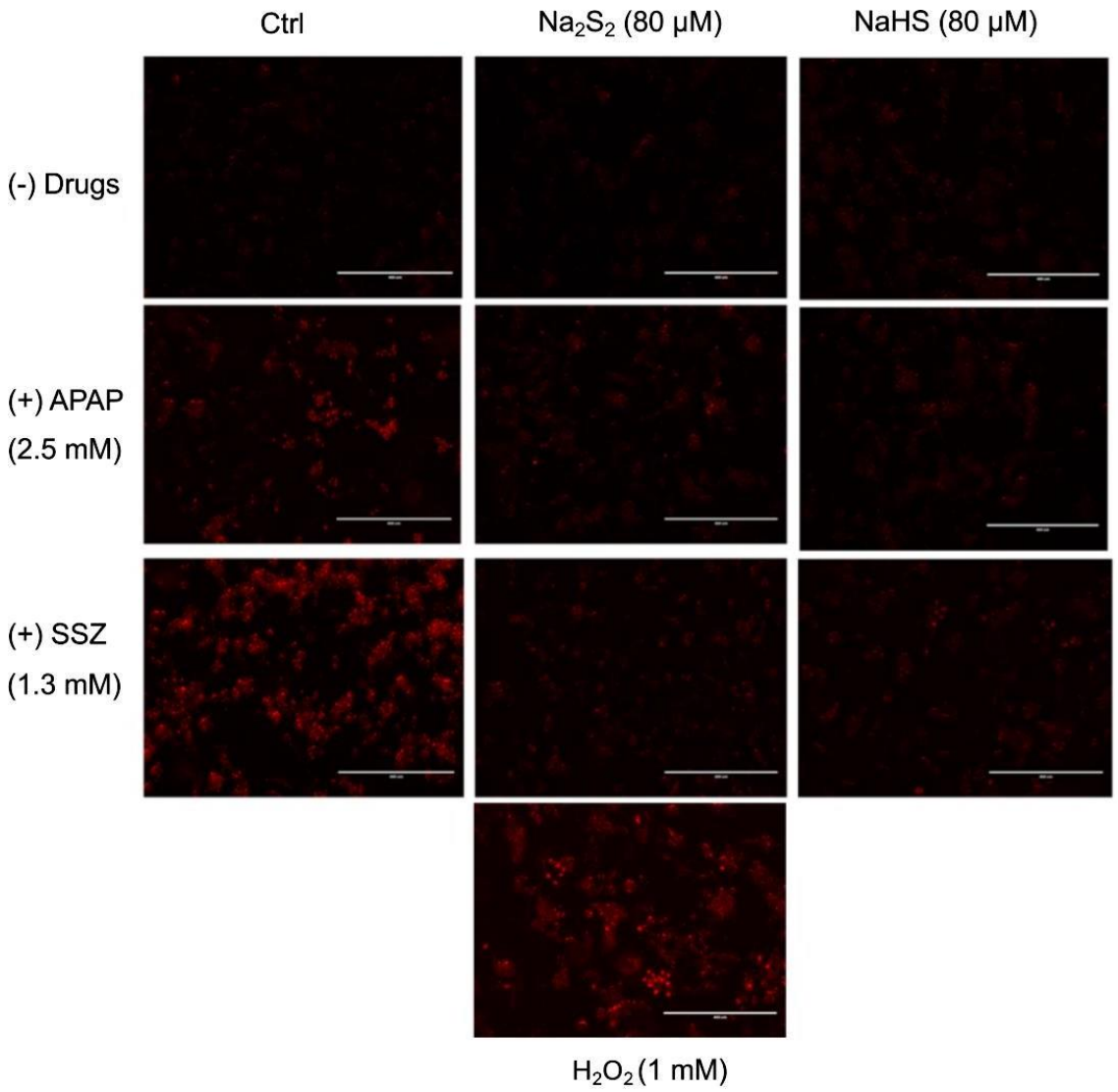


Figure 6. 8 Visualisation of mitochondrial (using MitoSOX™ probe) ROS formation in HepG2 cells exposed to control conditions (Ctrl, untreated cells), IC_{50} of drugs (APAP 2.5 mM or SSZ 1.3 mM) alone, H_2S donor alone (80 µM NaHS), H_2S_n donor alone (80 µM Na_2S_2), co-treatments of drugs with the donors or H_2O_2 (positive control, 1mM) for 120 minutes. Scale bar represents 400 µm.

As ROS production often occurs in parallel with lipid peroxidation (LPO) in cell membranes, we evaluated the ability of H_2S and H_2S_n to counteract APAP- and SSZ-induced LPO using the Image-iT® probe and by measuring Malondialdehyde (MDA) levels using Lipid Peroxidation (MDA) Assay Kit.

The Image-iT® Lipid Peroxidation Kit utilizes the BODIPY™(581 / 591) C11 reagent, a fluorescent molecule that detects lipid peroxidation. This indicator changes its fluorescence emission from red (590 nm) to green (510 nm) when lipids are oxidised. As shown in **Figure 6.9**, the IC_{50} concentrations of both APAP (2.5 mM) and SSZ (1.3 mM) increased LPO after 120 minutes of treatment, as evidenced by increased green fluorescence compared to untreated cells. This increase was comparable to the positive control (100 µM cumene hydroperoxide). However, when cells were co-treated with either 80 µM of NaHS or Na_2S_2 in addition to the drugs, the green fluorescence was reduced and was comparable to the untreated group at 120 minutes. These results can demonstrate that both sulphur-containing compounds mitigate APAP- and SSZ-induced LPO in HepG2 cells, suggesting this effect can be a potential component of their overall protective mechanism of NaHS and Na_2S_2. To confirm the observation, we further measured MDA levels when cells co-treated with NaHS or Na_2S_2. MDA is a major end product of LPO, formed ROS attack polyunsaturated fatty acids in cell membranes. The fluorescent images obtained from Image-iT® experiment were corelated with MDA levels. Indeed, accumulation of MDA levels reduced when HepG2 cells co-treated with NaHS and Na_2S_2 for 120 minutes compared to drug only group. MDA levels decreased from 4.49 ± 0.01 nmol / mg protein in the APAP-only group **to** 2.02 ± 0.01 nmol / mg protein with APAP + NaHS ($P = 0.021$) ($P < 0.01$)and **to** 1.1 ± 0.03 nmol / mg protein with APAP + Na_2S_2 ($P < 0.01$). Similarly, NaHS and Na_2S_2 reduced MDA in HepG2 cells treated with SSZ (4.35 ± 0.01 nmol / mg protein) **to** 2.12 ± 0.01 nmol / mg protein ($P = 0.03$) and 1.79 ± 0.01 nmol / mg protein ($P < 0.01$) at 120 minutes, respectively. Such time-course experiments would offer deeper insights into the protective mechanisms. However, we were unable to conduct these experiments in the current study due to time constraints.

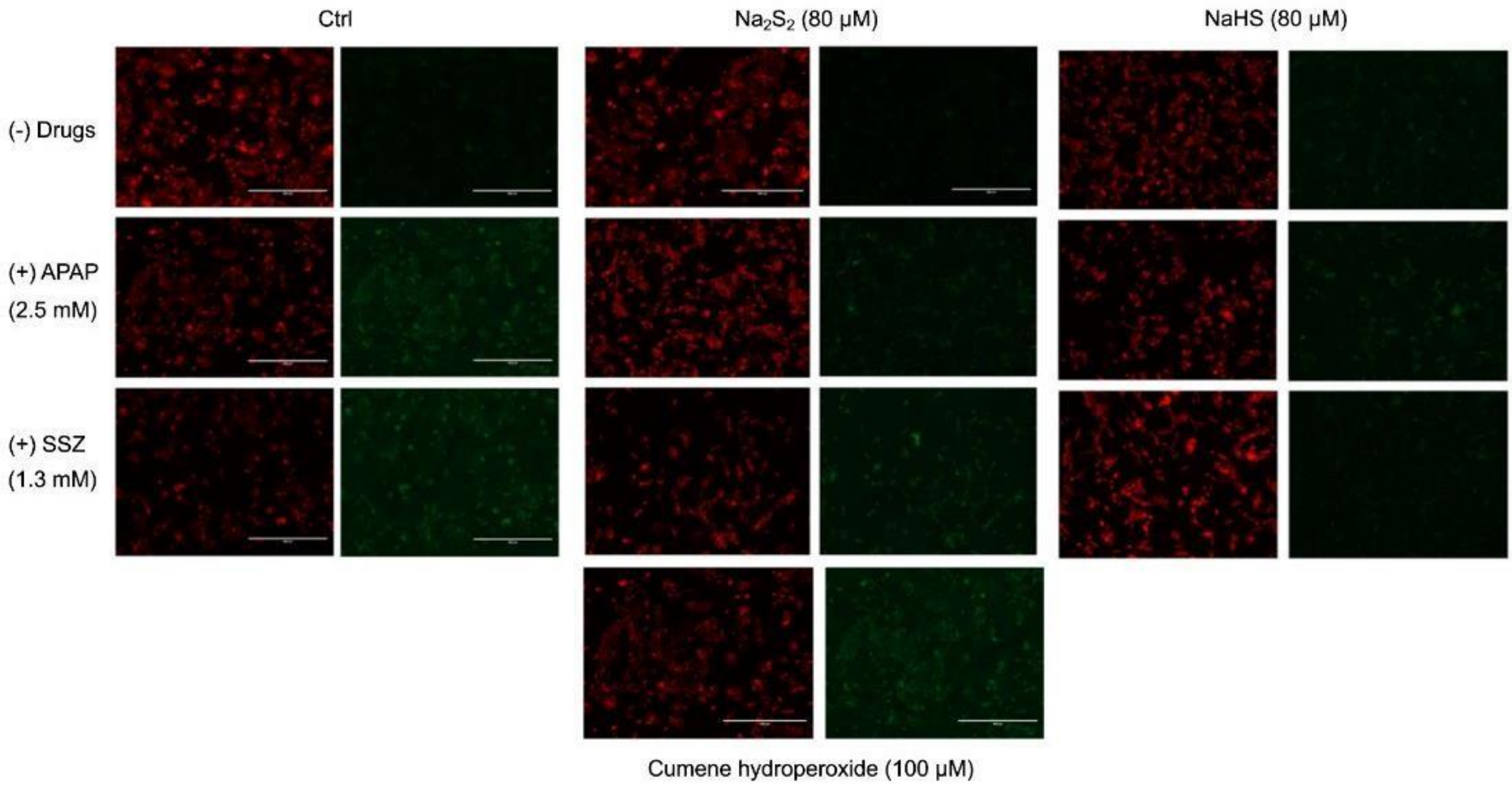


Figure 6. 9 Visualisation of lipid peroxidation (using Image-iT® probe) in HepG2 cells exposed to control conditions (Ctrl, untreated cells), IC_{50} of drugs (APAP 2.5 mM or SSZ 1.3 mM) alone, H_2S donor alone (80 µM NaHS), H_2S_n donor alone (80 µM Na_2S_2), co-treatments of drugs with the donors or cumene hydroperoxide (positive control, 100 µM) for 120 minutes. Scale bar represents 400 µm.

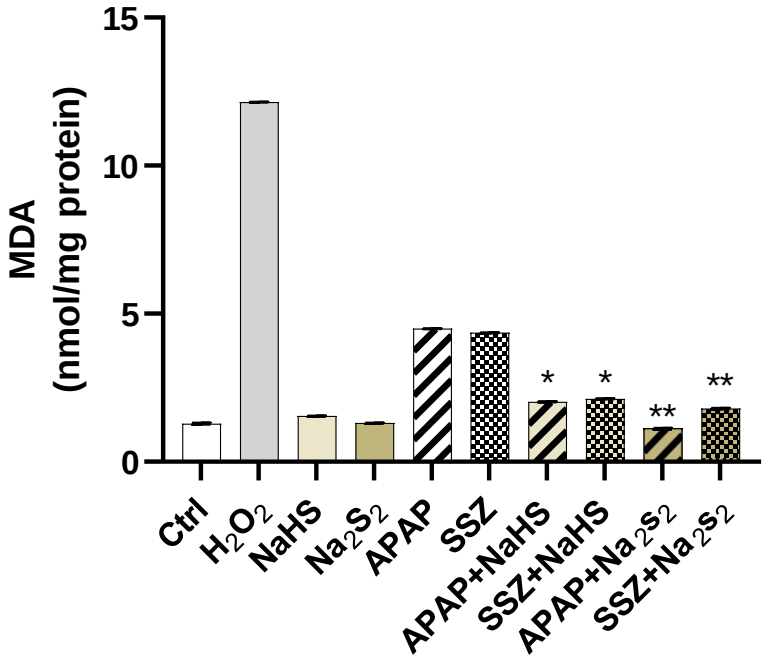


Figure 6.10 MDA levels in HepG2 cells at 120 minutes. Cells were exposed to control conditions (Ctrl, untreated cells), H_2O_2 (positive control, 1mM), IC_{50} of drugs (APAP 2.5 mM or SSZ 1.3 mM) alone, H_2S donor alone (80 µM NaHS), H_2S_n donor alone (80 µM Na_2S_2) or co-treatments of drugs with the donors. **(*)** $P < 0.05$ and **(**)** $P < 0.01$ for co-treatments compared to their respective drug-only treatments. Data represent means ± SEM of independent experiments (n = 3).

6.4.2.3 NaHS and Na_2S_2 ameliorate drugs induced mitochondrial depolarisation in HepG2 cells

The co-treatment of SSZ with either NaHS or Na_2S_2 alleviate the loss of mitochondrial membrane potential with the ratio significantly elevated **from** 0.73 ± 0.04 **to** 0.93 ± 0.07 and to 0.91 ± 0.04 (untreated cells **vs** SSZ + NaHS **vs** SSZ + Na_2S_2 ($P = 0.03$ and $P = 0.041$)), respectively (**Figure 6.11**). Results from this experiment suggested that H_2S and H_2S_n can inhibit the APAP- and SSZ-induced mitochondrial dysfunction in HepG2 cells. Possibly through a direct antioxidant effect or stimulation of other cellular protective mechanism. Time-dependent experiments would be helpful to better understand the role of NaHS and Na_2S_2 in preserving mitochondrial function.

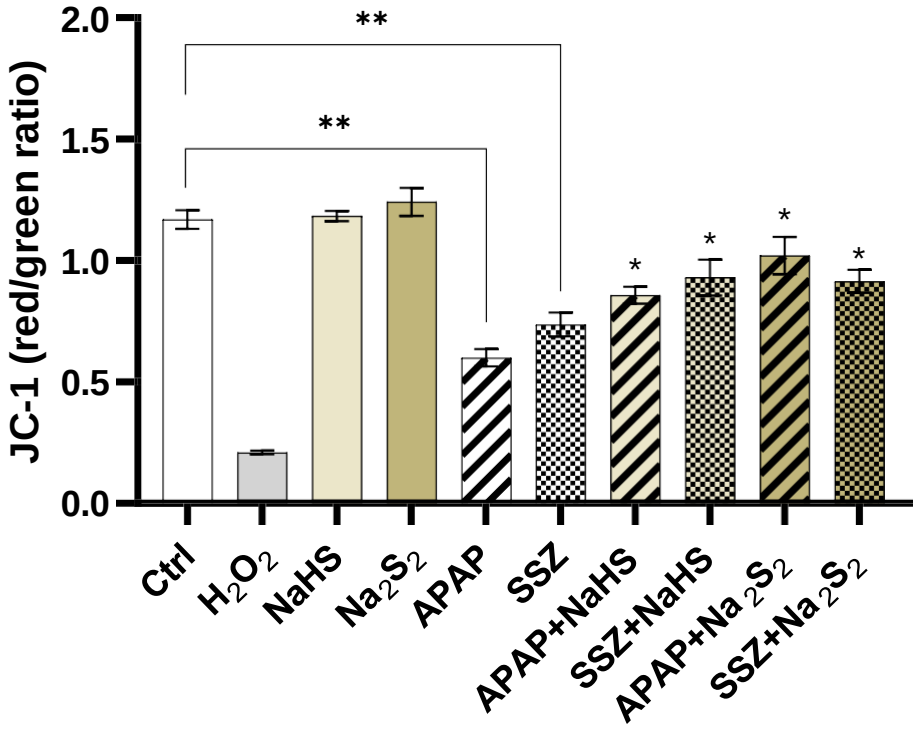


Figure 6.11 Mitochondrial membrane potential was assessed by JC-1 staining in HepG2 cells at 0, 30, 60 and 120 minutes after exposure to 2.5 mM APAP, 1.3 mM SSZ, 80 µM of Na_2S_2, drugs in combinations with 80 µM of Na_2S_2. **(*)** p <0 .05 **and (**)** p< 0.001 versus drug-only group. Data represent means ± SEM of independent experiments (n = 6).

6.4.2.4 NaHS and Na_2S_2 reduces drugs induced glutathione reductions in HepG2 cells

Following on from the early markers of oxidative stress results I next assessed intracellular glutathione levels. This was to aid in determining the molecular mechanisms by which H_2S and H_2S_n were promoting cytoprotection. To assess this, intracellular GSH levels was measured using a commercially available kit.

Figure 6.12 showed that 80 µM NaHS or Na_2S_2 significantly increased intracellular glutathione levels in HepG2 cells when co-treated with 2.5 mM APAP for 24 hours. Glutathione levels significantly elevated from 4.62 ± 0.03 nmol GSH / mg protein (APAP only) to 24.5 ± 0.22 nmol GSH / mg protein (APAP + NaHS) and 29.63 ± 1.21 nmol GSH / mg protein (APAP + Na_2S_2) ($P < 0.01$, respectively). Similarly for SSZ, NaHS or Na_2S_2 in combination increased intracellular GSH levels from 5.79 ± 0.16 nmol GSH / mg protein (SSZ only) to 23.7 ± 1.5 nmol GSH / mg protein (SSZ + NaHS) and 26.59 ± 0.06 nmol GSH / mg protein (SSZ + Na_2S_2) ($P < 0.01$, respectively). These results suggest that the elevation of GSH levels by NaHS or Na_2S_2 may be a potential mechanism for protecting HepG2 cells from drug-induced cell death.

In fact, we noticed significant elevation in GSH levels when cells treated with 80 µM Na_2S_2 alone, compared to control (untreated cells) (64.05 ± 0.17 nmol GSH / mg protein **vs** 54.9 ± 0.32 nmol GSH / mg protein, Na_2S_2 **vs** control, $P < 0.001$). However, 80 µM NaHS maintained intracellular GSH levels to a comparable levels to untreated cells (56.6 ± 1.16 nmol GSH / mg protein **vs** 54.9 ± 0.32 nmol GSH / mg protein, NaHS **vs** control, $P < 0.001$). This result could suggest that Na_2S_2 appears to have a more potent effect in elevating intracellular glutathione compared to NaHS.

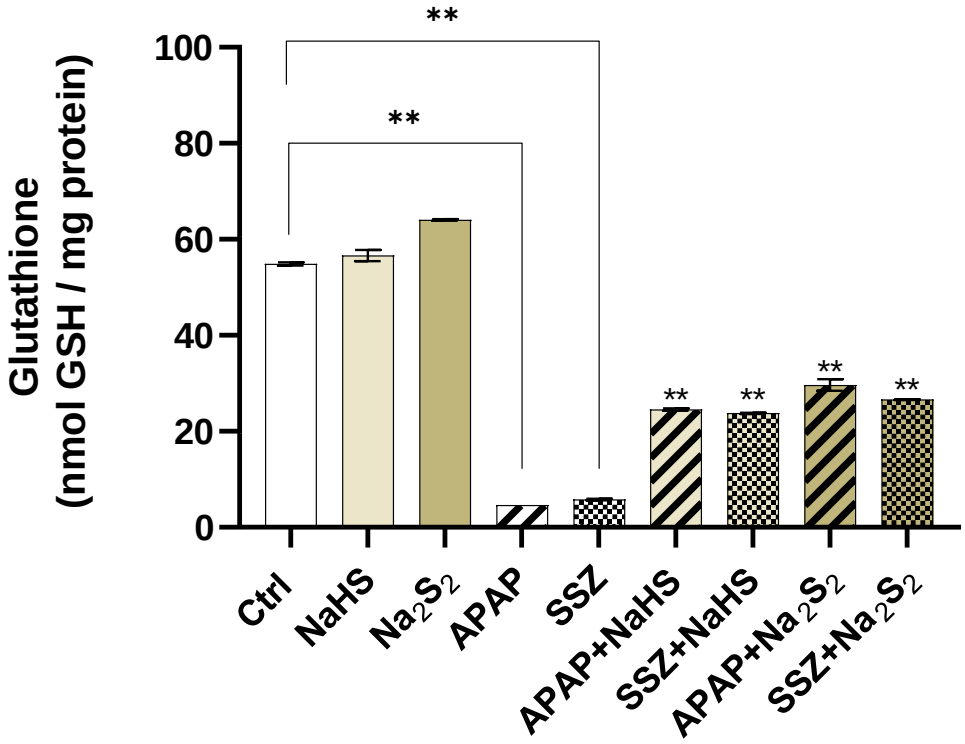


Figure 6.12 Intracellular total GSH levels measured in HepG2 cells treated with 2.5 mM APAP, 1.3 mM SSZ, 80 µM of Na_2S_2, 2.5 mM APAP + 80 µM Na_2S_2, 1.3 mM SSZ + 80 µM Na_2S_2 for 24 hours using a colorimetric assay. Cells were exposed to control conditions (Ctrl, untreated cells), H_2S donor alone (80 µM NaHS), H_2S_n donor alone (80 µM Na_2S_2), IC_{50} of drugs (APAP 2.5 mM or SSZ 1.3 mM) alone, or co-treatments of drugs with the donors. **(**)** $P < 0.01$ for co-treatments compared to their respective drug-only treatments. Data represent means ± SEM of independent experiments (n = 3).

6.4.2.5 NaHS and Na_2S_2 protect HepG2 cells against APAP- and SSZ-induced apoptosis

The protection effects of polysulphide were further assessed against apoptosis. To investigate this aspect of the study, I chose to examine three crucial indicators of apoptosis: LDH release, DNA damage and cleaved PARP levels.

To assess if H_2S and H_2S_n have roles in protecting cell membrane integrity, we measured LDH release. Almost all cells contain the cytosolic enzyme LDH, which is

leaked into the extracellular space when membranes are damaged and its release can be used to indicate apoptosis. We first noticed that HepG2 cells treated with 80 µM of either NaHS or Na_2S_2 for 24 hours have similar LDH levels compared to control (untreated cells) (5.0 ± 0.2 % **vs** 4.54 % ± 0.42 **vs** 5.20 % ± 0.41, NaHS **vs** Na_2S_2 **vs** control; $P > 0.05$). Following this, we further assessed the impact of H_2S and H_2S_n against APAP- and SSZ-mediated LDH release as shown in **Figure 6.13**. Indeed, compared to only APAP treated group (2.5 mM), extracellular LDH reduced from 20.6 ± 0.93 % **to** 12.0 ± 0.64 % when cells treated with 2.5 mM APAP + 80 µM NaHS and **to** 9.7 ± 0.40 % when cells treated with 2.5 mM APAP + 80 µM Na_2S_2 ($P < 0.05$ and $P < 0.01$, respectively), respectively. Similarly, a significant reduction in LDH release was noticed at 24 hours when HepG2 cells were co-treated with either NaHS or Na_2S_2 in addition to 1.3 mM SSZ, compared to cells treated with 1.3 mM SSZ only. The LDH release percentages were as follows: SSZ only group (30.9 ± 1.35 %), SSZ + NaHS group (19.4 ± 0.53 %) and SSZ + Na_2S_2 group (15.3 ± 0.49 %) ($P < 0.05$ and $P < 0.01$, respectively). These results indicate that both H_2S and H_2S_n can protect cell membrane damages in HepG2 cells treated with the drugs, suggesting a possible mechanism of cytorpotection.

Then, I looked at the second key hallmark of apoptosis, DNA damage. The impact of H_2S and H_2S_n on DNA damages caused by the drugs was assessed by looking at nucleus condensation using DAPI staining. As shown in **Figure 6.14**, HepG2 cells treated with IC_{50} concentrations of APAP (2.5 mM) or SSZ (1.3 mM) exhibited nuclear condensation and blebbing at 24 hours comparable to the positive control (1 mM H_2O_2). In contrast, cells treated with either 80 µM NaHS or Na_2S_2 showed blue fluorescence similar to untreated control cells. This result suggests that H_2S and H_2S_n donors may protect HepG2 cells from the nuclear damage induced by APAP and SSZ.

To test this, cells were treated with APAP or SSZ in combination with either H_2S or H_2S_n donors (NaHS and Na_2S_2, respectively). The co-treatments resulted in less intense blue DAPI staining and reduced nuclear condensation compared to cells treated with APAP or SSZ alone. These findings suggest a possible mechanism by which both H_2S and H_2S_n alleviate apoptosis via protection against DNA damage.

The last marker of apoptosis I assessed was PARP cleavage. PARP cleavage fragments serve as reliable indicators of apoptotic activity in cells. **Figure 6.15** showed that at 80 µM concentration, both NaHS and Na_2S_2 alone maintained cleaved PARP levels similar to those in untreated control cells at 24 hours treatment (0.53 ± 0.01 cleaved PARP/ mg protein **vs** 0.65 ± 0.02 cleaved PARP/ mg protein **vs** 0.83 ± 0.02 cleaved PARP/ mg protein; control **vs** NaHS **vs** Na_2S_2; $P > 0.05$). Then we assess the impact of these sulphur containing compounds on PARP cleavage levels when cells exposed to the drugs. PARP cleavage by APAP and SSZ was blocked with NaHS and Na_2S_2 at 24 hours of co-treatment (**Figure 5.15**). Indeed, 80 µM of either NaHS or Na_2S_2 in combination with 2.5 mM APAP significantly alleviated PARP cleavage from 4.16 ± 0.04 cleaved PARP/ mg protein to 2.38 ± 0.01 cleaved PARP/ mg protein and to 2.30 ± 0.02 cleaved PARP/ mg protein (2.5 mM APAP only **vs** APAP + NaHS **vs** APAP + Na_2S_2 ($P < 0.001$)), respectively. Similar protective effects of both NaHS and Na_2S_2 against SSZ-induced PARP cleavage in HepG2 cells was notices at 24 hours (from 8.31 ± 0.06 cleaved PARP/ mg protein vs 5.5 ± 0.04 cleaved PARP/ mg protein v**s** 3.42 ± 0.02 cleaved PARP/ mg protein (1.3 mM SSZ only **vs** SSZ + NaHS **vs** SSZ + Na_2S_2 ($P = 0.007$ and $P < 0.01$)), respectively. This suggests that reducing PARP cleavage is part of NaHS and Na_2S_2 protection effects against the apoptotic cascade induced by the drugs. It would be interesting to explore whether this protective effect occurs in a concentration-dependent manner. However, due to time limitations, I could

not further assess this, which would be intriguing to investigate in the future. Moreover, given that activated caspases cleave PARP during apoptosis, a western blot analysis of caspases in cells exposed to NaHS and Na_2S_2 in combination with the drugs would further extend our understanding of H_2S and H_2S_n's impact on apoptosis. Unfortunately, time constraints prevented us from conducting this set of experiments. Additionally, it should be noted that there was a different in cell density between the conducted assays, however, these were normalised to their respective controls in each assay.

Figure 6.16 represents a summary of a possible protective mechanism of NaHS and Na_2S_2 against APAP and SSZ in HepG2 cells based our results.

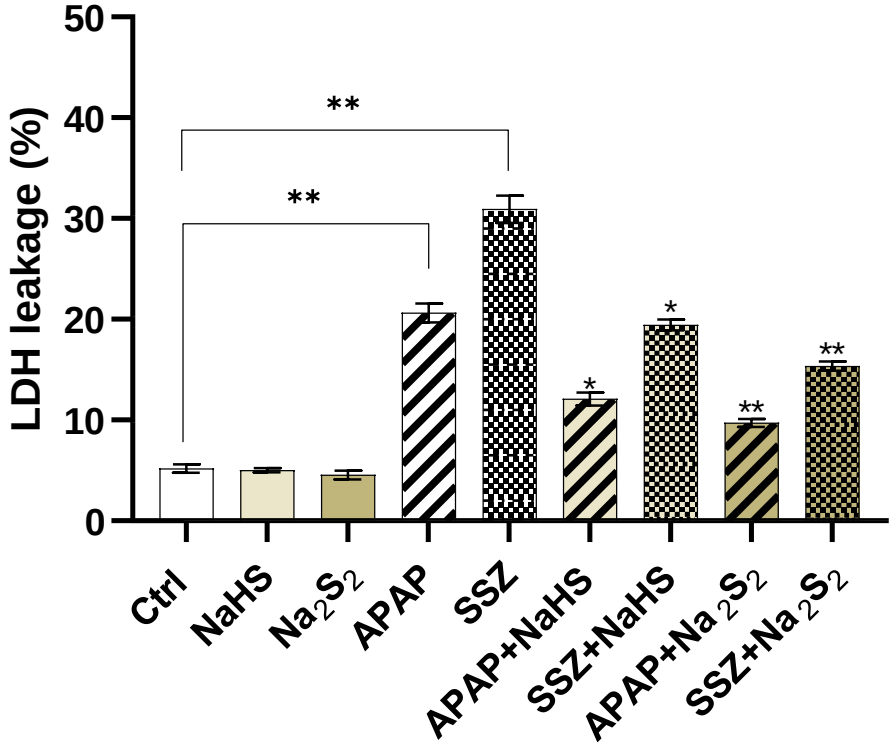


Figure 6.13 (A) LDH leakage (%)at 24 hours in HepG2 cells exposed to control conditions (Ctrl, untreated cells), IC_{50} of drugs (APAP 2.5 mM or SSZ 1.3 mM) alone, H_2S donor alone (80 µM NaHS), H_2S_n donor alone (80 µM Na_2S_2) or co-treatments of drugs with the donors. Data represent as % of means ± SEM of independent experiments (n = 6).

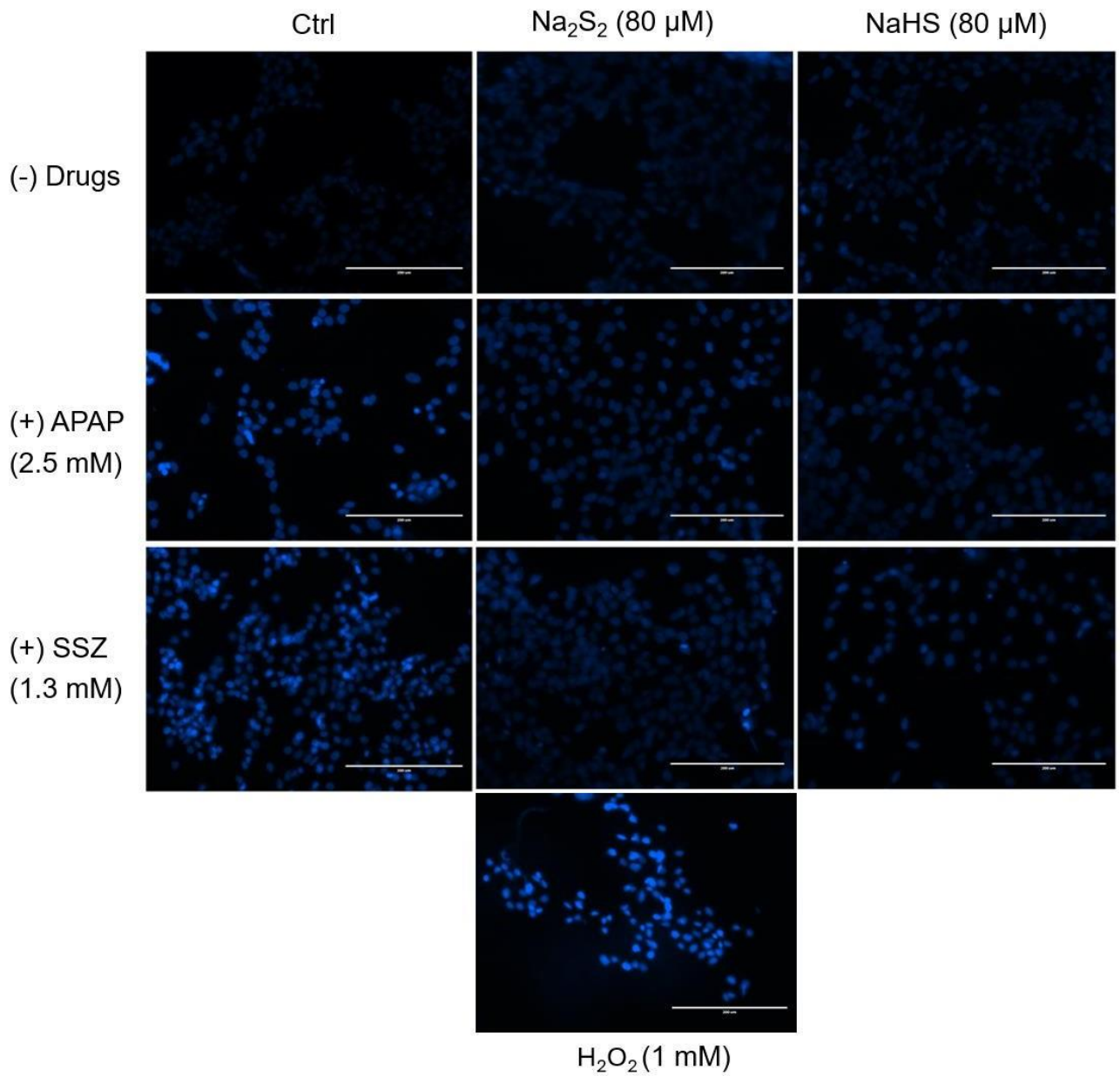


Figure 6. 14 DNA damage (using DAPI staining) in HepG2 cells exposed to control conditions (Ctrl, untreated cells), IC50 of drugs (APAP 2.5 mM or SSZ 1.3 mM) alone, H_2S donor alone (80 µM NaHS), H_2S_n donor alone (80 µM Na_2S_2), co-treatments of drugs with the donors or cumene hydroperoxide (positive control, 100 µM) for 24 hours prior to staining with DAPI.

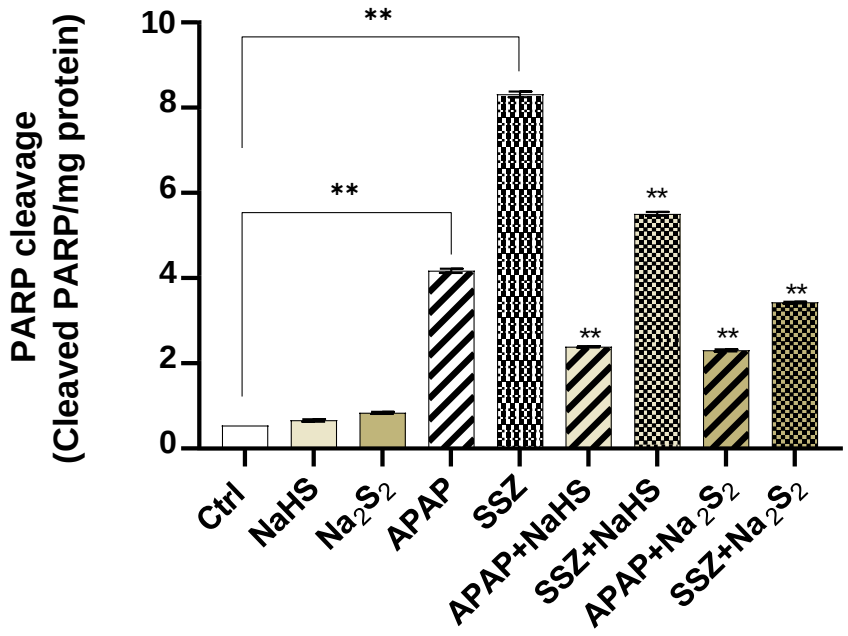


Figure 6.15 PARP cleavage was detected at 24 hours of in HepG2 cells exposed to control conditions (Ctrl, untreated cells), IC_{50} of drugs (APAP 2.5 mM or SSZ 1.3 mM) alone, H_2S donor alone (80 µM NaHS), H_2S_n donor alone (80 µM Na_2S_2) or co-treatments of drugs with the donors. Data represent means ± SEM **(**)** $p < 0.001$ versus drug only group (n = 3).

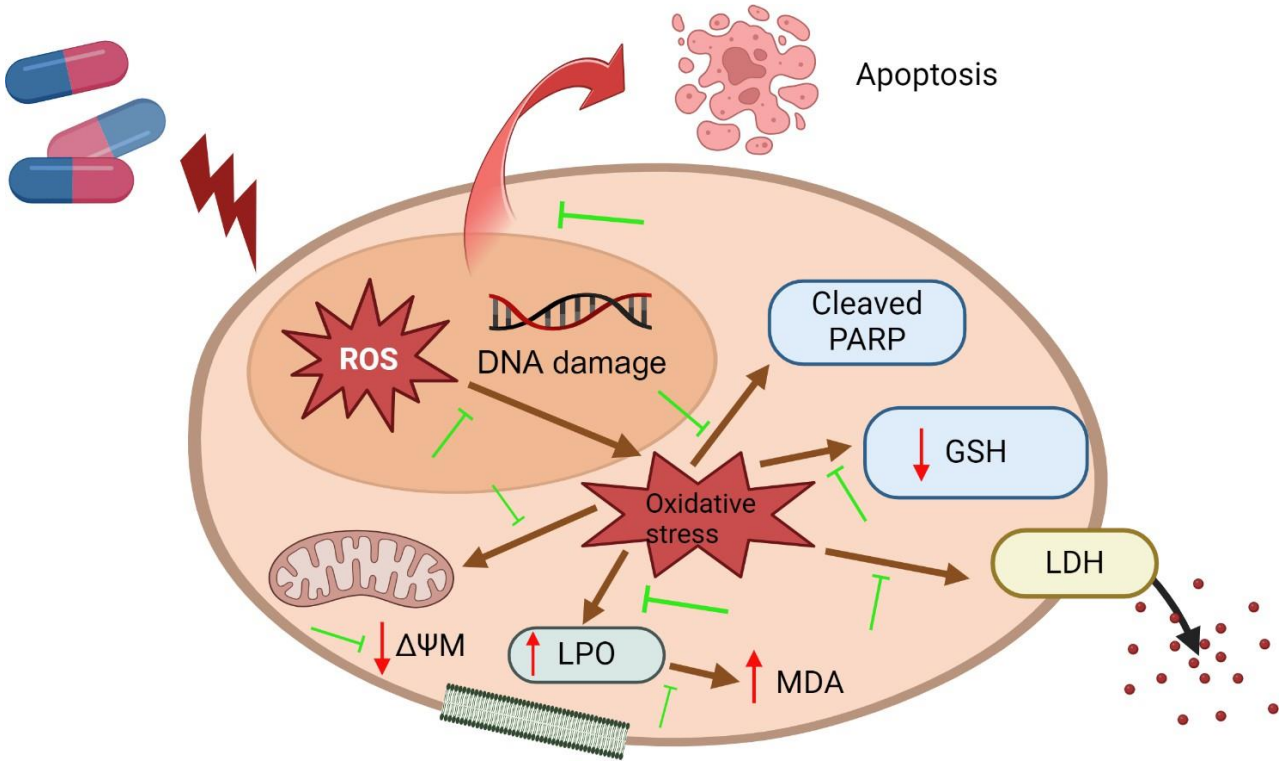


Figure 6.16 Model for possible mechanisms of NaHS and Na_2S_2 in protecting HepG2 cells treated with APAP and SSZ against oxidative stress markers and apoptosis. Based on our results, this figure showing NaHS and Na_2S_2 can reduce ROS formation, LPO and MDA, mitochondrial depolarisation, LDH leakage, DNA damage, GSH reduction, PARP cleavage and eventually cell death. **Abbreviations** used are; **ROS**, reactive oxygen species; **GSH**, glutathione; **LPO**, lipid peroxidation, **MDA**, malondialdehyde, **Δ Ψ m**, mitochondrial membrane potential , **LDH**, Lactate dehydrogenase, **PARP**, poly-ADP ribose polymeras . Green arrows represent NaHS and Na_2S_2 blocking action (illustration created with BioRender.com).

6.4.3 Antioxidant assays

During the assessment of the impact of H_2S and H_2S_n on the drugs-induced oxidative stress in HepG2 cell, we noted that these compounds mitigate the early oxidative events that drive apoptotic induction in HepG2 cells. This made us consider whether these compounds were having a direct antioxidant impact. We evaluated the antioxidant properties of NaHS and Na_2S_2 using two methods: ABTS assay and a copper reduction assay (by a commercially available kit (antioxidant assay kit).

The copper reduction assay measures total antioxidant capacity (TAC) by quantifying the reduction of Cu^{2+} to Cu^{+} by antioxidants in the sample. The resulting Cu^{+} forms a coloured complex, with the intensity of the colour being proportional to the antioxidant capacity. As shown in **Figure 6.17 (A)**, there was a concentration-dependent elevation in TAC for both NaHS and Na_2S_2. The ABTS assay quantifies the decolourisation of the ABTS radical cation (ABTS•+) as it is lowered by antioxidants. As shown in **Figure 6.17 (B)**, both NaHS and Na_2S_2 exhibit concentration-dependent ABTS inhibition, indicating increasing antioxidant activity ($P < 0.01$). At 80 µM, the inhibition reaches ≈ 64 % and ≈ 68 % for 80 µM NaHS and Na_2S_2, respectively. These antioxidant capabilities could explain, partially, their protective effects observed in cell culture experiments against APAP and SSZ.

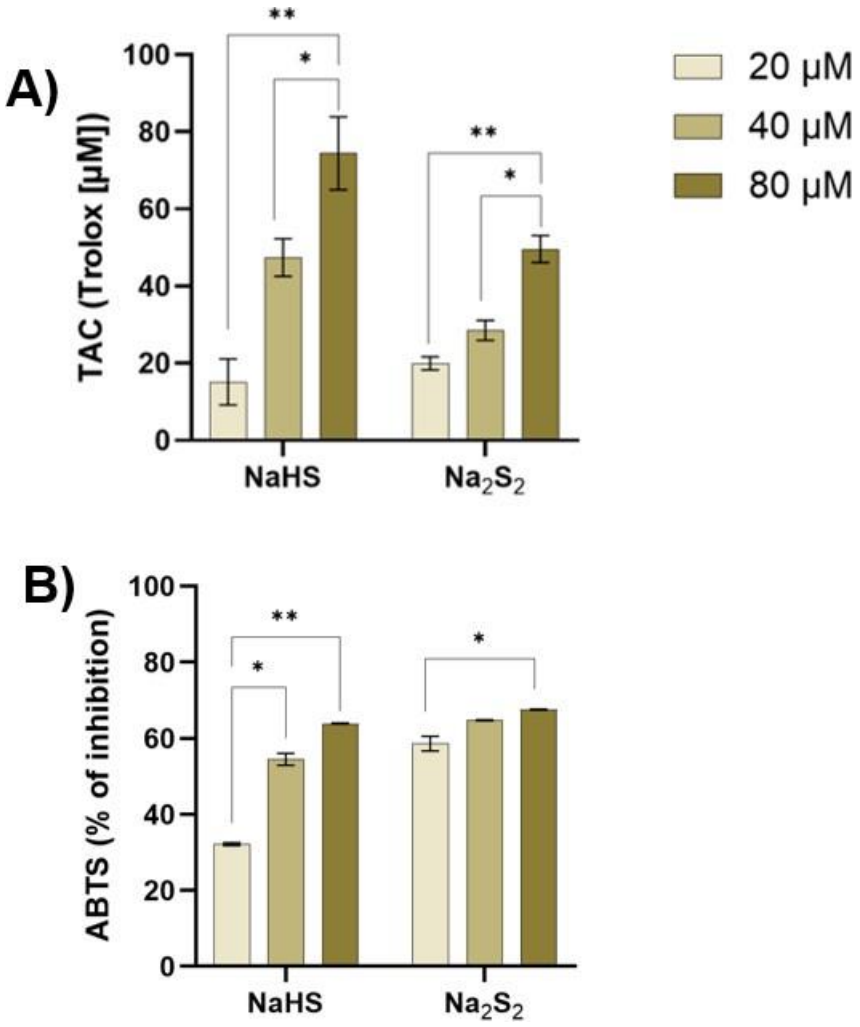


Figure 6.17 (A) Total antioxidant capacity of NaHS and Na_2S_2 at the three indicative concentrations expressed in Trolox equivalents **(B)** radical scavenging capacity of NaHS and Na_2S_2 determined using ABTS assay (n=3 for TAC and ABTS). Data represent means ± SEM **(**)** $p < 0.01$ **(*)** $p < 0.05$.

6.5 Discussion

In **chapter 5**, we have showed that APAP and SSZ induced cytotoxicity and mediated oxidative stress in HepG2 hepatoma cells. Hydrogen sulphide (H_2S) and polysulphide (H_2S_n) play critical roles in cells and tissues of mammals, supporting a number of processes including homeostasis, cellular antioxidant activity, cryoprotection and mitochondrial function (Uba et al., 2021; Sun et al., 2021; Yang et al., 2014). This made us considered whether APAP and SSZ also affect H_2S and H_2S_n levels. This is the first study, to our knowledge, that assess the impacts of APAP and SSZ on H_2S / H_2S_n production in HepG2 cells. We also assessed protein expression of H_2S production associated enzymes, CBS and CSE, as well as H_2S_n levels in HepG2 cells. Following this, we evaluate the impact of H_2S and H_2S_n donors (NaHS and Na_2S_2, respectively) on drug-induced oxidative stress markers, including cell death, ROS formation, LPO, mitochondrial depolarisation, LDH leakage, DNA damage, GSH depletion and PARP cleavage. Then, we investigate the potential antioxidant properties of NaHS and Na_2S_2 against drug-induced oxidative stress to further understand possible protective mechanism.

H_2S was previously only recognised as a toxic gas with a rotten egg odour, it has been now identified as the third endogenous signalling gasotransmitter (Rose, Moore and Zhu, 2017). H_2S produced in all mammalian cells, largely produced in the liver tissues (Sun et al., 2021). We (**Figure 6.1** and **6.2**) and others (Fiorucci et al., 2005) have confirmed the production of H_2S in hepatocytes. CBS and CSE are the primary producers of H_2S. There is a noticeable degree of tissue specificity, but CBS and CSE are widely distributed in tissues. The brain has a higher concentration of CBS, while peripheral tissues like blood vessels exhibit the highest levels of CSE activity (Łowicka and Bełtowski, 2007). Both CSE and CBS have been identified in the liver (Kimura,

2002), which were both detectable in our cell line. H_2S_n is produced, partially, by the oxidation of H_2S or its rection with NO (Kimura, 2021; Miyamoto et al., 2017). They are known as endogenous sulphur-containing molecules that many physiological roles have been recently attributed to this molecule. Polysulphides have been proposed to be an antioxidant (Kaneko et al. 2022) and cytoprotective (Takahashi et al., 2018). There are two main types of polysulphide that have been reported: organic and anions polysulphide. Organic polysulphide like garlic derived diallyl disulphides (DADS) and diallyltrisulphides (DATS) (Bradley, Organ and Lefer, 2016). The anion form, have the general formula S_n^{2-} which is the chemical basis of hydrogen polysulphide H_2S_n and sodium polysulphide Na_2S_n.

Previous investigations have demonstrated the potential impact of APAP and SSZ on H_2S levels and its key enzymes in mammalian systems, however with very limited evidence. Our study, focusing on APAP and SSZ influences on H_2S production in HepG2 cells, contributes to and expands upon the existing body of knowledge in this field of research. Results from the first set of experiments in this chapter suggest that APAP could indirectly affect H_2S production in HepG2 cell line by significantly reduced the expressions of its key producing enzymes namely CBS and CSE, while we have not observed significant direct reductions on both H_2S and H_2S_n, probably due to the short exposure time (up to 4 hours). SSZ showed interesting findings of significant elevations the expression of CSE enzymes at low concentrations (0.12 and 0.2 mM) at 24 hours, with non-significant reduction pattern regarding CBS. While cell studies are scarce, our results align with *in vivo* findings from Elshazly, El- Moselhy and Barakat (2014) in Wistar rats, who found APAP (1.5 g / kg i.p.) for 12 hours significantly reduced the expression of CBS enzyme in the liver. Similarly, Li et al. (2019) evaluated the involvement of JNK / MAPK signalling in APAP-induced acute liver failure, found that

APAP (150 mg / kg) for 12 hours treatment decreased the expression of both CBS and CSE proteins in liver tissues in male C57BL/6J mice. However, while these studies did not measure H_2S levels, Wiliński et al. (2011) reported that, in female CBA-strain mice, after five days of treatment with 30 mg / kg / day of APAP, the brain H_2S concentration was significantly lower in the treated group than in the control group (1.47 ± 0.02 µg / g **vs.** 0.80 ± 0.02 µg / g; $P < 0.01$: control **vs** 30 mg). While H_2S levels were higher in liver, kidney and heart compared to control group. A recent *in vitro* study showed that 200 µM SSZ for 12 hours significantly supressed the expression of CBS in BV2 microglial cells compared to the control ($P < 0.01$) (Wang et al., 2021). In contrast, when we used a similar concentration (250 µM) concentration of SSZ, no significant reduction was observed ($P = 0.132$) (**Figure 6.4 (B)**). Discrepancies in significance findings may be due to using different cell line, antibody or treatment regimen used. Although we report a non-significant pattern of CBS expression reduction with SSZ, previous *in vivo* studies showed significant reduction in CBS expression with other anti-inflammatory drugs such as aspirin (Cipriani et al., 2013) and diclofenac (Wallace et al., 2007). Reductions in H_2S levels were also mirrored in a wider range of medications, reviewed extensively (Alsaeedi et al., 2023).

Previous *in vivo* studies showed reduction in CSE and / or CBS induced by other medications lead to H_2S reduction (Ca et al., 2018; di Villa Bianca et al., 2015). These reduction was associated with health issues including increase blood pressure, inflammation and nephrotoxicity. Moreover, in gastric mucosal tissues, aspirin (125 mg

/ kg / ig) decreased the expression of CSE protein and the production of H_2S, which resulted in gastric lesions (Magierowski et al., 2016). In fact, CBS and CSE reduction has been associated with increased potential diseases such as atherosclerosis and bladder cancer (Renga, 2011) and hyperhomocystinuria leading to increased risk of CVDs (Nandi and Mishra, 2017), which can be contributed to decreased H_2S (Liu et al., 2018). Molecular interrogation showed that APAP can activate p-JNK (Yiang et al., 2015), which lead to inhibition in the expression of CBS and CSE enzymes. APAP does not significantly alter Specificity Protein 1 (SP1) levels directly (Malsy, Graf and Bundscherer, 2019), a critical transcription factor for CBS and CSE regulation, APAP can induce oxidative stress, as we have seen in **chapter 5**, that ultimately can reduce overall SP1 bindings (Chhunchha et al., 2018). SP1 reduction can lead to decreased expression of CSE and CBS enzymes. While SSZ, inhibiting NF-κB activation (Weber et al., 2000) and SP1 reduction (Liggett et al., 2014); leading to reduce the expression of CBS (Conan et al., 2023; Wu, Siow and Karmin, 2010).

Interestingly, we showed significant elevation in CSE expression at low concentrations (0.12 and 0.2 mM), although full time course analysis needed to further understand the picture, this result suggests that while lower SSZ concentrations trigger a compensatory upregulation of CSE to counteract the reduction in H_2S, although our study could not definitively confirm this mechanism. However, at high SSZ concentrations, the adaptive response fails, leading to decreased expression of the enzyme. Moreover, to the best our knowledge, we attempted for the first time assessing the impact of drugs on polysulphide levels. Our results showed no significant changes in H_2S_n and mirrored the H_2S production. Long time exposure of treatment is needed to fully understand the impact. There may be direct or indirect mechanisms involved in the complex effects on polysulphide levels. Although H_2S is

the main subject of most research, H_2S_n are produced naturally from H_2S in the presence of oxygen. Numerous effects are connected to changes in H_2S levels or H_2S -producing enzymes, which can impact the formation of polysulphides, which need further investigations.

Furthermore, building on the findings from **chapter 5**, which showed that APAP and SSZ induced cytotoxicity and mediated oxidative stress in HepG2 hepatoma cells, another set of results in this chapter provide evidence of the protective effects of both H_2S and H_2S_n against APAP- and SSZ-induced oxidative stress markers. We observed that NaHS and Na_2S_2 treatment significantly alleviated the cell viability reduction caused by the IC_{50} of APAP and SSZ in a concentration dependent manner ($P < 0.01$). Our mechanistic studies showed that both of the sulphur containing molecules reduce ROS formation, lipid peroxidation and mitochondrial depolarisation. This attenuation of oxidative stress correlated with decreased LDH leakage, DNA damage and PARP cleavage, as well as preservation of GSH levels. The findings of this chapter are consistent with those of Li et al. (2019), who observed that, after 12 hours of exposure to 20 mM APAP, NaHS significantly increased the viability (by 50 %) of human hepatocyte cell line (HL-7702), although the specific concentration of NaHS used was not stated. In comparison, our data show that treatment with 80 µM NaHS in the presence of either 2.5 mM APAP or 1.3 mM SSZ increased HepG2 cell viability by about 72 % and 7 0%, respectively, after a 24-hour period. Variations in exposure duration and drug concentrations may be the cause of the discrepancies seen across these investigations. However, their mechanistic findings indicate that NaHS lowered the expression of caspase-3 and attenuated APAP-induced increases in phosphorylated JNK (p-JNK). While Morsy et al. (2010) reported that 3 mg / kg, i.p. of NaHS administered to mice 30 minutes after an injection with a single dose (650 mg /

kg, i.p.) of APAP alleviated APAP-induced hepatotoxicity through antioxidant effects; enhancing GSH levels and reducing MDA levels, which was in agreement with our findings. In N2A cells, glutathione upregulation after polysulphide treatment was attributed to the alleviation in oxidative stress markers such as reductions in LDH leakage (Koike et al., 2013). H_2S may enhance the activity of cystine / glutamate antiporter, as well as the activity of glutamate-cysteine ligase, the rate-limiting enzyme in GSH synthesis. Together, these effects can lead to higher intracellular levels of GSH, which helps protect cells from oxidative stress (Kimura, Goto and Kimura, 2010).

Although studies on the effects of H_2S and H_2S_n on APAP and SSZ are limited, our results parallel those investigating other drugs such as cisplatin toxicity. Cao et al. (2018) demonstrated that Na_2S_4 (80 µM) pretreatment ameliorated cisplatin-induced cell death, caspase-3 cleavage and LDH leakage in renal proximal tubular cells. Compared to our findings, while both studies used the same concentration of polysulphide donor (80 µM), Cao et al. (2018) observed a higher % of LDH reduction (approximately 60%) compared to ours ≈ 50 % (for both drugs). This discrepancy can be attributed to **i)** the higher number of sulphur atoms in Na_2S_4 compared to Na_2S_2 used in our study. In fact, sodium polysulphides (Na_2S_n) tend to have higher antioxidant and cytoprotective potency with more sulphur atoms (Noguchi, Saito and Niki, 2023), and **ii)** different medications used. Despite this, our observations indicate that polysulphides with fewer sulphur atoms can still confer significant protection, supporting the cytoprotective role of polysulphide. Cao et al. (2018) also observed reduced ROS formation, as indicated by DCFDA fluorescence and suppressed MAPK activation; suggesting a common mechanism of polysulphide-mediated cytoprotection in mammalian cells. Polysulphides can modify specific cysteine residues in various functional proteins through sulphhydration, thereby regulating their activity and levels

(Kimura, 2015). Previous work suggested that the potential protective effects of polysulphide attributed to their direct ability of scavenging. Koike et al. (2016), reported that polysulphide alleviates cytotoxicity in SH-SY5Y cells against Methylglyoxal (MG), highly reactive dicarbonyl compound through the direct scavenging effects against MG. This work was in line with our results (the antioxidant assays (**Figure 6.17**)). The concentration-dependent increases in TAC and ABTS radical scavenging ability shown by NaHS and Na_2S_2 are consistent with earlier research that found 55.60 % DPPH radical scavenging activity with 50 µM NaHS (Ahmad et al., 2015), which is similar to our work (ABTS inhibition reached 54 % with 40 µM NaHS). Our findings are in parallel with other studies who found polysulphide alleviate oxidative stress markers. Indeed, Takahashi et al. (2018) reported that polysulphide significantly reduced ROS formation in rat midbrain slice cultures *via* enhancing glutathione synthesis. A recent animal study showed that polysulphide alleviates mitochondria dysfunction in diabetic mice (Xiong et al., 2023). Sun et al. (2021) reported that polysulphide alleviates apoptosis in HK-2 cells by reducing cleaved PARP and cleaved caspase-3. Kaneko et al. (2022) reported that polysulphide can reduce lipid peroxidation in human plasma.

Conclusion

Collectively, our results showed that APAP and SSZ could indirectly affect H_2S production as it reduces CBS and / or CSE. However, our current study has limitations i) short term drugs exposure which may contributed not the lack of effects ii) SSZ interferes with the probs we used (AzMC and SSP4), if time allowed, we could have tried another method such as Methylene blue (Olson, 2012). When discussing clinical applications, the *in vitro* concentrations of the drugs used in the current study should be interpreted with cautions, considering APAP and SSZ plasma concentrations in human. Moreover, our results with the aforementioned studies suggest that NaHS and

Na_2S_2 could be employed as a tool to alleviate drugs induced oxidative stress. It offers a means to manipulate cell protective systems to prevent cellular damage. More research may be necessary to determine whether polysulphides could be a promising therapeutic treatment for the drug toxicity in clinical trials. In the future, studies are needed to explore if polysulphide affecting the drugs anti-inflammatory (SSZ) and analgesic (APAP) activities. Our findings might also shed light on the potential benefits of polysulphide rich foods in APAP- and SSZ-induced oxidative stress. We expect that our present evidence would provide a basis for the further application of polysulphide in a wider range of commonly used drugs that induced oxidative stress.

Chapter 7 Overall discussion

7 General discussion

Global projections indicate a rise in life expectancy, potentially leading to increased medication use for managing multiple chronic conditions. This trend is further amplified by growing self-medication practices and economic pressures from the cost of living crisis. The escalating use of medications may expand the population at risk of micronutrient deficiencies or inadequacies. Despite the prevalence of medication use, research on drug-nutrient interactions remains limited. This is particularly concerning for essential metabolic processes, such as the trans-sulphuration pathway (TSP), which relies on vitamin B_6 for its function. Our research employed a diverse approach aiming to shed light on the impact of commonly-prescribed medications on the TSP.

Maintaining the flux of TSP is critical to production of metabolites playing important roles in cellular redox status, such as GSH, H_2S and taurine (Sbodio, Snyder and Paul, 2019). In **chapter 3**, we conducted a scoping review to assess the current evidence on the impacts of medications on TSP metabolites in humans, using paracetamol (APAP) as a model therapeutic. Our findings showed that, despite a lack of risk factors for dysregulate in GSH homeostasis among participants in this scoping review, significant GSH depletion was reported upon short-term APAP use (≤ 4 days); however no definitive conclusion could be made for other TSP metabolites, due to a lack of human studies which met the inclusion criteria. Although we acknowledge these metabolic targets are largely identified as GSH-related due to the known detoxification pathway for this compound, it has been reported that many people in the general population are now self-medicating with APAP. This review reinforces the importance of nutritional assessment when prescribing APAP, especially among vulnerable populations, such as the elderly. We highlighted animal studies that showed APAP can affect a wider range of TSP metabolites, not just GSH, but it would also be interesting

to measure these metabolites – such as Tau and H_2S – in humans using APAP and find out whether or not their reductions contributed to some side effects of APAP.

In **chapter 3** we evaluated the impact of drugs on the TSP flux by focusing on its metabolites; then, in **chapter 4**, we looked at the side reactions involved in the TSP (vitamin B_6), which could also impact on the TSP flux and potentially increase the relative risk of age-related diseases such as cognitive dysfunction. We carried out a secondary analysis using the NDNS dataset (2008 - 2017) to assess dietary vitamin B_6 and pyridoxal 5′-phosphate (PLP) levels among UK adults aged ≥ 19 years and to investigate the impact of common medications on vitamin B_6 status. Our results showed that median dietary vitamin B_6 intake of the UK population met the RNI, while median plasma PLP were higher than the threshold for vitamin B_6 deficiency. However, our data highlighted a trend of declining dietary vitamin B_6 intake and plasma PLP levels with advancing age. These findings raise concerns for the elderly population, as such a decline could potentially exacerbate age-related diseases (Porter et al., 2016), contribute to malnutrition (Zanetti et al., 2023) and lead to an overall decline in quality of life (Cantlay, Glyn and Barton, 2016). We also reported seven drug classes were associated with reduction of plasma PLP concentrations, namely lipid-lowering drugs, analgesics, antibacterials, antidiabetics, antidepressants, Ca^{2+} blocker and prescribed asthma medications. Previous evidence from cell culture and animal studies showed the impact of changing vitamin B_6 status on alteration of the TSP metabolites (Zhang et al., 2009; DeRatt et al., 2014). These studies suggested that low vitamin B_6 status reduced the enzymatic activity of CBS and CSE enzymes and H_2S production. Although we could not make a causal link between PLP reduction and TSP enzymes and metabolites in this study, due to restrictions imposed during COVID-19, our findings align with the possibility that restoration of vitamin B_6 homeostasis among medication

users may positively influence the disruption in TSP induced by these medications and thus overall health. Further investigations in humans using medications are needed to investigate whether vitamin B_6 reduction induced by medication use can be linked with TSP metabolites dysregulation: an interesting area to explore.

In **Chapter 5,** our research then focused on two representative medications, APAP and SSZ, to gain insights into their cellular-level impacts using an *in vitro* approach. We validated a human hepatoma HepG2 model to assess the cytotoxicity profiles of these drugs. Our results demonstrated that both APAP and SSZ reduce cell viability through several potential mechanisms, including ROS formation, LPO, mitochondrial depolarisation, as well as, and late apoptosis markers such as LDH leakage, PARP cleavage and DNA damage. Notably, APAP reduced GSH levels significantly without affecting cell viability; a crucial finding, as GSH depletion may accelerate other forms of oxidative stress, including DNA damage. This *in vitro* observation aligns with our findings in **chapter 3**, where APAP was shown to reduce GSH levels in human-based studies. The significance of these results is particularly concerning for vulnerable populations, such as the elderly, who often have naturally-reduced GSH levels (Lang et al., 1992) and are at increased risk of adverse effects. This susceptibility is further exacerbated by the common practice in this population of polypharmacy, highlighting the need for careful consideration when prescribing these medications to older adults.

In chapter 6, we explored the impact of APAP and SSZ on H_2S and H_2S_n levels in HepG2 cells. We hypothesised that APAP and SSZ would affect the TSP metabolite namely H_2S (and its oxidised form, H_2S_n), in the HepG2 cell line. Although we observed no direct impacts of these drugs on H_2S and H_2S_n reductions, SSZ interferes with the probes we used to measure H_2S and H_2S_n (AzMC and SSP4); this being the case, we could try another method in the future, such as Methylene blue.

We also reported that APAP significantly reduced the expressions of the key enzymes of H_2S synthesis (CBS and CSE) in a concentration-dependent manner. SSZ, for instance, led to a non-significant concentration-dependent reduction in the expression of CBS enzymes. Such reductions in CBS and CSE expression could reduce H_2S production, although this was not the case in our study, possibly due to the short treatment duration; we can therefore partially accept our hypothesis that APAP and SSZ affect H_2S production. Previous work linked the reduction of the key H_2S - producing enzymes (CBS and CSE) to H_2S reduction when medications were administered over longer periods, and potentially increased risk of chronic diseases such as high blood pressure (di Villa Bianca et al., 2015). To provide a more comprehensive understanding, future studies should include a full-time course analysis of CBS and CSE expression and enzymatic activity. This would help elucidate the temporal dynamics of drug impacts on the H_2S production pathway and potentially reveal changes in H_2S levels that were not apparent in our short-term exposure study.

Also, in **chapter 6**, we built on our findings from **chapter 5**, where we observed that APAP and SSZ induce early markers of oxidative stress alongside the later markers of apoptosis, leading to cell death. This study assessed whether or not these cellular damages could be alleviated by co-treatment with NaHS and Na_2S_2. Our study showed, to the best of our knowledge for the first time, that both H_2S and H_2S_n can alleviate oxidative stress in the HepG2 cell line induced by APAP and SSZ. Our mechanistic findings showed that both sulphur-containing compounds reduce ROS formation, lipid peroxidation, mitochondrial depolarisation, LDH leakage, DNA damage and PARP cleavage, and both NaHS and Na_2S_2 preserve GSH levels in HepG2 cells. We also reported that these donors exhibit antioxidant properties; as their antioxidant could also contributed to the potential protection

mechanism. These studies might shed light on the potential benefits of H_2S- and H_2S_n-rich foods such as cruciferous vegetables (e.g. broccoli, cauliflower, cabbage, kale) and alliums (e.g. garlic, onions, leeks, chives) in APAP- and SSZ-induced oxidative stress. We expect that our present evidence would provide a basis for the further application of hydrogen sulphide and polysulphide in a wider range of commonly-used drugs that induce oxidative stress.

Together, our overarching hypothesis – 'common medications, exemplified by APAP and SSZ, would reduce the TSP metabolites (H_2S, H_2S_n and GSH) and decrease the expression of the key enzymes (CBS and CSE) in HepG2 cells' – has been addressed and can be partially accepted. As a result of the short-time drugs exposure, we could not observe any reduction in H_2S and H_2S_n. However, we accept that both APAP and SSZ reduced CBS and / or CSE protein expressions in HepG2 cells. The cytotoxicity data (**chapter 5**) and scoping review (**chapter 3**) showed APAP significantly depletes another TSP metabolite, namely glutathione. However, additional long time drug exposure would help to get a bigger picture regarding the impact on H_2S / H_2S_n production alongside a full-time course of western blot, to better assess CBS and CSE protein expressions.

7.1 Potential future work

Although we could not conduct our original study due to COVID-19, the adaptation in our methodology not only allowed for the continuation of our research, but also opens up new areas for investigation:

- Our scoping review raised a knowledge gap which would be interesting to investigate; **i)** advancing age coupled with malnutrition with low protein intakes and other specific dietary patterns with low SAAs (such as vegetarian) are reported in animal models as risk factors for APAP-induced GSH depletion leading to toxicity;

whether or not this phenomenon and other health consequences, e.g. sarcopenia, would mirror such studies in humans needs further investigation; and **ii)** previous *in vitro* studies have demonstrated that a decrease in GSH levels can result in diminished H_2S production (Olson and Gao, 2019). Hence, we propose the hypothesis that the cardiovascular risks associated with APAP (Roberts et al., 2016) might be linked to reduced H_2S production, which may occur as a consequence of GSH depletion caused by APAP. Exploring these ideas further may provide new insights into the pharmacological profile of APAP and may also influence clinical practice for patients with cardiovascular comorbidities or those in vulnerable groups, such as the elderly. Moreover, future studies should also take into consideration the nutritional status / SAAs dietary intake of participants, as it was overlooked by most of the included studies in our scoping review. Data from humans is limited, and most of the included studies were for short-term APAP exposure; therefore, there is a need for longitudinal human studies.

- The NDNS findings draw attention to the possibility that a wider range of medications could affect vitamin B_6 status. As such, it would also be interesting to assess vitamin B_6 status among a wider range of drugs, as this would help healthcare professionals targeting at-risk populations to prevent vitamin B_6 deficiency / marginal deficiency. Future testing of our original hypothesis (that 'some commonly used medications can affect vitamin B_6 status among the elderly population in homecare in the UK, leading to alteration in the TSP metabolites i.e. H_2S and GSH') will help us gain a wider and more valuable picture in regards to the relationship between vitamin B_6, medication and TSP metabolites. This would assist in lowering potential health-related comorbidities.
- A comprehensive assessment of caspase activation, PARP cleavage and other

oxidative stress markers; examining both time course and concentration effects, would offer a more detailed understanding of the cell death mechanisms triggered by APAP and SSZ. This would provide valuable insights into how these drugs induce toxicity in hepatic cells. Moreover, future studies should be focused on the therapeutic ranges of the drugs to enhance the real-world applicability of these findings and offer a useful insight for clinical practice. For example, the therapeutic plasma APAP concentration in humans ranged from 66 to 132 µmol/L (Ahmed et al., 2024); this mirrored the low-usage concentrations (0.06 and 0.12 mM), while most of the concentration levels in the current work is considered supratherapeutic or potentially toxic levels (0.25 to 4 mM).

- A full-time course analysis of CBS and CSE expression and enzymatic activity. This would help elucidate the temporal dynamics of drug impacts on the H_2S production pathway. Long-term treatment with APAP could potentially reveal changes in H_2S levels that were not apparent in our short-term exposure study; this needs to be investigated.

7.2 Final conclusion

Our multi-faceted investigation provides evidence for medication-induced dysregulation of the TSP metabolites, enzymes and vitamin B_6 cofactor. This thesis therefore raises a call for human-based research to investigate whether reduced vitamin B_6 levels caused by medication usage are associated with TSP metabolites reduction. Research is also needed to measure other TSP metabolites in humans using medications such as Tau and H_2S and whether or not their reductions contributed to some side effects of APAP.

8 References

Abe, K. and Kimura, H., 1996. The possible role of hydrogen sulfide as an endogenous neuromodulator. *Journal of Neuroscience*, *16*(3), pp.1066-1071.

Adams, J.D., Lauterburg, B.H. and Mitchell, J.R., 1983. Plasma glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress. *Journal of Pharmacology and Experimental Therapeutics*, *227*(3), pp.749-754.

Ahmad, A., Sattar, M.Z., Rathore, H.A., Hussain, A.I., Khan, S.A., Fatima, T., Afzal, S., Abdullah, N.A. and Johns, E.J., 2015. Antioxidant activity and free radical scavenging capacity of L-arginine and NaHS: A comparative in vitro study. *Acta Pol Pharm*, *72*(2), pp.245-252.

Ahmed, H.M., Shehata, H.H., Mohamed, G.S., Abo-Gabal, H.H. and El-Daly, S.M., 2023. Paracetamol overdose induces acute liver injury accompanied by oxidative stress and inflammation. *Egyptian Journal of Chemistry*, *66*(3), pp.399-408.

Ahmed, Y.M., El-Zanaty, M.R., Galal, A. and Atta, N.F., 2024. New insight for simultaneous detection of methocarbamol and paracetamol in human serum samples based on poly (L-methionine)/graphene-graphene oxide nanocomposite. *Microchemical Journal*, *205*, p.111370.

Alabdallat, N.G., 2021. Antioxidant Properties of Orally Administered of Aqueous Extracts of Selected Medicinal Plants and Paracetamol in Human Volunteers: In vivo. *Journal of Contemporary Medical Sciences*, *7*(3), pp.179-182.

AlKhamees, O.A., AlNemer, K.A., Maneea, M.W.B., AlSugair, F.A., AlEnizi, B.H. and Alharf, A.A., 2018. Top 10 most used drugs in the Kingdom of Saudi Arabia 2010–2015. *Saudi Pharmaceutical Journal*, *26*(2), pp.211-216.

Allgood, V.E. and Cidlowski, J.A., 1992. Vitamin B6 modulates transcriptional activation by multiple members of the steroid hormone receptor superfamily. *Journal of Biological Chemistry*, *267*(6), pp.3819-3824.

Alorfi, N.M. 2023. Pharmacological methods of pain management: Narrative review of medication used. *International Journal of General Medicine*, pp.3247-3256.

Alsaeedi, A., Welham, S., Rose, P. and Zhu, Y.Z., 2023. The Impact of Drugs on Hydrogen Sulfide Homeostasis in Mammals. *Antioxidants*, *12*(4), p.908.

Amaral, F.M., Miranda-Vilela, A.L., Lordelo, G.S., Ribeiro, I.F., Daldegan, M.B. and Grisolia, C.K., 2017. Interactions among methylenetetrahydrofolate reductase (mthfr) and cystathionine β-synthase (cbs) polymorphisms-a cross-sectional study: multiple heterozygosis as a risk factor for higher homocysteine levels and vaso-occlusive episodes. *Genet Mol Res*, *16*(1), p.16019374.

Aminzadeh, M.A. and Vaziri, N.D., 2012. Downregulation of the renal and hepatic hydrogen sulfide (H2S)-producing enzymes and capacity in chronic kidney disease. *Nephrology Dialysis Transplantation*, *27*(2), pp.498-504.

Andrés, C.M.C., Pérez de la Lastra, J.M., Andrés Juan, C., Plou, F.J. and Pérez-Lebeña, E., 2023. Chemistry of Hydrogen Sulfide—Pathological and Physiological Functions in Mammalian Cells. *Cells*, *12*(23), p.2684.

Anishchenko, E., Vigorito, C., Mele, L., Lombari, P., Perna, A.F. and Ingrosso, D., 2019. Novel applications of lead acetate and flow cytometry methods for detection of sulfur-containing molecules. *Methods and Protocols*, *2*(1), p.13.

Ankar, A. and Kumar, A., 2022. Vitamin B12 deficiency. In *StatPearls [Internet]*. StatPearls Publishing.

Asmat, U., Abad, K. and Ismail, K., 2016. Diabetes mellitus and oxidative stress—A concise review. *Saudi pharmaceutical journal*, *24*(5), pp.547-553.

Australian Institute of Health and Welfare (AIHW) (2024). Medicines in the health system.

Available at: https://www.aihw.gov.au/reports/medicines/medicines-in-the-health-system (Accessed April 2022).

Averill-Bates, D.A., 2023. The antioxidant glutathione. In *Vitamins and hormones* (Vol. 121, pp. 109-141). Academic Press.

Ayoub, S.S., 2021. Paracetamol (acetaminophen): A familiar drug with an unexplained mechanism of action. *Temperature*, *8*(4), pp.351-371.

Baba, S.P. and Bhatnagar, A., 2018. Role of thiols in oxidative stress. *Current opinion in toxicology*, *7*, pp.133-139.

Bader, A., Petters, O., Keller, M. and Pavlica, S., 2011. Paracetamol treatment increases telomerase activity in rat embryonic liver cells. *Pharmacological Reports*, *63*(6), pp.1435-1441.

Badr, H., Kozai, D., Sakaguchi, R., Numata, T. and Mori, Y., 2016. Different contribution of redox-sensitive transient receptor potential channels to acetaminophen-induced death of human hepatoma cell line. *Frontiers in pharmacology*, *7*, p.19.

Bai, J. and Cederbaum, A.I., 2004. Adenovirus mediated overexpression of CYP2E1 increases sensitivity of HepG2 cells to acetaminophen induced cytotoxicity. *Molecular and cellular biochemistry*, *262*, pp.165-176.

Baines, M., Kredan, M.B., Davison, A., Higgins, G., West, C., Fraser, W.D. and Ranganath, L.R., 2007. The association between cysteine, bone turnover, and low bone mass. *Calcified Tissue International*, *81*, pp.450-454.

Balboa-Castillo, T., Struijk, E.A., Lopez-Garcia, E., Banegas, J.R., Rodríguez-Artalejo, F. and Guallar-Castillon, P., 2018. Low vitamin intake is associated with risk of frailty in older adults. *Age and ageing*, *47*(6), pp.872-879.

Ballatori, N., Krance, S.M., Notenboom, S., Shi, S., Tieu, K. and Hammond, C.L., 2009. Glutathione dysregulation and the etiology and progression of human diseases.

Bangyuan, W., Hengmin, C., Xi, P., Jing, F., Wei, C. and Xiaodong, L., 2012. Pathology of spleen in chickens fed on a diet deficient in methionine. *Health*, *2012*.

Bardel, A., Wallander, M.A. and Svärdsudd, K., 2000. Reported current use of prescription drugs and some of its determinants among 35 to 65-year-old women in mid-Sweden: A population-based study. *Journal of Clinical Epidemiology*, *53*(6), pp.637-643.

Bartali, B., Semba, R.D., Frongillo, E.A., Varadhan, R., Ricks, M.O., Blaum, C.S., Ferrucci, L., Guralnik, J.M. and Fried, L.P., 2006. Low micronutrient levels as a predictor of incident disability in older women. *Archives of internal medicine*, *166*(21), pp.2335-2340.

Basu, C. and Sur, R., 2018. S-allyl cysteine alleviates hydrogen peroxide induced oxidative injury and apoptosis through upregulation of Akt/Nrf-2/HO-1 signaling pathway in HepG2 cells. *BioMed Research International*, *2018*.

Baum, F., Musolino, C., Gesesew, H.A. and Popay, J., 2021. New perspective on why women live longer than men: An exploration of power, gender, social determinants, and capitals. *International journal of environmental research and public health*, *18*(2), p.661.

Beard Jr, R.S. and Bearden, S.E., 2011. Vascular complications of cystathionine β-synthase deficiency: Future directions for homocysteine-to-hydrogen sulfide research. *American Journal of Physiology-Heart and Circulatory Physiology*, *300*(1), pp.H13-H26.

Beaudart, C., Sanchez-Rodriguez, D., Locquet, M., Reginster, J.Y., Lengelé, L. and Bruyère, O., 2019. Malnutrition as a strong predictor of the onset of sarcopenia. *Nutrients*, *11*(12), p.2883.

Becquet, P., Vazquez-Anon, M., Mercier, Y., Batonon-Alavo, D.I., Yan, F., Wedekind, K. and Mahmood, T., 2023. Absorption of methionine sources in animals—is there more to know?. *Animal Nutrition*, *12*, pp.159-170.

Behrends, V., Giskeødegård, G.F., Bravo-Santano, N., Letek, M. and Keun, H.C., 2019. Acetaminophen cytotoxicity in HepG2 cells is associated with a decoupling of glycolysis from the TCA cycle, loss of NADPH production, and suppression of anabolism. *Archives of toxicology*, *93*(2), pp.341-353.

Bennadi, D., 2013. Self-medication: A current challenge. *Journal of basic and clinical pharmacy*, *5*(1), p.19.

Bennett Institute for Applied Data Science. (2020, March). What are the most commonly prescribed medicines? Top 10 prescribed medicines in NHS England primary care for 2019. University of Oxford. Available at: https://www.bennett.ox.ac.uk/blog/2020/03/what-are-the-most-commonly-prescribed-medicines-top-10-prescribed-medicines-in-nhs-england-primary-care-for-2019/ (accessed April 2022)

Berger, M.M., Shenkin, A., Schweinlin, A., Amrein, K., Augsburger, M., Biesalski, H.K., Bischoff, S.C., Casaer, M.P., Gundogan, K., Lepp, H.L. and De Man, A.M., 2022. ESPEN micronutrient guideline. *Clinical Nutrition*, *41*(6), pp.1357-1424.

Berry, T.M. and Moustafa, A.A., 2021. Osteoporosis and the effect of dysregulation of the transsulfuration pathway via taurine on intracellular calcium homeostasis, vitamin D absorption and vitamin K absorption. *Clinical nutrition ESPEN*, *43*, pp.191-196.

Bianchi, G., Brizi, M., Rossi, B., Ronchi, M., Grossi, G. and Marchesini, G., 2000. Synthesis of glutathione in response to methionine load in control subjects and in patients with cirrhosis. *Metabolism-Clinical and Experimental*, *49*(11), pp.1434-1439.

Bilski, P., Li, M.Y., Ehrenshaft, M., Daub, M.E. and Chignell, C.F., 2000. Vitamin B6 (pyridoxine) and its derivatives are efficient singlet oxygen quenchers and potential fungal antioxidants. *Photochemistry and photobiology*, *71*(2), pp.129-134.

Bio-rad, L. 2012. A guide to polyacrylamide gel electrophoresis and detection. *Laboratories Bio-Rad.*

Blidisel, A., Marcovici, I., Coricovac, D., Hut, F., Dehelean, C.A. and Cretu, O.M., 2021. Experimental models of hepatocellular carcinoma—a preclinical perspective. *Cancers*, *13*(15), p.3651.

Blom, H.J. and Smulders, Y., 2011. Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects. *Journal of inherited metabolic disease*, *34*, pp.75-81.

Bonaga, B., Sánchez-Jurado, P.M., Martínez-Reig, M., Ariza, G., Rodríguez-Mañas, L., Gnjidic, D., Salvador, T. and Abizanda, P., 2018. Frailty, polypharmacy, and health outcomes in older adults: the frailty and dependence in albacete study. *Journal of the American Medical Directors Association*, *19*(1), pp.46-52.

Bouras, H., Roig, S.R., Kurstjens, S., Tack, C.J., Kebieche, M., de Baaij, J.H. and Hoenderop, J.G., 2020. Metformin regulates TRPM6, a potential explanation for magnesium imbalance in type 2 diabetes patients. *Canadian journal of physiology and pharmacology*, *98*(6), pp.400-411.

Bradley, J.M., Organ, C.L. and Lefer, D.J., 2016. Garlic-derived organic polysulfides and myocardial protection. *The Journal of Nutrition*, *146*(2), pp.403S-409S.

Braunstein, I., Engelman, R., Yitzhaki, O., Ziv, T., Galardon, E. and Benhar, M., 2020. Opposing effects of polysulfides and thioredoxin on apoptosis through caspase persulfidation. *Journal of Biological Chemistry*, *295*(11), pp.3590-3600.

Bridges, R.J., Natale, N.R. and Patel, S.A., 2012. System xc-cystine/glutamate antiporter: an update on molecular pharmacology and roles within the CNS. *British journal of pharmacology*, *165*(1), pp.20-34.

British Heart Foundation. (2024). UK Factsheet. Available at: https://www.bhf.org.uk/-/media/files/for-professionals/research/heart-statistics/bhf-cvd-statistics-uk-factsheet.pdf (Accessed on 14 April 2023)

British Nutrition Foundation (2021), Nutrition requirements https://www.nutrition.org.uk/media/1z2ekndj/nutrition-requirements-update.pdf (Accessed in April 2020)

British Nutrition Foundation (BNF) (2021).Vitamins and minerals Available at: https://www.nutrition.org.uk/media/nmmewdug/nutrition-requirements.pdf (Accessed April 2022).

British Nutrition Foundation (BNF) (2023).Vitamins and minerals Available at: https://www.nutrition.org.uk/nutritional-information/vitamins-and-minerals/ (Accessed April 2022).

Broer, S., 2008. Amino acid transport across mammalian intestinal and renal epithelia. *Physiological reviews*, *88*(1), pp.249-286.

Brosnan, J.T. and Brosnan, M.E., 2006. The sulfur-containing amino acids: an overview. *The Journal of nutrition*, *136*(6), pp.1636S-1640S.

Brown, M.J., Ameer, M.A., Daley, S.F. and Beier, K., 2017. Vitamin B6 deficiency.

Burdo, J., Dargusch, R. and Schubert, D., 2006. Distribution of the cystine/glutamate antiporter system x− c in the brain, kidney, and duodenum. *Journal of Histochemistry & Cytochemistry*, *54*(5), pp.549-557.

Burgunder, J.M. and Lauterburg, B.H., 1987. Decreased production of glutathione in patients with cirrhosis. *European journal of clinical investigation*, *17*(5), pp.408-414.

Burgunder, J.M., Varriale, A. and Lauterburg, B.H., 1989. Effect of N-acetylcysteine on plasma cysteine and glutathione following paracetamol administration. *European journal of clinical pharmacology*, *36*, pp.127-131.

Busch, M., Göbert, A., Franke, S., Ott, U., Gerth, J., Müller, A., Stein, G., Bitsch, R. and Wolf, G., 2010. Vitamin B6 metabolism in chronic kidney disease–relation to transsulfuration, advanced glycation and cardiovascular disease. *Nephron Clinical Practice*, *114*(1), pp.c38-c46.

Cahyani, D.I., Puryatni, A. and Permatasari, N., 2017. Cysteine, Malondyaldehide (MDA) and Glutathione (GSH) Levels in Marasmic Type Malnutrition. *Journal of Tropical Life Science*, *7*(2), pp.151-157.

Calderón-Ospina, C.A. and Nava-Mesa, M.O., 2020. B Vitamins in the nervous system: Current knowledge of the biochemical modes of action and synergies of thiamine, pyridoxine, and cobalamin. *CNS neuroscience & therapeutics*, *26*(1), pp.5-13.

Cantlay, A., Glyn, T. and Barton, N., 2016. Polypharmacy in the elderly. *InnovAiT*, *9*(2), pp.69-77.

Cao, X., Nie, X., Xiong, S., Cao, L., Wu, Z., Moore, P.K. and Bian, J.S., 2018. Renal protective effect of polysulfide in cisplatin-induced nephrotoxicity. *Redox biology*, *15*, pp.513-521.

Cao, X., Zhang, W., Moore, P.K. and Bian, J., 2019. Protective smell of hydrogen sulfide and polysulfide in cisplatin-induced nephrotoxicity. *International journal of molecular sciences*, *20*(2), p.313.

Cattaneo, M., Lombardi, R., Lecchi, A., Bucciarelli, P. and Mannucci, P.M., 2001. Low plasma levels of vitamin B6 are independently associated with a heightened risk of deep-vein thrombosis. *Circulation*, *104*(20), pp.2442-2446.

Ceballos-Picot, I., Witko-Sarsat, V., Merad-Boudia, M., Nguyen, A.T., Thévenin, M., Jaudon, M.C., Zingraff, J., Verger, C., Jingers, P. and Descamps-Latscha, B., 1996. Glutathione antioxidant system as a marker of oxidative stress in chronic renal failure. *Free Radical Biology and Medicine*, *21*(6), pp.845-853.

Cederholm, T., Bosaeus, I., Barazzoni, R., Bauer, J., Van Gossum, A., Klek, S., Muscaritoli, M., Nyulasi, I., Ockenga, J., Schneider, S.M. and de van der Schueren, M.A., 2015. Diagnostic criteria for malnutrition–an ESPEN consensus statement. *Clinical nutrition*, *34*(3), pp.335-340.

Cena, H. and Calder, P.C., 2020. Defining a healthy diet: evidence for the role of contemporary dietary patterns in health and disease. *Nutrients*, *12*(2), p.334.

Chaitanya, G.V. and Babu, P.P., 2008. Activation of calpain, cathepsin-b and caspase-3 during transient focal cerebral ischemia in rat model. *Neurochemical Research*, *33*, pp.2178-2186.

Chaitanya, G.V., Alexander, J.S. and Babu, P.P., 2010. PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. *Cell communication and signaling*, *8*, pp.1-11.

Charlesworth, C.J., Smit, E., Lee, D.S., Alramadhan, F. and Odden, M.C., 2015. Polypharmacy among adults aged 65 years and older in the United States: 1988–2010. *Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences*, *70*(8), pp.989-995.

Chatterjee, S., 2016. Oxidative stress, inflammation, and disease. In *Oxidative stress and biomaterials* (pp. 35-58). Academic Press.

Chazotte, B., 2011. Labeling nuclear DNA using DAPI. *Cold Spring Harb Protoc*, *2011*(1), p.pdb.

Chen, X. and Liu, X.S., 2016. Hydrogen sulfide from a NaHS source attenuates dextran sulfate sodium (DSS)-induced inflammation via inhibiting nuclear factor-κB. *Journal of Zhejiang University. Science. B*, *17*(3), p.209.

Chen, X., Zhao, X., Lan, F., Zhou, T., Cai, H., Sun, H., Kong, W. and Kong, W., 2017. Hydrogen sulphide treatment increases insulin sensitivity and improves oxidant metabolism through the CaMKKbeta-AMPK pathway in PA-induced IR C2C12 cells. *Scientific Reports*, *7*(1), p.13248.

Cheng, C.M., Chang, W.H., Chiu, Y.C., Sun, Y., Lee, H.J., Tang, L.Y., Wang, P.N., Chiu, M.J., Yang, C.H., Tsai, S.J. and Tsai, C.F., 2018. Association of polypharmacy with mild cognitive impairment and cognitive ability: a nationwide survey in Taiwan. *The Journal of clinical psychiatry*, *79*(6), p.14680.

Cheng, S.B., Lin, P.T., Liu, H.T., Peng, Y.S., Huang, S.C. and Huang, Y.C., 2016. Vitamin B-6 supplementation could mediate antioxidant capacity by reducing plasma homocysteine concentration in patients with hepatocellular carcinoma after tumor resection. *BioMed research international*, *2016*.

Chhunchha, B., Kubo, E., Singh, P. and Singh, D.P., 2018. Sumoylation-deficient Prdx6 repairs aberrant Sumoylation-mediated Sp1 dysregulation-dependent Prdx6 repression and cell injury in aging and oxidative stress. *Aging (Albany NY)*, *10*(9), p.2284.

Choe, H., Hwang, J.Y., Yun, J.A., Kim, J.M., Song, T.J., Chang, N., Kim, Y.J. and Kim, Y., 2016. Intake of antioxidants and B vitamins is inversely associated with ischemic stroke and cerebral atherosclerosis. *Nutrition research and practice*, *10*(5), p.516.

Choi, E.Y. and Cho, Y.O., 2009. Effect of vitamin B6 deficiency on antioxidative status in rats with exercise-induced oxidative stress. *Nutrition Research and Practice*, *3*(3), pp.208-211.

Choi, J. and Fenando, A., 2020. Sulfasalazine.

Choi, Y.H., Lee, H.S., Chung, C.K., Kim, E.J. and Kang, I.J., 2017. Protective effects of an ethanol extract of Angelica keiskei against acetaminophen-induced hepatotoxicity in HepG2 and HepaRG cells. *Nutrition Research and Practice*, *11*(2), pp.97-104.

Christensen, L.D., Reilev, M., Juul-Larsen, H.G., Jørgensen, L.M., Kaae, S., Andersen, O., Pottegård, A. and Petersen, J., 2019. Use of prescription drugs in the older adult population—a nationwide pharmacoepidemiological study. *European journal of clinical pharmacology*, *75*, pp.1125-1133.

Chung, H.Y., Kim, D.H., Lee, E.K., Chung, K.W., Chung, S., Lee, B., Seo, A.Y., Chung, J.H., Jung, Y.S., Im, E. and Lee, J., 2019. Redefining chronic inflammation in aging and age-related diseases: proposal of the senoinflammation concept. *Aging and disease*, *10*(2), p.367.

Cipriani, S., Mencarelli, A., Bruno, A., Renga, B., Distrutti, E., Santucci, L., Baldelli, F. and Fiorucci, S., 2013. Activation of the bile acid receptor GPBAR 1 protects against gastrointestinal injury caused by non-steroidal anti-inflammatory drugs and aspirin in mice. *British Journal of Pharmacology*, *168*(1), pp.225-237.

Cirino, G., Szabo, C. and Papapetropoulos, A., 2023. Physiological roles of hydrogen sulfide in mammalian cells, tissues, and organs. *Physiological Reviews*, *103*(1), pp.31-276.

Clauson, K.A., Shields, K.M., McQueen, C.E. and Persad, N., 2008. Safety issues associated with commercially available energy drinks. *Journal of the American Pharmacists Association*, *48*(3), pp.e55-e67.

Comas, F. and Moreno-Navarrete, J.M., 2021. The impact of H2S on obesity-associated metabolic disturbances. *Antioxidants*, *10*(5), p.633.

Conan, P., Léon, A., Caroff, N., Rollet, C., Chaïr, L., Martin, J., Bihel, F., Mignen, O., Voisset, C. and Friocourt, G., 2023. New insights into the regulation of Cystathionine beta synthase (CBS), an enzyme involved in intellectual deficiency in Down syndrome. *Frontiers in Neuroscience*, *16*, p.1110163.

Corcoran, C., Murphy, C., Culligan, E.P., Walton, J. and Sleator, R.D., 2019. Malnutrition in the elderly. *Science progress*, *102*(2), pp.171-180.

Corona-Trejo, A., Gonsebatt, M.E., Trejo-Solis, C., Campos-Peña, V., Quintas-Granados, L.I., Villegas-Vázquez, E.Y., Daniel Reyes-Hernández, O., Hernández-Abad, V.J., Figueroa-González, G. and Silva-Adaya, D., 2023. Transsulfuration pathway: a targeting neuromodulator in Parkinson's disease. *Reviews in the Neurosciences*, *34*(8), pp.915-932.

Corona-Trejo, A., Gonsebatt, M.E., Trejo-Solis, C., Campos-Peña, V., Quintas-Granados, L.I., Villegas-Vázquez, E.Y., Daniel Reyes-Hernández, O., Hernández-Abad, V.J., Figueroa-González, G. and Silva-Adaya, D., 2023. Transsulfuration pathway: a targeting neuromodulator in Parkinson's disease. *Reviews in the Neurosciences*, *34*(8), pp.915-932.

Craig, D.G., Bates, C.M., Davidson, J.S., Martin, K.G., Hayes, P.C. and Simpson, K.J., 2011. Overdose pattern and outcome in paracetamol-induced acute severe hepatotoxicity. *British journal of clinical pharmacology*, *71*(2), pp.273-282.

Craig, W.J., 2009. Health effects of vegan diets. *The American journal of clinical nutrition*, *89*(5), pp.1627S-1633S.

Cruikshank, A., Reed, M.C. and Nijhout, H.F., 2024. Sex differences in glutathione metabolism and acetaminophen toxicity.

D'Alessandro, C., Benedetti, A., Di Paolo, A., Giannese, D. and Cupisti, A., 2022. Interactions between food and drugs, and nutritional status in renal patients: a narrative review. *Nutrients*, *14*(1), p.212.

da Silva, V.R., Rios-Avila, L., Lamers, Y., Ralat, M.A., Midttun, Ø., Quinlivan, E.P., Garrett, T.J., Coats, B., Shankar, M.N., Percival, S.S. and Chi, Y.Y., 2013. Metabolite profile analysis reveals functional effects of 28-day vitamin B-6 restriction on one-carbon metabolism

and tryptophan catabolic pathways in healthy men and women. *The Journal of nutrition*, *143*(11), pp.1719-1727.

Dai, Z., Wang, R., Ang, L.W., Yuan, J.M. and Koh, W.P., 2013. Dietary B vitamin intake and risk of hip fracture: the Singapore Chinese Health Study. *Osteoporosis International*, *24*, pp.2049-2059.

Damião, C.P., Rodrigues, A.O., Pinheiro, M.F.M.C., Cruz Filho, R.A.D., Cardoso, G.P., Taboada, G.F. and Lima, G.A.B., 2016. Prevalence of vitamin B12 deficiency in type 2 diabetic patients using metformin: a cross-sectional study. *Sao Paulo Medical Journal*, *134*(06), pp.473-479.

Deavall, D.G., Martin, E.A., Horner, J.M. and Roberts, R., 2012. Drug-induced oxidative stress and toxicity. *Journal of toxicology*, *2012*.

Deijen, J.B., Van der Beek, E.J., Orlebeke, J.F. and Van den Berg, H., 1992. Vitamin B-6 supplementation in elderly men: effects on mood, memory, performance and mental effort. *Psychopharmacology*, *109*, pp.489-496.

DeMichele, J., Rezaizadeh, H. and Goldstein, J.I., 2012. Sulfasalazine crystalluria-induced anuric renal failure. *Clinical Gastroenterology and Hepatology*, *10*(2), p.A32.

d'Emmanuele di Villa Bianca, R., Sorrentino, R., Maffia, P., Mirone, V., Imbimbo, C., Fusco, F., De Palma, R., Ignarro, L.J. and Cirino, G., 2009. Hydrogen sulfide as a mediator of human corpus cavernosum smooth-muscle relaxation. *Proceedings of the National Academy of Sciences*, *106*(11), pp.4513-4518.

den Bakker, E., Smith, D.E., Finken, M.J., Wamelink, M.M., Salomons, G.S., van de Kamp, J.M. and Bökenkamp, A., 2024. Sulfate: a neglected (but potentially highly relevant) anion. *Essays in Biochemistry*, p.EBC20230097.

Deng, J., Wei, W., Chen, Z., Lin, B., Zhao, W., Luo, Y. and Zhang, X., 2019. Engineered liver-on-a-chip platform to mimic liver functions and its biomedical applications: A review. *Micromachines*, *10*(10), p.676.

Dent, E., Wright, O.R., Woo, J. and Hoogendijk, E.O., 2023. Malnutrition in older adults. *The Lancet*, *401*(10380), pp.951-966.

DeRatt, B.N., Ralat, M.A., Kabil, O., Chi, Y.Y., Banerjee, R. and Gregory III, J.F., 2014. Vitamin B-6 restriction reduces the production of hydrogen sulfide and its biomarkers by the transsulfuration pathway in cultured human hepatoma cells. *The Journal of nutrition*, *144*(10), pp.1501-1508.

Desouza, C., Keebler, M., McNamara, D.B. and Fonseca, V., 2002. Drugs affecting homocysteine metabolism: impact on cardiovascular risk. *Drugs*, *62*, pp.605-616.

di Villa Bianca, R.D.E., Mitidieri, E., Donnarumma, E., Tramontano, T., Brancaleone, V., Cirino, G., Bucci, M. and Sorrentino, R., 2015. Hydrogen sulfide is involved in dexamethasone-induced hypertension in rat. *Nitric Oxide*, *46*, pp.80-86.

Dickerhof, N., Pearson, J.F., Hoskin, T.S., Berry, L.J., Turner, R., Sly, P.D. and Kettle, A.J., 2017. Oxidative stress in early cystic fibrosis lung disease is exacerbated by airway glutathione deficiency. *Free Radical Biology and Medicine*, *113*, pp.236-243.

Dierkes, J., Luley, C. and Westphal, S., 2007. Effect of lipid-lowering and anti-hypertensive drugs on plasma homocysteine levels. *Vascular Health and Risk Management*, *3*(1), pp.99-108.

Dikis, O.S., Acat, M., Casim, H., Haskul, I., Neselioglu, S., Simsek, A. and Erel, O., 2019. The relationship of thiol/disulfide homeostasis in the etiology of patients with obstructive sleep apnea: a case-control study. *The Aging Male*.

Dimova, S., Hoet, P.H., Dinsdale, D. and Nemery, B., 2005. Acetaminophen decreases intracellular glutathione levels and modulates cytokine production in human alveolar macrophages and type II pneumocytes in vitro. *The international journal of biochemistry & cell biology*, *37*(8), pp.1727-1737.

Ding, R., Lin, S. and Chen, D., 2012. The association of cystathionine β synthase (CBS) T833C polymorphism and the risk of stroke: a meta-analysis. *Journal of the neurological sciences*, *312*(1-2), pp.26-30.

Distrutti, E., Sediari, L., Mencarelli, A., Renga, B., Orlandi, S., Antonelli, E., Roviezzo, F., Morelli, A., Cirino, G., Wallace, J.L. and Fiorucci, S., 2006. Evidence that hydrogen sulfide exerts antinociceptive effects in the gastrointestinal tract by activating KATP channels. *Journal of Pharmacology and Experimental Therapeutics*, *316*(1), pp.325-335.

DoH (Ed.), 2008. Dietary reference values for food energy and nutrients for the United Kingdom: report, 18. Impression. ed, Report on health and social subjects. TSO, London.

DoH, 1991. Dietary Reference Values A Guide 1991. Crown copyright, London.

Dong, Z., Gao, X., Chinchilli, V.M., Sinha, R., Muscat, J., Winkels, R.M. and Richie, J.P., 2020. Association of sulfur amino acid consumption with cardiometabolic risk factors: cross-sectional findings from NHANES III. *EClinicalMedicine*, *19*.

Dröge, W., 2005. Oxidative stress and ageing: is ageing a cysteine deficiency syndrome?. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *360*(1464), pp.2355-2372.

Dubois-Deruy, E., Peugnet, V., Turkieh, A. and Pinet, F., 2020. Oxidative stress in cardiovascular diseases. *Antioxidants*, *9*(9), p.864.

Dutta, S., Sengupta, P. and Samrot, A.V., 2024. Physiological and Pathological Functions of Reactive Nitrogen Species (RNS) and Reactive Sulphur Species (RSS) on Male Reproductive Functions. *Journal of Integrated Science and Technology*, *12*(3), pp.755-755.

Eichholzer, M., 2003. Micronutrient deficiencies in Switzerland: causes and consequences. *Journal of food engineering*, *56*(2-3), pp.171-179.

Elengoe, A. and Hamdan, S., 2017. Evaluation of MCF-7 cell viability by LDH, trypan blue and crystal violet staining assays. *Malaysian Journal of Medical Research (MJMR)*, *1*(3), pp.37-42.

Elkhateeb, N., Hyde, S., Hogg, S.L., Allsop, D., Shankar, A., Deegan, P. and Tan, C.Y., 2023. Paracetamol toxicity in classic homocystinuria: Effect of N-acetylcysteine on total homocysteine. *JIMD reports*, *64*(3), pp.238-245.

Elrod, J.W., Calvert, J.W., Morrison, J., Doeller, J.E., Kraus, D.W., Tao, L., Jiao, X., Scalia, R., Kiss, L., Szabo, C. and Kimura, H., 2007. Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. *Proceedings of the National Academy of Sciences*, *104*(39), pp.15560-15565.

Elshazly, S.M., El-Moselhy, M.A. and Barakat, W., 2014. Insights in the mechanism underlying the protective effect of α-lipoic acid against acetaminophen-hepatotoxicity. *European journal of pharmacology*, *726*, pp.116-123.

Engle-Stone, R., Vosti, S.A., Luo, H., Kagin, J., Tarini, A., Adams, K.P., French, C. and Brown, K.H., 2019. Weighing the risks of high intakes of selected micronutrients compared with the risks of deficiencies. *Annals of the New York Academy of Sciences*, *1446*(1), pp.81-101.

Erden-İnal, M., Sunal, E. and Kanbak, G., 2002. Age-related changes in the glutathione redox system. *Cell biochemistry and function*, *20*(1), pp.61-66.

Ershad, M., Naji, A. and Vearrier, D., 2019. N acetylcysteine.

Eryilmaz, M.A., Kozanhan, B., Solak, I., Cetinkaya, C.D., Neselioglu, S. and Erel, Ö., 2019. Thiol-disulfide homeostasis in breast cancer patients. *Journal of Cancer Research and Therapeutics*, *15*(5), pp.1062-1066.

Espinosa-Salas, S. and Gonzalez-Arias, M., 2023. Nutrition: Micronutrient Intake, Imbalances, and Interventions. In *StatPearls [Internet]*. StatPearls Publishing.

Eto, K., Asada, T., Arima, K., Makifuchi, T. and Kimura, H., 2002. Brain hydrogen sulfide is severely decreased in Alzheimer's disease. *Biochemical and biophysical research communications*, *293*(5), pp.1485-1488.

Fakurazi, S., Hairuszah, I. and Nanthini, U., 2008. Moringa oleifera Lam prevents acetaminophen induced liver injury through restoration of glutathione level. *Food and chemical toxicology*, *46*(8), pp.2611-2615.

Fan, J., Pung, E., Lin, Y. and Wang, Q., 2022. Recent development of hydrogen sulfide-releasing biomaterials as novel therapies: a narrative review. *Biomaterials Translational*, *3*(4), p.250.

Fang, Y.Z., Yang, S. and Wu, G., 2002. Free radicals, antioxidants, and nutrition. *Nutrition*, *18*(10), pp.872-879.

Farhat, Z., Browne, R.W., Bonner, M.R., Tian, L., Deng, F., Swanson, M. and Mu, L., 2018. How do glutathione antioxidant enzymes and total antioxidant status respond to air pollution exposure?. *Environment international*, *112*, pp.287-293.

Fauzy, N.K.M., Harith, S., Wan Taib, W.R., Yahaya, R., Sita, A. and Khasanah, F., 2023. Malnutrition, Quality of Life and Their Relationship among Older Adults: A Scoping Review. *Malaysian Journal of Medicine & Health Sciences*, *19*.

Feoktistova, M., Geserick, P. and Leverkus, M., 2016. Crystal violet assay for determining viability of cultured cells. Cold Spring Harbor Protocols, 2016(4), pp.pdb-prot087379.

Fernández-Liz, E., Modamio, P., Catalán, A., Lastra, C.F., Rodríguez, T. and Mariño, E.L., 2008. Identifying how age and gender influence prescription drug use in a primary health care environment in Catalonia, Spain. *British journal of clinical pharmacology*, *65*(3), pp.407-417.

Filip, A., Veliceasa, B., Bordeianu, G., Iancu, C., Cuciureanu, M. and Badulescu, O.V., 2024. The Relationship between Cysteine, Homocysteine, and Osteoporosis.

Filip, C. and Albu, E. eds., 2018. *Reactive oxygen species (ROS) in living cells*. BoD–Books on Demand.

Finkelstein, J.D. and Chalmers, F.T., 1970. Pyridoxine effects on cystathionine synthase in rat liver. *The Journal of Nutrition*, *100*(4), pp.467-469.

Fiorucci, S., Antonelli, E., Mencarelli, A., Orlandi, S., Renga, B., Rizzo, G., Distrutti, E., Shah, V. and Morelli, A., 2005. The third gas: H2S regulates perfusion pressure in both the isolated and perfused normal rat liver and in cirrhosis. *Hepatology*, *42*(3), pp.539-548.

Food Standards Agency. (2003). Safe upper levels for vitamins and minerals. Available at: https://cot.food.gov.uk/sites/default/files/vitmin2003.pdf (Accessed April 2023)

Fra, A., Yoboue, E.D. and Sitia, R., 2017. Cysteines as redox molecular switches and targets of disease. *Frontiers in molecular neuroscience*, *10*, p.167.

Franco, R. and Cidlowski, J.A., 2009. Apoptosis and glutathione: beyond an antioxidant. *Cell Death & Differentiation*, *16*(10), pp.1303-1314.

Franco, R. and Cidlowski, J.A., 2009. Apoptosis and glutathione: beyond an antioxidant. *Cell Death & Differentiation*, *16*(10), pp.1303-1314.

Freo, U., Ruocco, C., Valerio, A., Scagnol, I. and Nisoli, E., 2021. Paracetamol: a review of guideline recommendations. *Journal of clinical medicine*, *10*(15), p.3420.

Friedman, P.A. and Bushinsky, D.A., 1999, November. Diuretic effects on calcium metabolism. In *Seminars in nephrology* (Vol. 19, No. 6, pp. 551-556).

Friso, S., Jacques, P.F., Wilson, P.W., Rosenberg, I.H. and Selhub, J., 2001. Low circulating vitamin B6 is associated with elevation of the inflammation marker C-reactive protein independently of plasma homocysteine levels. *Circulation*, *103*(23), pp.2788-2791.

Fu, Z., Liu, X., Geng, B., Fang, L. and Tang, C., 2008. Hydrogen sulfide protects rat lung from ischemia–reperfusion injury. *Life sciences*, *82*(23-24), pp.1196-1202.

Gajski, G., Gerić, M., Lovrenčić, M.V., Božičević, S., Rubelj, I., Nanić, L., Vidaček, N.Š., Bendix, L., Peraica, M., Rašić, D. and Domijan, A.M., 2018. Analysis of health-related biomarkers between vegetarians and non-vegetarians: A multi-biomarker approach. *Journal of functional foods*, *48*, pp.643-653.

George, I. and Meldrum, J., 2020. A study to assess the prevalence of unintentional paracetamol overdose among patients presenting in dental pain in primary care. *British dental journal*, pp.1-4.

Ghezzi, P., Lemley, K.V., Andrus, J.P., De Rosa, S.C., Holmgren, A., Jones, D., Jahoor, F., Kopke, R., Cotgreave, I., Bottiglieri, T. and Kaplowitz, N., 2019. Cysteine/glutathione deficiency: a significant and treatable corollary of disease. *The therapeutic use of N-Acetylcysteine (NAC) in Medicine*, pp.349-386.

Giuffrè, A. and Vicente, J.B., 2018. Hydrogen sulfide biochemistry and interplay with other gaseous mediators in mammalian physiology. *Oxidative medicine and cellular longevity*, *2018*(1), p.6290931.

Glaeske, G., Gerdau-Heitmann, C., Höfel, F. and Schicktanz, C., 2012. "Gender-specific drug prescription in Germany" results from prescriptions analyses. *Sex and gender differences in pharmacology*, pp.149-167.

Godswill, A.G., Somtochukwu, I.V., Ikechukwu, A.O. and Kate, E.C., 2020. Health benefits of micronutrients (vitamins and minerals) and their associated deficiency diseases: A systematic review. *International Journal of Food Sciences*, *3*(1), pp.1-32.

Gombart, A.F., Pierre, A. and Maggini, S., 2020. A review of micronutrients and the immune system–working in harmony to reduce the risk of infection. *Nutrients*, *12*(1), p.236.

Gommers, L.M., Hoenderop, J.G. and de Baaij, J.H., 2022. Mechanisms of proton pump inhibitor-induced hypomagnesemia. *Acta Physiologica*, *235*(4), p.e13846.

Gonzalez, D., Bejarano, I., Barriga, C., Rodriguez, A.B. and Pariente, J.A., 2010. Oxidative stress-induced caspases are regulated in human myeloid HL-60 cells by calcium signal. *Current Signal Transduction Therapy*, *5*(2), pp.181-186.

González, L.T., Minsky, N.W., Espinosa, L.E.M., Aranda, R.S., Meseguer, J.P. and Pérez, P.C., 2017. In vitro assessment of hepatoprotective agents against damage induced by acetaminophen and CCl 4. *BMC complementary and alternative medicine*, *17*, pp.1-10.

González, L.T., Minsky, N.W., Espinosa, L.E.M., Aranda, R.S., Meseguer, J.P. and Pérez, P.C., 2017. In vitro assessment of hepatoprotective agents against damage induced by acetaminophen and CCl 4. *BMC complementary and alternative medicine*, *17*, pp.1-10.

Gout, P.W., Buckley, A.R., Simms, C.R. and Bruchovsky, N., 2001. Sulfasalazine, a potent suppressor of lymphoma growth by inhibition of the xc− cystine transporter: a new action for an old drug. *Leukemia*, *15*(10), pp.1633-1640.

Green, D.R., 2022. Caspases and their substrates. *Cold Spring Harbor Perspectives in Biology*, *14*(3), p.a041012.

Green, R., Allen, L.H., Bjørke-Monsen, A.L., Brito, A., Guéant, J.L., Miller, J.W., Molloy, A.M., Nexo, E., Stabler, S., Toh, B.H. and Ueland, P.M., 2017. Vitamin B12 deficiency. *Nature reviews Disease primers*, *3*(1), pp.1-20.

Gregory, J.F., DeRatt, B.N., Rios-Avila, L., Ralat, M. and Stacpoole, P.W., 2016. Vitamin B6 nutritional status and cellular availability of pyridoxal 5′-phosphate govern the function of the transsulfuration pathway's canonical reactions and hydrogen sulfide production via side reactions. *Biochimie*, *126*, pp.21-26.

Gröber, U., Schmidt, J. and Kisters, K., 2020. Important drug-micronutrient interactions: A selection for clinical practice. *Critical reviews in food science and nutrition*, *60*(2), pp.257-275.

Grootswagers, P., Mensink, M., Berendsen, A.A., Deen, C.P., Kema, I.P., Bakker, S.J., Santoro, A., Franceschi, C., Meunier, N., Malpuech-Brugère, C. and Bialecka-Debek, A., 2021. Vitamin B-6 intake is related to physical performance in European older adults: results of the New Dietary Strategies Addressing the Specific Needs of the Elderly Population for Healthy Aging in Europe (NU-AGE) study. *The American journal of clinical nutrition*, *113*(4), pp.781-789.

Guillouzo, A. and Guguen-Guillouzo, C., 2020. Antibiotics-induced oxidative stress. *Current Opinion in Toxicology*, *20*, pp.23-28.

Gylling, B., Myte, R., Schneede, J., Hallmans, G., Häggström, J., Johansson, I., Ulvik, A., Ueland, P.M., Van Guelpen, B. and Palmqvist, R., 2017. Vitamin B-6 and colorectal cancer risk: a prospective population-based study using 3 distinct plasma markers of vitamin B-6 status. *The American Journal of Clinical Nutrition*, *105*(4), pp.897-904.

Haider, S.I., Ansari, Z., Vaughan, L., Matters, H. and Emerson, E., 2014. Prevalence and factors associated with polypharmacy in Victorian adults with intellectual disability. *Research in Developmental Disabilities*, *35*(11), pp.3071-3080.

Han, Y., Xi, Q.Q., Dai, W., Yang, S.H., Gao, L., Su, Y.Y. and Zhang, X., 2015. Abnormal transsulfuration metabolism and reduced antioxidant capacity in Chinese children with autism spectrum disorders. *International Journal of Developmental Neuroscience*, *46*, pp.27-32.

Hanna, M., Jaqua, E., Nguyen, V. and Clay, J.B., 2022. Vitamins: functions and uses in medicine. *Perm. J*, *26*(2), pp.89-97.

Hariz, A. and Bhattacharya, P.T., 2019. Megaloblastic anemia.

Harris, D.A., Guo, Y., Nakhla, N., Tadrous, M., Hogan, D.B., Hennessy, D., Langlois, K., Garner, R., Leslie, S., Bronskill, S.E. and Heckman, G., 2022. Prevalence of prescription and non-prescription polypharmacy by frailty and sex among middle-aged and older Canadians. *Health Reports*, *33*(6), pp.3-16.

Hazelton, G.A., Hjelle, J.J. and Klaassen, C.D., 1986. Effects of cysteine pro-drugs on acetaminophen-induced hepatotoxicity. *Journal of Pharmacology and Experimental Therapeutics*, *237*(1), pp.341-349.

He, L., He, T., Farrar, S., Ji, L., Liu, T. and Ma, X., 2017. Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. *Cellular Physiology and Biochemistry*, *44*(2), pp.532-553.

Heidari, R., Esmailie, N., Azarpira, N., Najibi, A. and Niknahad, H., 2016. Effect of thiol-reducing agents and antioxidants on sulfasalazine-induced hepatic injury in normotermic recirculating isolated perfused rat liver. *Toxicological Research*, *32*, pp.133-140.

Heller, C.A. and Friedman, P.A., 1983. Pyridoxine deficiency and peripheral neuropathy associated with long-term phenelzine therapy. *The American journal of medicine*, *75*(5), pp.887-888.

Henry, E.B., Carswell, A., Wirz, A., Fyffe, V. and McColl, K.E.L., 2005. Proton pump inhibitors reduce the bioavailability of dietary vitamin C. *Alimentary pharmacology & therapeutics*, *22*(6), pp.539-545.

Hickey, E.J., Raje, R.R., Reid, V.E., Gross, S.M. and Ray, S.D., 2001. Diclofenac induced in vivo nephrotoxicity may involve oxidative stress-mediated massive genomic DNA fragmentation and apoptotic cell death. *Free Radical Biology and Medicine*, *31*(2), pp.139-152.

Hizal, M., Bilgin, B., Sendur, M.A., Akinci, M., Neselioglu, S., Erel, O. and Yalcin, B., 2018. P2. 01-83 Evaluation of Dynamic Thiol/Disulphide Homeostasis in Advance Non-Small Cell Lung Cancer and Small Cell Lung Cancer. *Journal of Thoracic Oncology*, *13*(10), pp.S696-S697.

Ho, C.L., Quay, T.A., Devlin, A.M. and Lamers, Y., 2016. Prevalence and predictors of low vitamin B6 status in healthy young adult women in metro Vancouver. *Nutrients*, *8*(9), p.538.

Hoffman, R., 2017. Micronutrient deficiencies in the elderly–could ready meals be part of the solution?. *Journal of nutritional science*, *6*, p.e2.

Hou, C.T., Wu, Y.H., Cheng, C.H., Huang, P.N. and Huang, Y.C., 2012. Higher plasma homocysteine is associated with lower vitamin B6 status in critically ill surgical patients. *Nutrition in clinical practice*, *27*(5), pp.695-700.

Hu, L.F., Lu, M., Tiong, C.X., Dawe, G.S., Hu, G. and Bian, J.S., 2010. Neuroprotective effects of hydrogen sulfide on Parkinson's disease rat models. *Aging cell*, *9*(2), pp.135-146.

Huang, T.H., Lewis, J.L., Lin, H.S., Kuo, L.T., Mao, S.W., Tai, Y.S., Chang, M.S., Ables, G.P., Perrone, C.E. and Yang, R.S., 2014. A methionine-restricted diet and endurance exercise decrease bone mass and extrinsic strength but increase intrinsic strength in growing male rats. *The Journal of nutrition*, *144*(5), pp.621-630.

Hughes, C.F., Ward, M., Tracey, F., Hoey, L., Molloy, A.M., Pentieva, K. and McNulty, H., 2017. B-vitamin intake and biomarker status in relation to cognitive decline in healthy older adults in a 4-year follow-up study. *Nutrients*, *9*(1), p.53.

Hughes, M.J., Harrison, E.M., Jin, Y., Homer, N. and Wigmore, S.J., 2015. Acetaminophen metabolism after liver resection: A prospective case–control study. *Digestive and Liver Disease*, *47*(12), pp.1039-1046.

Hughes, M.N., Centelles, M.N. and Moore, K.P., 2009. Making and working with hydrogen sulfide: the chemistry and generation of hydrogen sulfide in vitro and its measurement in vivo: a review. *Free Radical Biology and Medicine*, *47*(10), pp.1346-1353.

Hum, S., Robitaille, L. and Hoffer, L.J., 1991. Plasma glutathione turnover in the rat: effect of fasting and buthionine sulfoximine. *Canadian journal of physiology and pharmacology*, *69*(5), pp.581-587.

Huskisson, E., Maggini, S. and Ruf, M., 2007. The role of vitamins and minerals in energy metabolism and well-being. *Journal of international medical research*, *35*(3), pp.277-289.

Hussy, N., Deleuze, C., Desarménien, M.G. and Moos, F.C., 2000. Osmotic regulation of neuronal activity: a new role for taurine and glial cells in a hypothalamic neuroendocrine structure. *Progress in neurobiology*, *62*(2), pp.113-134.

Ighodaro, O.M. and Akinloye, O.A., 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria journal of medicine*, *54*(4), pp.287-293.

Institute for Health Metrics and Evaluation (IHME) (2024). Global life expectancy to increase by nearly 5 years by 2050 despite geopolitical, metabolic, and environmental threats,

reports new global study . Available at: https://www.healthdata.org/news-events/newsroom/news-releases/global-life-expectancy-increase-nearly-5-years-2050-despite (Accessed January 2024)

Institute of Medicine. (2005). Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids. Washington, DC: The National Academies Press.

Ito, F., Sono, Y. and Ito, T., 2019. Measurement and clinical significance of lipid peroxidation as a biomarker of oxidative stress: oxidative stress in diabetes, atherosclerosis, and chronic inflammation. *Antioxidants*, *8*(3), p.72.

Itoh, N., Matsumura, S., Iwaki, T., Takenaka, S. and Kanouchi, H., 2024. Low cellular pyridoxal 5'-phosphate levels decrease neurotransmitter and glutathione concentrations and increase susceptibility to hydrogen peroxide toxicity in SH-SY5Y cells. *Clinical Nutrition Open Science*, *55*, pp.123-135.

Jackson, M.J., 1999. Diagnosis and detection of deficiencies of micronutrients: minerals. *British medical bulletin*, *55*(3), pp.634-642.

Jaeschke, H., McGill, M.R. and Ramachandran, A., 2012. Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. *Drug metabolism reviews*, *44*(1), pp.88-106.

Jakubowski, H., 2020. Proteomic exploration of cystathionine β-synthase deficiency: implications for the clinic. *Expert Review of Proteomics*, *17*(10), pp.751-765.

Jangra, A., Gola, P., Singh, J., Gond, P., Ghosh, S., Rachamalla, M., Dey, A., Iqbal, D., Kamal, M., Sachdeva, P. and Jha, S.K., 2024. Emergence of taurine as a therapeutic agent for neurological disorders. *Neural Regeneration Research*, *19*(1), pp.62-68.

Jannuzzi, A.T., Kara, M. and Alpertunga, B., 2018. Celastrol ameliorates acetaminophen-induced oxidative stress and cytotoxicity in HepG2 cells. *Human & Experimental Toxicology*, *37*(7), pp.742-751.

Jayedi, A. and Zargar, M.S., 2019. Intake of vitamin B6, folate, and vitamin B12 and risk of coronary heart disease: a systematic review and dose-response meta-analysis of prospective cohort studies. *Critical reviews in food science and nutrition*, *59*(16), pp.2697-2707.

Jeon, S., Kim, Y., Min, S., Song, M., Son, S. and Lee, S., 2021. Taste sensitivity of elderly people is associated with quality of life and inadequate dietary intake. *Nutrients*, *13*(5), p.1693.

Jiang, S. and Chen, Y., 2022. The role of sulfur compounds in chronic obstructive pulmonary disease. *Frontiers in Molecular Biosciences*, *9*, p.928287.

Johansen, D., Ytrehus, K. and Baxter, G.F., 2006. Exogenous hydrogen sulfide (H 2 S) protects against regional myocardial ischemia–reperfusion injury: Evidence for a role of K ATP channels. *Basic research in cardiology*, *101*, pp.53-60.

Jomova, K., Raptova, R., Alomar, S.Y., Alwasel, S.H., Nepovimova, E., Kuca, K. and Valko, M., 2023. Reactive oxygen species, toxicity, oxidative stress, and antioxidants: Chronic diseases and aging. *Archives of toxicology*, *97*(10), pp.2499-2574.

Jones, D.P., Park, Y., Gletsu-Miller, N., Liang, Y., Yu, T., Accardi, C.J. and Ziegler, T.R., 2011. Dietary sulfur amino acid effects on fasting plasma cysteine/cystine redox potential in humans. *Nutrition*, *27*(2), pp.199-205.

Jong, C.J., Azuma, J. and Schaffer, S., 2012. Mechanism underlying the antioxidant activity of taurine: prevention of mitochondrial oxidant production. *Amino acids*, *42*, pp.2223-2232.

Jong, C.J., Sandal, P. and Schaffer, S.W., 2021. The role of taurine in mitochondria health: more than just an antioxidant. *Molecules*, *26*(16), p.4913.

Jørring Pallesen, A.V., Kristiansen, M., Westendorp, R.G. and Mortensen, L.H., 2022. Polypharmacy occurrence and the related risk of premature death among older adults in Denmark: a nationwide register-based cohort study. *PLoS One*, *17*(2), p.e0264332.

Juan, C.A., Pérez de la Lastra, J.M., Plou, F.J. and Pérez-Lebeña, E., 2021. The chemistry of reactive oxygen species (ROS) revisited: outlining their role in biological macromolecules (DNA, lipids and proteins) and induced pathologies. *International journal of molecular sciences*, *22*(9), p.4642.

Kamiloglu, S., Sari, G., Ozdal, T. and Capanoglu, E., 2020. Guidelines for cell viability assays. Food Frontiers, 1(3), pp.332-349.

Kamyshny, A., Goifman, A., Rizkov, D. and Lev, O., 2003. Formation of carbonyl sulfide by the reaction of carbon monoxide and inorganic polysulfides. *Environmental science & technology*, *37*(9), pp.1865-1872.

Kaneko, T., Mita, Y., Nozawa-Kumada, K., Yazaki, M., Arisawa, M., Niki, E., Noguchi, N. and Saito, Y., 2022. Antioxidant action of persulfides and polysulfides against free radical-mediated lipid peroxidation. *Free Radical Research*, *56*(9-10), pp.677-690.

Kanemaru, E., Miyazaki, Y., Marutani, E., Ezaka, M., Goto, S., Ohshima, E., Bloch, D.B. and Ichinose, F., 2023. Intranasal administration of polysulfide prevents neurodegeneration in spinal cord and rescues mice from delayed paraplegia after spinal cord ischemia. *Redox Biology*, *60*, p.102620.

Kanthasamy, A.G., Anantharam, V., Zhang, D., Latchoumycandane, C., Jin, H., Kaul, S. and Kanthasamy, A., 2006. A novel peptide inhibitor targeted to caspase-3 cleavage site of a proapoptotic kinase protein kinase C delta (PKCδ) protects against dopaminergic neuronal degeneration in Parkinson's disease models. *Free Radical Biology and Medicine*, *41*(10), pp.1578-1589.

Kantor, E.D., Rehm, C.D., Haas, J.S., Chan, A.T. and Giovannucci, E.L., 2015. Trends in prescription drug use among adults in the United States from 1999- 2012. *Jama*, *314*(17), pp.1818-1830.

Kasamatsu, S. and Ihara, H., 2021. Regulation of redox signaling by reactive sulfur species. *Journal of clinical biochemistry and nutrition*, *68*(2), pp.111-115.

Katikaneni, A., Jelcic, M., Gerlach, G.F., Ma, Y., Overholtzer, M. and Niethammer, P., 2020. Lipid peroxidation regulates long-range wound detection through 5-lipoxygenase in zebrafish. *Nature cell biology*, *22*(9), pp.1049-1055.

Kato, N., Kimoto, A., Zhang, P., Bumrungkit, C., Karunaratne, S., Yanaka, N. and Kumrungsee, T., 2024. Relationship of Low Vitamin B6 Status with Sarcopenia, Frailty, and Mortality: A Narrative Review. *Nutrients*, *16*(1), p.177.

Kattoor, A.J., Pothineni, N.V.K., Palagiri, D. and Mehta, J.L., 2017. Oxidative stress in atherosclerosis. *Current atherosclerosis reports*, *19*, pp.1-11.

Kelly, P.J., Kistler, J.P., Shih, V.E., Mandell, R., Atassi, N., Barron, M., Lee, H., Silveira, S. and Furie, K.L., 2004. Inflammation, homocysteine, and vitamin B6 status after ischemic stroke. *Stroke*, *35*(1), pp.12-15.

Kesari, A. and Noel, J.Y., 2022. Nutritional Assessment. *StatPearls [Internet]; StatPearls Publishing: Treasure Island, FL, USA*.

Kiani, A.K., Dhuli, K., Donato, K., Aquilanti, B., Velluti, V., Matera, G., Iaconelli, A., Connelly, S.T., Bellinato, F., Gisondi, P. and Bertelli, M., 2022. Main nutritional deficiencies. *Journal of preventive medicine and hygiene*, *63*(2 Suppl 3), p.E93.

Kim, D.H., Abdullah, M.D. and Lee, S.J., 2019. Mechanisms of growth inhibition by sulfasalazine and erastin in hepatocellular carcinoma cell lines. 약학회지, *63*(3), pp.152-159.

Kim, Y.N. and Cho, Y.O., 2014. Evaluation of vitamin B6 intake and status of 20-to 64-year-old Koreans. *Nutrition research and practice*, *8*(6), p.688.

Kimura, H., 2002. Hydrogen sulfide as a neuromodulator. *Molecular neurobiology*, *26*, pp.13-19.

Kimura, H., 2011. Hydrogen sulfide: its production, release and functions. *Amino acids*, *41*, pp.113-121.

Kimura, H., 2015. Hydrogen sulfide and polysulfides as signaling molecules. *Proceedings of the Japan Academy, Series B*, *91*(4), pp.131-159.

Kimura, H., 2015. Signaling molecules: hydrogen sulfide and polysulfide. *Antioxidants & redox signaling*, *22*(5), pp.362-376.

Kimura, H., 2017. Hydrogen sulfide and polysulfide signaling. *Antioxidants & redox signaling*, *27*(10), pp.619-621.

Kimura, H., 2021. Hydrogen sulfide (H2S) and polysulfide (H2Sn) signaling: The first 25 years. *Biomolecules*, *11*(6), p.896.

Kimura, Y. and Kimura, H., 2004. Hydrogen sulfide protects neurons from oxidative stress. *The FASEB journal*, *18*(10), pp.1165-1167.

Kimura, Y., Goto, Y.I. and Kimura, H., 2010. Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. *Antioxidants & redox signaling*, *12*(1), pp.1-13.

Kjeldby, I.K., Fosnes, G.S., Ligaarden, S.C. and Farup, P.G., 2013. Vitamin B6 deficiency and diseases in elderly people–a study in nursing homes. *BMC geriatrics*, *13*, pp.1-8.

Koike, S., Kayama, T., Yamamoto, S., Komine, D., Tanaka, R., Nishimoto, S., Suzuki, T., Kishida, A. and Ogasawara, Y., 2016. Polysulfides protect SH-SY5Y cells from methylglyoxal-induced toxicity by suppressing protein carbonylation: A possible physiological scavenger for carbonyl stress in the brain. *Neurotoxicology*, *55*, pp.13-19.

Koike, S., Ogasawara, Y., Shibuya, N., Kimura, H. and Ishii, K., 2013. Polysulfide exerts a protective effect against cytotoxicity caused by t-buthylhydroperoxide through Nrf2 signaling in neuroblastoma cells. *FEBS letters*, *587*(21), pp.3548-3555.

Kok, W.E., Haverkort, E.B., Algra, Y.A., Mollema, J., Hollaar, V.R.Y., Naumann, E., De Van Der Schueren, M.A.E. and Jerković-Ćosić, K., 2022. The association between polypharmacy and malnutrition (risk) in older people: a systematic review. *Clinical Nutrition ESPEN*, *49*, pp.163-171.

Koklesova, L., Mazurakova, A., Samec, M., Biringer, K., Samuel, S.M., Büsselberg, D., Kubatka, P. and Golubnitschaja, O., 2021. Homocysteine metabolism as the target for predictive medical approach, disease prevention, prognosis, and treatments tailored to the person. *EPMA Journal*, *12*, pp.477-505.

Kolluru, G.K., Shen, X., Bir, S.C. and Kevil, C.G., 2013. Hydrogen sulfide chemical biology: pathophysiological roles and detection. *Nitric oxide*, *35*, pp.5-20.

Kondo, K., Yamada, N., Suzuki, Y., Toyoda, K., Hashimoto, T., Takahashi, A., Kobayashi, A., Shoda, T., Kuno, H. and Sugai, S., 2012. Enhancement of acetaminophen-induced chronic hepatotoxicity in restricted fed rats: a nonclinical approach to acetaminophen-induced chronic hepatotoxicity in susceptible patients. *The Journal of toxicological sciences*, *37*(5), pp.911-929.

Kowalczyk-Bołtuć, J., Wiórkowski, K. and Bełtowski, J., 2022. Effect of exogenous hydrogen sulfide and polysulfide donors on insulin sensitivity of the adipose tissue. *Biomolecules*, *12*(5), p.646.

Kozer, E., Evans, S., Barr, J., Greenberg, R., Soriano, I., Bulkowstein, M., Petrov, I., Chen-Levi, Z., Barzilay, B. and Berkovitch, M., 2003. Glutathione, glutathione-dependent enzymes and antioxidant status in erythrocytes from children treated with high-dose paracetamol. *British journal of clinical pharmacology*, *55*(3), pp.234-240.

Krishnamurthy, H.K., Pereira, M., Jayaraman, V., Krishna, K., Wang, T., Bei, K. and Rajasekaran, J.J., 2024. Oxidative Stress: Mechanisms, Quantification and its role in human aging. *ScienceOpen Preprints*.

Krok-Schoen, J.L., Price, A.A., Luo, M., Kelly, O.J. and Taylor, C.A., 2019. Low dietary protein intakes and associated dietary patterns and functional limitations in an aging population: a NHANES analysis. *The Journal of nutrition, health and aging*, *23*(4), pp.338-347.

Kubrak, C. and Jensen, L., 2007. Malnutrition in acute care patients: a narrative review. *International journal of nursing studies*, *44*(6), pp.1036-1054.

Kuffner, E.K., Green, J.L., Bogdan, G.M., Knox, P.C., Palmer, R.B., Heard, K., Slattery, J.T. and Dart, R.C., 2007. The effect of acetaminophen (four grams a day for three consecutive days) on hepatic tests in alcoholic patients–a multicenter randomized study. *BMC medicine*, *5*, pp.1-9.

Kükürt, A., Gelen, V., Başer, Ö.F., Deveci, H.A. and Karapehlivan, M., 2021. Thiols: Role in oxidative stress-related disorders. *Accenting Lipid Peroxidation. London: IntechOpen*, pp.27-47.

Kumar, P., Liu, C., Suliburk, J., Hsu, J.W., Muthupillai, R., Jahoor, F., Minard, C.G., Taffet, G.E. and Sekhar, R.V., 2023. Supplementing glycine and N-acetylcysteine (GlyNAC) in older adults improves glutathione deficiency, oxidative stress, mitochondrial dysfunction, inflammation, physical function, and aging hallmarks: a randomized clinical trial. *The Journals of Gerontology: Series A*, *78*(1), pp.75-89.

Kumrungsee, T., Zhang, P., Chartkul, M., Yanaka, N. and Kato, N., 2020. Potential role of vitamin B6 in ameliorating the severity of COVID-19 and its complications. *Frontiers in nutrition*, *7*, p.562051.

Kurstjens, S., de Baaij, J.H., Bouras, H., Bindels, R.J., Tack, C.J. and Hoenderop, J.G., 2017. Determinants of hypomagnesemia in patients with type 2 diabetes mellitus. *European journal of endocrinology*, *176*(1), pp.11-19.

Kuzniar, A., Mitura, P., Kurys, P., Szymonik-Lesiuk, S., Florianczyk, B. and Stryjecka-Zimmer, M., 2003. The influence of hypomagnesemia on erythrocyte antioxidant enzyme defence system in mice. *Biometals*, *16*, pp.349-357.

Lam, J.R., Schneider, J.L., Zhao, W. and Corley, D.A., 2013. Proton pump inhibitor and histamine 2 receptor antagonist use and vitamin B12 deficiency. *Jama*, *310*(22), pp.2435-2442.

Lamers, Y., 2011. Indicators and methods for folate, vitamin B-12, and vitamin B-6 status assessment in humans. *Current Opinion in Clinical Nutrition & Metabolic Care*, *14*(5), pp.445-454.

Lang, C.A., Naryshkin, S., Schneider, D.L., Mills, B.J. and Lindeman, R.D., 1992. Low blood glutathione levels in healthy aging adults. *The Journal of laboratory and clinical medicine*, *120*(5), pp.720-725.

Langford, R., Hurrion, E. and Dawson, P.A., 2017. Genetics and pathophysiology of mammalian sulfate biology. *Journal of Genetics and Genomics*, *44*(1), pp.7-20.

Lauterburg, B.H. and Mitchell, J.R., 1987. Therapeutic doses of acetaminophen stimulate the turnover of cysteine and glutathione in man. *Journal of hepatology*, *4*(2), pp.206-211.

Lauterburg, B.H. and Velez, M.E., 1988. Glutathione deficiency in alcoholics: risk factor for paracetamol hepatotoxicity. *Gut*, *29*(9), pp.1153-1157.

Lechner P. 2015 Important drug-micronutrient interactions to know. *Pharmacy Times*. http://www.pharmacytimes.com/contributor/petra-lechner-pharmd/2015/12/important-drug-micronutrient-interactions-to-know. (Accessed March 2022).

Lee, L.S., Kim, S.H., Kim, Y.B. and Kim, Y.C., 2014. Quantitative analysis of major constituents in green tea with different plucking periods and their antioxidant activity. *Molecules*, *19*(7), pp.9173-9186..

Leklem, James E. "Vitamin B-6: a status report." *The Journal of nutrition* 120 (1990): 1503-1507.

Li, B., Zhou, P., Xu, K., Chen, T., Jiao, J., Wei, H., Yang, X., Xu, W., Wan, W. and Xiao, J., 2020. Metformin induces cell cycle arrest, apoptosis and autophagy through ROS/JNK signaling pathway in human osteosarcoma. *International Journal of Biological Sciences*, *16*(1), p.74.

Li, L., Bhatia, M., Zhu, Y.Z., Zhu, Y.C., Ramnath, R.D., Wang, Z.J., Anuar, F.B.M., Whiteman, M., Salto-Tellez, M. and Moore, P.K., 2005. Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. *The FASEB Journal*, *19*(9), pp.1196-1198.

Li, L., Rose, P. and Moore, P.K., 2011. Hydrogen sulfide and cell signaling. *Annual review of pharmacology and toxicology*, *51*, pp.169-187.

Li, L., Whiteman, M., Guan, Y.Y., Neo, K.L., Cheng, Y., Lee, S.W., Zhao, Y., Baskar, R., Tan, C.H. and Moore, P.K., 2008. Characterization of a novel, water-soluble hydrogen sulfide–releasing molecule (GYY4137) new insights into the biology of hydrogen sulfide. *Circulation*, *117*(18), pp.2351-2360.

Li, S., Guo, Y., Men, J., Fu, H. and Xu, T., 2021. The preventive efficacy of vitamin B supplements on the cognitive decline of elderly adults: a systematic review and meta-analysis. *BMC geriatrics*, *21*(1), p.367.

Li, X., Lin, J., Lin, Y., Huang, Z., Pan, Y., Cui, P., Yu, C., Cai, C. and Xia, J., 2019. Hydrogen sulfide protects against acetaminophen-induced acute liver injury by inhibiting apoptosis via the JNK/MAPK signaling pathway. *Journal of cellular biochemistry*, *120*(3), pp.4385-4397.

Liang, Y.F., Zhang, D.D., Yu, X.J., Gao, H.L., Liu, K.L., Qi, J., Li, H.B., Yi, Q.Y., Chen, W.S., Cui, W. and Zhu, G.Q., 2017. Hydrogen sulfide in paraventricular nucleus attenuates blood pressure by regulating oxidative stress and inflammatory cytokines in high salt-induced hypertension. *Toxicology Letters*, *270*, pp.62-71.

Lieberman, M.W., Wiseman, A.L., Shi, Z.Z., Carter, B.Z., Barrios, R., Ou, C.N., Chévez-Barrios, P., Wang, Y., Habib, G.M., Goodman, J.C. and Huang, S.L., 1996. Growth retardation and cysteine deficiency in gamma-glutamyl transpeptidase-deficient mice. *Proceedings of the National*

Liggett, J.L., Zhang, X., Eling, T.E. and Baek, S.J., 2014. Anti-tumor activity of non-steroidal anti-inflammatory drugs: cyclooxygenase-independent targets. *Cancer letters*, *346*(2), pp.217-224.

Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., Gargiulo, G., Testa, G., Cacciatore, F., Bonaduce, D. and Abete, P., 2018. Oxidative stress, aging, and diseases. *Clinical interventions in aging*, pp.757-772.

Linares, V., Alonso, V. and Domingo, J.L., 2011. Oxidative stress as a mechanism underlying sulfasalazine-induced toxicity. *Expert opinion on drug safety*, *10*(2), pp.253-263.

Linjakumpu, T., Hartikainen, S., Klaukka, T., Veijola, J., Kivelä, S.L. and Isoaho, R., 2002. Use of medications and polypharmacy are increasing among the elderly. *Journal of clinical epidemiology*, *55*(8), pp.809-817.

Liptay, S., Fulda, S., Schanbacher, M., Bourteele, S., Ferri, K.F., Kroemer, G., Adler, G., Debatin, K.M. and Schmid, R.M., 2002. Molecular mechanisms of sulfasalazine-induced T-cell apoptosis. *British journal of pharmacology*, *137*(5), pp.608-620.

Litchford, M.D., 2020. Drug–nutrient interactions. *Integrative and functional medical nutrition therapy: Principles and practices*, pp.213-220.

Liu, J., Xia, X. and Huang, P., 2020. xCT: a critical molecule that links cancer metabolism to redox signaling. *Molecular Therapy*, *28*(11), pp.2358-2366.

Liu, M., Deng, M., Su, J., Lin, Y., Jia, Z., Peng, K., Wang, F. and Yang, T., 2018. Specific downregulation of cystathionine β-synthase expression in the kidney during obesity. *Physiological reports*, *6*(13), p.e13630.

Liu, Z., Han, Y., Li, L., Lu, H., Meng, G., Li, X., Shirhan, M., Peh, M.T., Xie, L., Zhou, S. and Wang, X., 2013. The hydrogen sulfide donor, GYY 4137, exhibits anti-atherosclerotic activity in high fat fed apolipoprotein E−/− mice. *British journal of pharmacology*, *169*(8), pp.1795-1809.

Lombardo, M., Aulisa, G., Padua, E., Annino, G., Iellamo, F., Pratesi, A., Caprio, M. and Bellia, A., 2020. Gender differences in taste and foods habits. *Nutrition & Food Science*, *50*(1), pp.229-239.

Long, A.N., Atwell, C.L., Yoo, W. and Solomon, S.S., 2012. Vitamin B12 deficiency associated with concomitant metformin and proton pump inhibitor use. *Diabetes Care*, *35*(12), pp.e84-e84.

Lotfy, M., Al-Hammadi, R., Palakkott, A.R., Yasin, J., Al-Hammadi, S. and Ksiksi, T., 2020. Hepatoprotective potentials of Acridocarpus orientalis in mice. *Clinical Phytoscience*, *6*, pp.1-9.

Lotto, V., Choi, S.W. and Friso, S., 2011. Vitamin B6: a challenging link between nutrition and inflammation in CVD. *British Journal of Nutrition*, *106*(2), pp.183-195.

Lowe, N.M., 2021. The global challenge of hidden hunger: perspectives from the field. *Proceedings of the Nutrition Society*, *80*(3), pp.283-289.

Łowicka, E. and Bełtowski, J., 2007. Hydrogen sulfide (H2S)-the third gas of interest for pharmacologists. *Pharmacological reports: PR*, *59*(1), pp.4-24.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *J biol Chem*, *193*(1), pp.265-275.

Lumeng, L. and Li, T.K., 1974. Vitamin B 6 Metabolism in Chronic Alcohol Abuse: Pyridoxal phosphate levels in plasma and the effects of acetaldehyde on pyridoxal phosphate synthesis and degradation in human erythrocytes. *The Journal of clinical investigation*, *53*(3), pp.693-704.

Lunsky, Y. and Modi, M., 2018. Predictors of psychotropic polypharmacy among outpatients with psychiatric disorders and intellectual disability. *Psychiatric services*, *69*(2), pp.242-246.

Lyon, P., Strippoli, V., Fang, B. and Cimmino, L., 2020. B vitamins and one-carbon metabolism: implications in human health and disease. Nutrients, 12(9), p.2867.

Lyons, J., Rauh-Pfeiffer, A., Yu, Y.M., Lu, X.M., Zurakowski, D., Tompkins, R.G., Ajami, A.M., Young, V.R. and Castillo, L., 2000. Blood glutathione synthesis rates in healthy adults

receiving a sulfur amino acid-free diet. *Proceedings of the National Academy of Sciences*, *97*(10), pp.5071-5076.

Mabuchi, T., Hosomi, K., Yokoyama, S. and Takada, M., 2020. Polypharmacy in elderly patients in Japan: Analysis of Japanese real-world databases. *Journal of Clinical Pharmacy and Therapeutics*, *45*(5), pp.991-996.

Macanas-Pirard, P., Yaacob, N.S., Lee, P.C., Holder, J.C., Hinton, R.H. and Kass, G.E., 2005. Glycogen synthase kinase-3 mediates acetaminophen-induced apoptosis in human hepatoma cells. *Journal of Pharmacology and Experimental Therapeutics*, *313*(2), pp.780-789.

Magierowski, M., Jasnos, K., Kwiecień, S. and Brzozowski, T., 2013. Role of hydrogen sulfide in the physiology of gastrointestinal tract and in the mechanism of gastroprotection. *Postepy higieny i medycyny doswiadczalnej (Online)*, *67*, pp.150- 156.

Magierowski, M., Magierowska, K., Hubalewska-Mazgaj, M., Adamski, J., Bakalarz, D., Sliwowski, Z., Pajdo, R., Kwiecien, S. and Brzozowski, T., 2016. Interaction between endogenous carbon monoxide and hydrogen sulfide in the mechanism of gastroprotection against acute aspirin-induced gastric damage. *Pharmacological Research*, *114*, pp.235-250.

Malsy, M., Graf, B. and Bundscherer, A., 2019. The effects of analgesics and local anesthetics on gene transcription mediated by NFATc2 and Sp1 in pancreatic carcinoma. *Anticancer Research*, *39*(9), pp.4721-4728.

Mangoni, A.A. and Zinellu, A., 2024. Transsulfuration and folate pathways in rheumatoid arthritis: A systematic review and meta-analysis. *European Journal of Clinical Investigation*, p.e14158.

Manley, R.C., 2019. *Comparing Glutathione in the Plasma of Vegetarian and Omnivore Populations*. Arizona State University.

Mannery, Y.O., Ziegler, T.R., Park, Y. and Jones, D.P., 2010. Oxidation of plasma cysteine/cystine and GSH/GSSG redox potentials by acetaminophen and sulfur amino acid insufficiency in humans. *Journal of Pharmacology and Experimental Therapeutics*, *333*(3), pp.939-947.

Margaritelis, N.V., Chatzinikolaou, P.N., Bousiou, F.V., Malliou, V.J., Papadopoulou, S.K., Potsaki, P., Theodorou, A.A., Kyparos, A., Geladas, N.D., Nikolaidis, M.G. and Paschalis, V., 2021. Dietary cysteine intake is associated with blood glutathione levels and isometric strength. *International Journal of Sports Medicine*, *42*(05), pp.441-447.

Markovich, D., 2001. Physiological roles and regulation of mammalian sulfate transporters. *Physiological reviews*, *81*(4), pp.1499-1533.

Martin, C.B., Hales, C.M., Gu, Q. and Ogden, C.L., 2019. Prescription drug use in the United States, 2015–2016.

MASELLA, R. and MAZZA, G., 2009. Glutathione and Sulfur Amino Acids in Human Health and Disease.

Masnoon, N., Shakib, S., Kalisch-Ellett, L. and Caughey, G.E., 2017. What is polypharmacy? A systematic review of definitions. *BMC geriatrics*, *17*, pp.1-10.

Mason, P., 2010. Important drug–nutrient interactions. *Proceedings of the Nutrition Society*, *69*(4), pp.551-557.

Mast, C., Pourpe, C., Voyard, G., Rémond, D., Migné, C., Centeno, D., Dardevet, D., Savary-Auzeloux, I. and Papet, I., 2017. Dietary supplementation with cysteine prevents adverse metabolic outcomes of repeated cures with paracetamol in old rats. *British journal of nutrition*, *118*(11), pp.889-896.

Masubuchi, Y., Nakayama, J. and Watanabe, Y., 2011. Sex difference in susceptibility to acetaminophen hepatotoxicity is reversed by buthionine sulfoximine. *Toxicology*, *287*(1-3), pp.54-60.

Mathai, J.C., Missner, A., Kügler, P., Saparov, S.M., Zeidel, M.L., Lee, J.K. and Pohl, P., 2009. No facilitator required for membrane transport of hydrogen sulfide. *Proceedings of the National Academy of Sciences*, *106*(39), pp.16633-16638.

Matthews, D.R., Li, H., Zhou, J., Li, Q., Glaser, S., Francis, H., Alpini, G. and Wu, C., 2021. Methionine-and choline-deficient diet–induced nonalcoholic steatohepatitis is associated with increased intestinal inflammation. *The American Journal of Pathology*, *191*(10), pp.1743-1753.

Mayengbam, S., Raposo, S., Aliani, M. and House, J.D., 2016. A vitamin B-6 antagonist from flaxseed perturbs amino acid metabolism in moderately vitamin B-6–deficient male rats. *The Journal of Nutrition*, *146*(1), pp.14-20.

McCarty, M.F., 2004. Sub-optimal taurine status may promote platelet hyperaggregability in vegetarians. *Medical hypotheses*, *63*(3), pp.426-433.

McColl, K.E., 2009. Effect of proton pump inhibitors on vitamins and iron. *Official journal of the American College of Gastroenterology| ACG*, *104*, pp.S5-S9.

McCrae, J.C., Morrison, E.E., MacIntyre, I.M., Dear, J.W. and Webb, D.J., 2018. Long-term adverse effects of paracetamol–a review. *British journal of clinical pharmacology*, *84*(10), pp.2218-2230.

McDowell, L.R., Wilkinson, N., Madison, R. and Felix, T., 2007, January. Vitamins and minerals functioning as antioxidants with supplementation considerations. In *Florida ruminant nutrition symposium* (Vol. 352, No. 3, pp. 1-17). Gainesville, FL, USA: Best Western Gateway Grand.

Meadows, J., Montano, M., Alfar, A.J., Başkan, Ö.Y., De Brún, C., Hill, J., McClatchey, R., Kallfa, N. and Fernandes, G.S., 2024. The impact of the cost-of-living crisis on population health in the UK: rapid evidence review. *BMC Public Health*, *24*(1), p.561.

Medici, V., Peerson, J.M., Stabler, S.P., French, S.W., Gregory III, J.F., Virata, M.C., Albanese, A., Bowlus, C.L., Devaraj, S., Panacek, E.A. and Rahim, N., 2010. Impaired homocysteine transsulfuration is an indicator of alcoholic liver disease. *Journal of hepatology*, *53*(3), pp.551-557.

Meier, M., Oliveriusova, J., Kraus, J.P. and Burkhard, P., 2003. Structural insights into mutations of cystathionine β-synthase. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1647*(1-2), pp.206-213.

Meng, G., Ma, Y., Xie, L., Ferro, A. and Ji, Y., 2015. Emerging role of hydrogen sulfide in hypertension and related cardiovascular diseases. *British journal of pharmacology*, *172*(23), pp.5501-5511.

Merigliano, C., Mascolo, E., Burla, R., Saggio, I. and Vernì, F., 2018. The relationship between vitamin B6, diabetes and cancer. *Frontiers in Genetics*, *9*, p.388.

Meyers, L.D., Hellwig, J.P. and Otten, J.J. eds., 2006. *Dietary reference intakes: the essential guide to nutrient requirements*. National Academies Press.

Mintzer, S., Skidmore, C.T. and Sperling, M.R., 2012. B-vitamin deficiency in patients treated with antiepileptic drugs. *Epilepsy & behavior*, *24*(3), pp.341-344.

Miyamoto, R., Koike, S., Takano, Y., Shibuya, N., Kimura, Y., Hanaoka, K., Urano, Y., Ogasawara, Y. and Kimura, H., 2017. Polysulfides (H2Sn) produced from the interaction of hydrogen sulfide (H2S) and nitric oxide (NO) activate TRPA1 channels. *Scientific reports*, *7*(1), p.45995.

Mocellin, S., Briarava, M. and Pilati, P., 2017. Vitamin B6 and cancer risk: a field synopsis and meta-analysis. *Journal of the National Cancer Institute*, *109*(3), p.djw230.

Mohn, E.S., Kern, H.J., Saltzman, E., Mitmesser, S.H. and McKay, D.L., 2018. Evidence of drug–nutrient interactions with chronic use of commonly prescribed medications: An update. *Pharmaceutics*, *10*(1), p.36.

Molnár, T., Farkas, K., Nagy, F., Iványi, B. and Wittmann, T., 2010. Sulfasalazine-induced nephrotic syndrome in a patient with ulcerative colitis. *Inflammatory bowel diseases*, *16*(4), pp.552-553.

Moore, K., Hughes, C.F., Hoey, L., Ward, M., Cunningham, C., Molloy, A.M., Strain, J.J., McCarroll, K., Casey, M.C., Tracey, F. and Laird, E., 2019. B-vitamins in relation to depression in older adults over 60 years of age: the Trinity Ulster Department of Agriculture (TUDA) Cohort Study. *Journal of the American Medical Directors Association*, *20*(5), pp.551-557.

Morris, M.S., Picciano, M.F., Jacques, P.F. and Selhub, J., 2008. Plasma pyridoxal 5′-phosphate in the US population: the National Health and Nutrition Examination Survey, 2003–2004. *The American journal of clinical nutrition*, *87*(5), pp.1446-1454.

Morsy, M.A., Ibrahim, S.A., Abdelwahab, S.A., Zedan, M.Z. and Elbitar, H.I., 2010. Curative effects of hydrogen sulfide against acetaminophen-induced hepatotoxicity in mice. *Life sciences*, *87*(23-26), pp.692-698.

Mukherjee, S., Banerjee, O. and Singh, S., 2023. The role of B vitamins in protecting mitochondrial function. In *Molecular Nutrition and Mitochondria* (pp. 167-193). Academic Press.

Mukhopadhyay, M., Banerjee, A., Majumder, R., Chattopadhyay, A. and Bandyopadhyay, D., 2024. Ameliorative role of melatonin against adrenaline induced oxidative stress mediated cardiac and hepatic tissue injuries through preserving pyridoxine metabolism in male Wistar rats: A mechanistic insight. *Advances in Redox Research*, *11*, p.100101.

Munteanu, C., Rotariu, M., Turnea, M., Dogaru, G., Popescu, C., Spînu, A., Andone, I., Postoiu, R., Ionescu, E.V., Oprea, C. and Albadi, I., 2022. Recent advances in molecular research on hydrogen sulfide (H2S) role in diabetes mellitus (DM)—a systematic review. *International Journal of Molecular Sciences*, *23*(12), p.6720.

Murphy, B., Bhattacharya, R. and Mukherjee, P., 2019. Hydrogen sulfide signaling in mitochondria and disease. *The FASEB Journal*, *33*(12), p.13098.

Muth, C., Blom, J.W., Smith, S.M., Johnell, K., Gonzalez-Gonzalez, A.I., Nguyen, T.S., Brueckle, M.S., Cesari, M., Tinetti, M.E. and Valderas, J.M., 2019. Evidence supporting the best clinical management of patients with multimorbidity and polypharmacy: a systematic guideline review and expert consensus. *Journal of internal medicine*, *285*(3), pp.272-288.

Myszkowska, J., Derevenkov, I., Makarov, S.V., Spiekerkoetter, U. and Hannibal, L., 2021. Biosynthesis, quantification and genetic diseases of the smallest signaling thiol metabolite: hydrogen sulfide. *Antioxidants*, *10*(7), p.1065.

Nakamura, T., Itoh, T., Yabe, A., Imai, S., Nakamura, Y., Mizokami, Y., Okouchi, Y., Ikeshita, A. and Kominato, H., 2021. Polypharmacy is associated with malnutrition and activities of daily living disability among daycare facility users: A cross-sectional study. *Medicine*, *100*(34), p.e27073.

Nandi, S.S. and Mishra, P.K., 2017. H2S and homocysteine control a novel feedback regulation of cystathionine beta synthase and cystathionine gamma lyase in cardiomyocytes. *Scientific reports*, *7*(1), p.3639.

Narang, V.S., Pauletti, G.M., Gout, P.W., Buckley, D.J. and Buckley, A.R., 2007. Sulfasalazine-induced reduction of glutathione levels in breast cancer cells: enhancement of growth-inhibitory activity of Doxorubicin. *Chemotherapy*, *53*(3), pp.210-217.

Naser, A.Y., Alwafi, H., Al-Daghastani, T., Hemmo, S.I., Alrawashdeh, H.M., Jalal, Z., Paudyal, V., Alyamani, N., Almaghrabi, M. and Shamieh, A., 2022. Drugs utilization profile in England and Wales in the past 15 years: a secular trend analysis. *BMC primary care*, *23*(1), p.239.

National Institute for Health and Care Excellence *(NICE) paracetamol* (2024) *NICE*. Available at: https://bnf.nice.org.uk/drugs/paracetamol/ (Accessed: 24 June 2023).

National Institute of Health (NIH) (2022). Magnesium. Available at: https://ods.od.nih.gov/factsheets/Magnesium-HealthProfessional/#h8 (Accessed April 2021)

National Institute of Health (NIH) (2023). Iron. Available at: https://ods.od.nih.gov/factsheets/Iron-HealthProfessional/#h7 Accessed April 2021)

National Institute of Health (NIH) (2023). Vitamin B6. Available at: https://ods.od.nih.gov/factsheets/VitaminB6-HealthProfessional/ (Accessed April 2023)

National Institute of Health (NIH) (2024). Iodine. Available at: https://ods.od.nih.gov/factsheets/Iodine-HealthProfessional/#h11 (Accessed April 2021)

Navik, U., Sheth, V.G., Khurana, A., Jawalekar, S.S., Allawadhi, P., Gaddam, R.R., Bhatti, J.S. and Tikoo, K., 2021. Methionine as a double-edged sword in health and disease: Current perspective and future challenges. *Ageing research reviews*, *72*, p.101500.

Ngowi, E.E., Sarfraz, M., Afzal, A., Khan, N.H., Khattak, S., Zhang, X., Li, T., Duan, S.F., Ji, X.Y. and Wu, D.D., 2020. Roles of hydrogen sulfide donors in common kidney diseases. *Frontiers in pharmacology*, *11*, p.564281.

NHS Business Services Authority (NHSBSA) (2023), Prescribing Costs in Hospitals and the Community - England 2018/19 to 2022/23. Available at: https://www.nhsbsa.nhs.uk/statistical-collections/prescribing-costs-hospitals-and-community-england (Accessed on 25 April 2022)

Nimni, M.E., Han, B. and Cordoba, F., 2007. Are we getting enough sulfur in our diet?. *Nutrition & metabolism*, *4*, pp.1-12.

Noguchi, N., Saito, Y. and Niki, E., 2023. Actions of thiols, persulfides, and polysulfides as free radical scavenging antioxidants. *Antioxidants & redox signaling*, *39*(10-12), pp.728-743.

Nykolaichuk, I. and Kopylchuk, H., 2022. Effect of Acetaminophen Against the Background of Alimentary Protein Deficiency on the Features of Sulfur-Containing Amino Acids Metabolism in Rats.

Odai, T., Terauchi, M., Suzuki, R., Kato, K., Hirose, A. and Miyasaka, N., 2020. Depressive Symptoms in Middle-Aged and Elderly Women Are Associated with a Low Intake of Vitamin B6: A Cross-Sectional Study. *Nutrients*, *12*(11), p.3437.

OGAWA, M., TAKAHARA, A., ISHIJIMA, M. and TAZAKI, S., 1985. Decrease of plasma sulfur amino acids in essential hypertension. *Japanese circulation journal*, *49*(12), pp.1217-1224.

Oguntibeju, O.O., 2019. Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links. *International journal of physiology, pathophysiology and pharmacology*, *11*(3), p.45.

Oktora, M.P., Denig, P., Bos, J.H., Schuiling-Veninga, C.C. and Hak, E., 2019. Trends in polypharmacy and dispensed drugs among adults in the Netherlands as compared to the United States. *PloS one*, *14*(3), p.e0214240.

Olatunji, L.A., Badmus, O.O., Abdullahi, K.O., Usman, T.O. and Adejare, A., 2024. Depletion of hepatic glutathione and adenosine by glucocorticoid exposure in Wistar rats is pregnancy-independent. *Toxicology Reports*, *12*, pp.485-491.

Oleszkiewicz, P., Krysinski, J., Religioni, U. and Merks, P., 2021, January. Access to medicines via non-pharmacy outlets in European countries—a review of regulations and the influence on the self-medication phenomenon. In *Healthcare* (Vol. 9, No. 2, p. 123). MDPI.

Olsen, T., Vinknes, K.J., Barvíková, K., Stolt, E., Lee-Ødegård, S., Troensegaard, H., Johannessen, H., Elshorbagy, A., Sokolová, J., Krijt, J. and Křížková, M., 2024. Dietary sulfur amino acid restriction in humans with overweight and obesity: Evidence of an altered plasma and urine sulfurome, and a novel metabolic signature that correlates with loss of fat mass and adipose tissue gene expression. *Redox Biology*, *73*, p.103192.

Olson, K.R., 2012. A practical look at the chemistry and biology of hydrogen sulfide. *Antioxidants & redox signaling*, *17*(1), pp.32-44.

Olson, K.R. and Gao, Y., 2019. Effects of inhibiting antioxidant pathways on cellular hydrogen sulfide and polysulfide metabolism. *Free Radical Biology and Medicine*, *135*, pp.1-14.

Olson, K.R. and Straub, K.D., 2016. The role of hydrogen sulfide in evolution and the evolution of hydrogen sulfide in metabolism and signaling. *Physiology*, *31*(1), pp.60-72.

Olson, K.R., 2005. Vascular actions of hydrogen sulfide in nonmammalian vertebrates. *Antioxidants & redox signaling*, *7*(5-6), pp.804-812.

Olson, K.R., 2018. H2S and polysulfide metabolism: Conventional and unconventional pathways. *Biochemical pharmacology*, *149*, pp.77-90.

Ozturk, A., Baltaci, A.K., Mogulkoc, R., Oztekin, E., Sivrikaya, A., Kurtoglu, E. and Kul, A., 2003. Effects of zinc deficiency and supplementation on malondialdehyde and glutathione levels in blood and tissues of rats performing swimming exercise. *Biological trace element research*, *94*, pp.157-166.

Öztürk, E., Ganidağlı, S. and Öztürk, Z.A., 2023. Polypharmacy in the Elderly: A Double-Edged Sword. *Medical Records*, *5*(2), pp.255-61.

Page, J.H., Ma, J., Chiuve, S.E., Stampfer, M.J., Selhub, J., Manson, J.E. and Rimm, E.B., 2009. Plasma vitamin B6 and risk of myocardial infarction in women. *Circulation*, *120*(8), pp.649-655.

Pajares, M.A. and Perez-Sala, D., 2018. Mammalian sulfur amino acid metabolism: a nexus between redox regulation, nutrition, epigenetics, and detoxification. *Antioxidants & redox signaling*, *29*(4), pp.408-452.

Palabiyik, S.S., Karakus, E., Halici, Z., Cadirci, E., Bayir, Y., Ayaz, G. and Cinar, I., 2016. The protective effects of carvacrol and thymol against paracetamol–induced toxicity on human hepatocellular carcinoma cell lines (HepG2). *Human & Experimental Toxicology*, *35*(12), pp.1252-1263.

Palacios, N., Scott, T., Sahasrabudhe, N., Gao, X. and Tucker, K.L., 2019. Lower plasma vitamin B-6 is associated with 2-year cognitive decline in the Boston Puerto Rican Health Study. *The Journal of nutrition*, *149*(4), pp.635-641.

Parikh, H., Pandita, N. and Khanna, A., 2015. Phytoextract of Indian mustard seeds acts by suppressing the generation of ROS against acetaminophen-induced hepatotoxicity in HepG2 cells. *Pharmaceutical biology*, *53*(7), pp.975-984.

Park, W.H., 2016. Exogenous H2O2 induces growth inhibition and cell death of human pulmonary artery smooth muscle cells via glutathione depletion. *Molecular medicine reports*, *14*(1), pp.936-942.

Parra, M., Stahl, S. and Hellmann, H., 2018. Vitamin B6 and its role in cell metabolism and

physiology. *Cells*, *7*(7), p.84.

Parvez, S., Tabassum, H., Banerjee, B.D. and Raisuddin, S., 2008. Taurine prevents tamoxifen-induced mitochondrial oxidative damage in mice. *Basic & clinical pharmacology & toxicology*, *102*(4), pp.382-387.

Pascual, C., Asghar, Z., Boateng, I. and Grassby, P., 2020. The Impact of Poly-Pharmacy on Mortality, Hospitalisation, Adverse Drug Reactions (ADR) and Falls in the Elderly.

Paul, B.D. and Snyder, S.H., 2014. Neurodegeneration in Huntington's disease involves loss of cystathionine γ-lyase. *Cell Cycle*, *13*(16), pp.2491-2493.

Paul, B.D., Sbodio, J.I., Xu, R., Vandiver, M.S., Cha, J.Y., Snowman, A.M. and Snyder, S.H., 2014. Cystathionine γ-lyase deficiency mediates neurodegeneration in Huntington's disease. *Nature*, *509*(7498), pp.96-100.

Payne, R.A., Avery, A.J., Duerden, M., Saunders, C.L., Simpson, C.R. and Abel, G.A., 2014. Prevalence of polypharmacy in a Scottish primary care population. *European journal of clinical pharmacology*, *70*, pp.575-581.

Pfau, A., López-Cayuqueo, K.I., Scherer, N., Wuttke, M., Wernstedt, A., Fassrainer, D.G., Smith, D.E., van de Kamp, J.M., Ziegeler, K., Eckardt, K.U. and Luft, F.C., 2023. SLC26A1 is a major determinant of sulfate homeostasis in humans. *The Journal of Clinical Investigation*, *133*(3).

Pickering, G., Macian, N., Papet, I., Dualé, C., Coudert, C. and Pereira, B., 2019. N-acetylcysteine prevents glutathione decrease and does not interfere with paracetamol antinociceptive effect at therapeutic dosage: A randomized double-blind controlled trial in healthy subjects. *Fundamental & Clinical Pharmacology*, *33*(3), pp.303-311.

Pickering, G., Schneider, E., Papet, I., Pujos-Guillot, E., Pereira, B., Simen, E., Dubray, C. and Schoeffler, P., 2011. Acetaminophen metabolism after major surgery: a greater challenge with increasing age. *Clinical Pharmacology & Therapeutics*, *90*(5), pp.707-711.

Picó, C., Serra, F., Rodríguez, A.M., Keijer, J. and Palou, A., 2019. Biomarkers of nutrition and health: new tools for new approaches. *Nutrients*, *11*(5), p.1092.

Piragine, E., Malanima, M.A., Lucenteforte, E., Martelli, A. and Calderone, V., 2023. Circulating Levels of Hydrogen Sulfide (H2S) in Patients with Age-Related Diseases: A Systematic Review and Meta-Analysis. *Biomolecules*, *13*(7), p.1023.

Pirouzeh, R., Heidarzadeh-Esfahani, N., Morvaridzadeh, M., Izadi, A., Yosaee, S., Potter, E., Heshmati, J., Pizarro, A.B., Omidi, A. and Heshmati, S., 2020. Effect of DASH diet on oxidative stress parameters: A systematic review and meta-analysis of randomized clinical trials. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, *14*(6), pp.2131-2138.

Pizzorno, J., 2014. Glutathione!. *Integrative Medicine: A Clinician's Journal*, *13*(1), p.8.

Polhemus, D.J., Calvert, J.W., Butler, J. and Lefer, D.J., 2014. The cardioprotective actions of hydrogen sulfide in acute myocardial infarction and heart failure. *Scientifica*, *2014*(1), p.768607.

Porter, K., Hoey, L., Hughes, C.F., Ward, M. and McNulty, H., 2016. Causes, consequences and public health implications of low B-vitamin status in ageing. *Nutrients*, *8*(11), p.725.

Porter, K.M., Ward, M., Hughes, C.F., O'Kane, M., Hoey, L., McCann, A., Molloy, A.M., Cunningham, C., Casey, M.C., Tracey, F. and Strain, S., 2019. Hyperglycemia and metformin use are associated with B vitamin deficiency and cognitive dysfunction in older adults. *The Journal of Clinical Endocrinology & Metabolism*, *104*(10), pp.4837-4847.

Powers, H.J., 2021. Approaches to setting dietary reference values for micronutrients, and translation into recommendations. *Proceedings of the Nutrition Society*, *80*(3), pp.365-372.

Prescott, J.D., Drake, V.J. and Stevens, J.F., 2018. Medications and micronutrients: identifying clinically relevant interactions and addressing nutritional needs. *Journal of Pharmacy Technology*, *34*(5), pp.216-230.

Prescott, L.F., 2000. Paracetamol: past, present, and future. *American journal of therapeutics*, *7*(2), pp.143-148.

Price, V.F. and Jollow, D.J., 1989. Effects of sulfur-amino acid-deficient diets on acetaminophen metabolism and hepatotoxicity in rats. *Toxicology and applied pharmacology*, *101*(2), pp.356-369.

Public Health England (2021) *Composition of Foods Integrated Dataset (CoFID)*, *GOV.UK*. Available at: https://www.gov.uk/government/publications/composition-of-foods-integrated-dataset-cofid (Accessed: 24 August 2022).

Pujos-Guillot, E., Pickering, G., Lyan, B., Ducheix, G., Brandolini-Bunlon, M., Glomot, F., Dardevet, D., Dubray, C. and Papet, I., 2012. Therapeutic paracetamol treatment in older persons induces dietary and metabolic modifications related to sulfur amino acids. *Age*, *34*, pp.181-193.

Qabazard, B., Li, L., Gruber, J., Peh, M.T., Ng, L.F., Kumar, S.D., Rose, P., Tan, C.H., Dymock, B.W., Wei, F. and Swain, S.C., 2014. Hydrogen sulfide is an endogenous regulator of aging in Caenorhabditis elegans. *Antioxidants & redox signaling*, *20*(16), pp.2621-2630.

Qian, L.L., Liu, X.Y., Chai, Q. and Wang, R.X., 2018. Hydrogen sulfide in diabetic complications: Focus on molecular mechanisms. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders)*, *18*(5), pp.470-476.

Qiu, G.H., Xie, X., Xu, F., Shi, X., Wang, Y. and Deng, L., 2015. Distinctive pharmacological differences between liver cancer cell lines HepG2 and Hep3B. *Cytotechnology*, *67*, pp.1-12.

Ramgoolie, P. and Nichols, S., 2016. Polypharmacy and the Risk of Malnutrition among Independently-living Elderly Persons in Trinidad. *West Indian Medical Journal*, *65*(2).

Rawle, M.J., Richards, M., Davis, D. and Kuh, D., 2018. The prevalence and determinants of polypharmacy at age 69: a British birth cohort study. *BMC geriatrics*, *18*, pp.1-12.

Raza, H. and John, A., 2012. Implications of altered glutathione metabolism in aspirin-induced oxidative stress and mitochondrial dysfunction in HepG2 cells. *PloS one*, *7*(4), p.e36325.

Read, E., Zhu, J. and Yang, G., 2021. Disrupted H2S signaling by cigarette smoking and alcohol drinking: evidence from cellular, animal, and clinical studies. *Antioxidants*, *10*(1), p.49.

Rehman, T., Shabbir, M.A., Inam-Ur-Raheem, M., Manzoor, M.F., Ahmad, N., Liu, Z.W., Ahmad, M.H., Siddeeg, A., Abid, M. and Aadil, R.M., 2020. Cysteine and homocysteine as biomarker of various diseases. *Food science & nutrition*, *8*(9), pp.4696-4707.

Reicks, M., Calvert, R.J. and Hathcock, J.N., 1988. Effects of prolonged acetaminophen ingestion and dietary methionine on mouse liver glutathione. *Drug-nutrient interactions*, *5*(4), pp.351-363.

Reid, M. and Jahoor, F., 2001. Glutathione in disease. *Current Opinion in Clinical Nutrition & Metabolic Care*, *4*(1), pp.65-71.

Renga, B., 2011. Hydrogen sulfide generation in mammals: the molecular biology of cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE). *Inflammation & Allergy-Drug Targets (Formerly Current Drug Targets-Inflammation & Allergy)(Discontinued)*, *10*(2), pp.85-91.

Reuter, S., Gupta, S.C., Chaturvedi, M.M. and Aggarwal, B.B., 2010. Oxidative stress, inflammation, and cancer: how are they linked?. *Free radical biology and medicine*, *49*(11), pp.1603-1616.

Ribeiro, B., 2023. Glutathione: the master antioxidant. *Revista Española de Ozonoterapia*, *13*(1), pp.175-197.

Rinaldi, A., Cippà, P.E., Nemazanyy, I., Anglicheau, D. and Pallet, N., 2024. Taurine Deficiency Is a Hallmark of Injured Kidney Allografts. *Transplantation*, pp.10-1097.

Roberts, E., Nunes, V.D., Buckner, S., Latchem, S., Constanti, M., Miller, P., Doherty, M., Zhang, W., Birrell, F., Porcheret, M. and Dziedzic, K., 2016. Paracetamol: not as safe as we thought? A systematic literature review of observational studies. *Annals of the rheumatic diseases*, *75*(3), pp.552-559.

Roe, C.M., McNamara, A.M. and Motheral, B.R., 2002. Gender-and age-related prescription drug use patterns. *Annals of Pharmacotherapy*, *36*(1), pp.30-39.

Rojo-Sebastián, A., González-Robles, C. and de Yébenes, J.G., 2020. Vitamin B6 deficiency in patients with Parkinson disease treated with levodopa/carbidopa. *Clinical Neuropharmacology*, *43*(5), pp.151-157.

Rolls, B.J., 1993. Appetite, hunger, and satiety in the elderly. *Critical reviews in food science and nutrition*, *33*(1), pp.39-44.

Root, E.J. and Longenecker, J.B., 1983. Brain cell alterations suggesting premature aging induced by dietary deficiency of vitamin B6 and/or copper. *The American journal of clinical nutrition*, *37*(4), pp.540-552.

Rose, C.S., György, P., Butler, M., Andres, R., Norris, A.H., Shock, N.W., Tobin, J., Brin, M. and Spiegel, H., 1976. Age differences in vitamin B6 status of 617 men. *The American Journal of Clinical Nutrition*, *29*(8), pp.847-853.

Rose, P., Moore, P.K. and Zhu, Y.Z., 2017. H 2 S biosynthesis and catabolism: new insights from molecular studies. *Cellular and Molecular Life Sciences*, *74*, pp.1391-1412.

Rose, P., Moore, P.K., Whiteman, M., Kirk, C. and Zhu, Y.Z., 2021. Diet and hydrogen sulfide production in mammals. *Antioxidants & Redox Signaling*, *34*(17), pp.1378-1393.

Rose, P., Whiteman, M., Huang, S.H., Halliwell, B. and Ong, C.N., 2003. β-phenylethyl isothiocyanate-mediated apoptosis in hepatoma HepG2 cells. *Cellular and Molecular Life Sciences CMLS*, *60*, pp.1489-1503.

Rowicka, G., Klemarczyk, W., Ambroszkiewicz, J., Strucińska, M., Kawiak-Jawor, E., Weker, H. and Chełchowska, M., 2023. Assessment of Oxidant and Antioxidant Status in Prepubertal Children following Vegetarian and Omnivorous Diets. *Antioxidants*, *12*(3), p.682.

Rubí, B., 2012. Pyridoxal 5'-phosphate (PLP) deficiency might contribute to the onset of type I diabetes. *Medical hypotheses*, *78*(1), pp.179-182.

Rybak, M.E. and Pfeiffer, C.M., 2004. Clinical analysis of vitamin B6: determination of pyridoxal 5′-phosphate and 4-pyridoxic acid in human serum by reversed-phase high-performance liquid chromatography with chlorite postcolumn derivatization. *Analytical biochemistry*, *333*(2), pp.336-344.

Rybka, J., Kupczyk, D., Kędziora-Kornatowska, K., Motyl, J., Czuczejko, J., Szewczyk-Golec, K., Kozakiewicz, M., Pawluk, H., Carvalho, L.A. and Kędziora, J., 2011. Glutathione-related antioxidant defense system in elderly patients treated for hypertension. *Cardiovascular toxicology*, *11*, pp.1-9.

Salimi, A., Bahreini, F., Jamali, Z. and Pourahmad, J., 2020. Mesalazine induces oxidative stress and cytochrome c release in isolated rat heart mitochondria: an analysis of cardiotoxic effects. *International Journal of Toxicology*, *39*(3), pp.241-247.

Sandikci, S.C., Colak, S., Omma, A., Enecik, M.E., Ozbalkan, Z., Neselioglu, S. and Erel, O., 2019. An investigation of thiol/disulfide homeostasis in patients with Behçet's disease. *Archives of Medical Science*, *15*(1).

Santo, L. and Kang, K., 2023. National hospital ambulatory medical care survey: 2019 national summary tables. Available online: **https://stacks.cdc.gov/view/cdc/123251** (accessed on 21 February 2022).

Santulli, G., Kansakar, U., Varzideh, F., Mone, P., Jankauskas, S.S. and Lombardi, A., 2023. Functional role of taurine in aging and cardiovascular health: an updated overview. *Nutrients*, *15*(19), p.4236.

Sarwar, M.F., Sarwar, M.H. and Sarwar, M., 2021. Deficiency of vitamin B-Complex and its relation with body disorders. *B-complex vitamins-sources, intakes and novel applications*, pp.79-100.

Saunders, J. and Smith, T., 2010. Malnutrition: causes and consequences. *Clinical medicine*, *10*(6), p.624.

Sawa, T., Motohashi, H., Ihara, H. and Akaike, T., 2020. Enzymatic regulation and biological functions of reactive cysteine persulfides and polysulfides. *Biomolecules*, *10*(9), p.1245.

Sawyer, D.B., Peng, X., Chen, B., Pentassuglia, L. and Lim, C.C., 2010. Mechanisms of anthracycline cardiac injury: can we identify strategies for cardioprotection?. *Progress in cardiovascular diseases*, *53*(2), pp.105-113.

Sayedali, E., Yalin, A.E. and Yalin, S., 2023. Association between metformin and vitamin B12 deficiency in patients with type 2 diabetes. *World journal of diabetes*, *14*(5), p.585.

Sbodio, J.I., Snyder, S.H. and Paul, B.D., 2019. Regulators of the transsulfuration pathway. *British journal of pharmacology*, *176*(4), pp.583-593.

Scammahorn, J.J., Nguyen, I.T., Bos, E.M., Van Goor, H. and Joles, J.A., 2021. Fighting oxidative stress with sulfur: Hydrogen sulfide in the renal and cardiovascular systems. *Antioxidants*, *10*(3), p.373.

Schaffer, S.W., Solodushko, V. and Kakhniashvili, D., 2002. Beneficial effect of taurine depletion on osmotic sodium and calcium loading during chemical hypoxia. *American Journal of Physiology-Cell Physiology*, *282*(5), pp.C1113-C1120.

Schmidt, J.A., Rinaldi, S., Scalbert, A., Ferrari, P., Achaintre, D., Gunter, M.J., Appleby, P.N., Key, T.J. and Travis, R.C., 2016. Plasma concentrations and intakes of amino acids in male meat-eaters, fish-eaters, vegetarians and vegans: a cross-sectional analysis in the EPIC-Oxford cohort. *European journal of clinical nutrition*, *70*(3), pp.306-312.

Schüpbach, R., Wegmüller, R., Berguerand, C., Bui, M. and Herter-Aeberli, I., 2017. Micronutrient status and intake in omnivores, vegetarians and vegans in Switzerland. *European journal of nutrition*, *56*, pp.283-293.

Seifert, C.F. and Anderson, D.C., 2007. Acetaminophen Usage Patterns and Concentrations of Glutathione and γ-Glutamyl Transferase in Alcoholic Subjects. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, *27*(11), pp.1473-1482.

Sekhar, R.V., Patel, S.G., Guthikonda, A.P., Reid, M., Balasubramanyam, A., Taffet, G.E. and Jahoor, F., 2011. Deficient synthesis of glutathione underlies oxidative stress in aging and can be corrected by dietary cysteine and glycine supplementation. *The American journal of clinical nutrition*, *94*(3), pp.847-853.

Sendo, K., Seino, M., Ohta, T. and Nagase, S., 2022. Impact of the glutathione synthesis pathway on sulfasalazine-treated endometrial cancer. *Oncotarget*, *13*, p.224.

Sergi, G., Rui, M.D., Sarti, S. and Manzato, E., 2011. Polypharmacy in the elderly: can comprehensive geriatric assessment reduce inappropriate medication use?. *Drugs & aging*, *28*, pp.509-518.

Sganga, F., Vetrano, D.L., Volpato, S., Cherubini, A., Ruggiero, C., Corsonello, A., Fabbietti, P., Lattanzio, F., Bernabei, R. and Onder, G., 2014. Physical performance measures and polypharmacy among hospitalized older adults: results from the CRIME study. *The Journal of nutrition, health and aging*, *18*(6), pp.616-621.

Shabbir, U., McNulty, H., Hughes, C., Caffrey, A., Clements, M., Dooley, J. and Hoey, L., 2024, February. B-Vitamin Biomarkers in Relation to Immune Function in Older Adults: Preliminary Analysis from the TUDA Study. In *Proceedings* (Vol. 91, No. 1, p. 285). MDPI.

Shamaa, M.M., 2021. Sulfasalazine synergistically enhances the inhibitory effects of imatinib against hepatocellular carcinoma (HCC) cells by targeting NFκB, BCR/ABL, and PI3K/AKT signaling pathway-related proteins. *FEBS Open bio*, *11*(3), pp.588-597.

Sharif, A.H., Iqbal, M., Manhoosh, B., Gholampoor, N., Ma, D., Marwah, M. and Sanchez-Aranguren, L., 2023. Hydrogen sulphide-based therapeutics for neurological conditions: Perspectives and challenges. *Neurochemical Research*, *48*(7), pp.1981-1996.

Sharifi-Rad, M., Anil Kumar, N.V., Zucca, P., Varoni, E.M., Dini, L., Panzarini, E., Rajkovic, J., Tsouh Fokou, P.V., Azzini, E., Peluso, I. and Prakash Mishra, A., 2020. Lifestyle, oxidative stress, and antioxidants: Back and forth in the pathophysiology of chronic diseases. *Frontiers in physiology*, *11*, p.694.

Shen, J., Lai, C.Q., Mattei, J., Ordovas, J.M. and Tucker, K.L., 2010. Association of vitamin B-6 status with inflammation, oxidative stress, and chronic inflammatory conditions: the Boston Puerto Rican Health Study. *The American journal of clinical nutrition*, *91*(2), pp.337-342.

Shen, Y., Shen, Z., Luo, S., Guo, W. and Zhu, Y.Z., 2015. The cardioprotective effects of hydrogen sulfide in heart diseases: from molecular mechanisms to therapeutic potential. *Oxidative Medicine and Cellular Longevity*, *2015*(1), p.925167.

Shenkin, A., 2006. The key role of micronutrients. *Clinical nutrition*, *25*(1), pp.1-13.

Shere, M., Bapat, P., Nickel, C., Kapur, B. and Koren, G., 2015. Association between use of oral contraceptives and folate status: a systematic review and meta-analysis. *Journal of Obstetrics and Gynaecology Canada*, *37*(5), pp.430-438.

Shergill-Bonner, R., 2013. Micronutrients. *Paediatrics and Child Health*, *23*(8), pp.331-336.

Shi, H., Yang, S., Liu, Y., Huang, P., Lin, N., Sun, X., Yu, R., Zhang, Y., Qin, Y. and Wang, L., 2015. Study on environmental causes and SNPs of MTHFR, MS and CBS genes related to congenital heart disease. *PloS one*, *10*(6), p.e0128646.

Shimizu, H., Kiyohara, Y., Kato, I., Kitazono, T., Tanizaki, Y., Kubo, M., Ueno, H., Ibayashi, S., Fujishima, M. and Iida, M., 2004. Relationship between plasma glutathione levels and cardiovascular disease in a defined population: the Hisayama study. *Stroke*, *35*(9), pp.2072-2077.

Shin, W.Y., Go, T.H. and Kim, J.H., 2024. Trends in commonly used and potentially inappropriate medications in older Korean patients with polypharmacy. *BMC Geriatrics*, *24*(1), pp.1-9.

Shojania, A.M., 1982. Oral contraceptives: effect of folate and vitamin B12 metabolism. *Canadian Medical Association Journal*, *126*(3), p.244.

Shukla, K., Thomas, A.G., Ferraris, D.V., Hin, N., Sattler, R., Alt, J., Rojas, C., Slusher, B.S. and Tsukamoto, T., 2011. Inhibition of xc-transporter-mediated cystine uptake by

sulfasalazine analogs. *Bioorganic & medicinal chemistry letters*, *21*(20), pp.6184-6187.

Sies, H., Stahl, W. and Sundquist, A.R., 1992. Antioxidant functions of vitamins. Vitamins E and C, beta-carotene, and other carotenoids. *Annals of the New York Academy of Sciences*, *669*, pp.7-20.

Singh, A., Kukreti, R., Saso, L. and Kukreti, S., 2019. Oxidative stress: a key modulator in neurodegenerative diseases. *Molecules*, *24*(8), p.1583.

Singh, P., Gollapalli, K., Mangiola, S., Schranner, D., Yusuf, M.A., Chamoli, M., Shi, S.L., Lopes Bastos, B., Nair, T., Riermeier, A. and Vayndorf, E.M., 2023. Taurine deficiency as a driver of aging. *Science*, *380*(6649), p.eabn9257.

Sinthorn, W., Chatuphonprasert, W., Chulasiri, M. and Jarukamjorn, K., 2016. Thai red rice extract provides liver protection in paracetamol-treated mice by restoring the glutathione system. *Pharmaceutical Biology*, *54*(5), pp.770-779.

Sitdikova, G.F., Weiger, T.M. and Hermann, A., 2010. Hydrogen sulfide increases calcium-activated potassium (BK) channel activity of rat pituitary tumor cells. *Pflügers Archiv-European Journal of Physiology*, *459*, pp.389-397.

Sivandzade, F., Bhalerao, A. and Cucullo, L., 2019. Analysis of the mitochondrial membrane potential using the cationic JC-1 dye as a sensitive fluorescent probe. *Bio-protocol*, *9*(1), pp.e3128-e3128.

Smith, A.D. and Refsum, H., 2016. Homocysteine, B vitamins, and cognitive impairment. *Annual review of nutrition*, *36*, pp.211-239.

Smith, A.D., Smith, S.M., De Jager, C.A., Whitbread, P., Johnston, C., Agacinski, G., Oulhaj, A., Bradley, K.M., Jacoby, R. and Refsum, H., 2010. Homocysteine-lowering by B vitamins slows the rate of accelerated brain atrophy in mild cognitive impairment: a randomized controlled trial. *PloS one*, *5*(9), p.e12244.

Snell, E.E. and Pearson, P.B., 1942. Effect of heat sterilization on growth-promoting activity of pyridoxine for streptococcus lactis R. *Proceedings of the Society for Experimental Biology and Medicine*, *51*(3), pp.356-358.

Šola, I., Vujčić Bok, V., Dujmović, M. and Rusak, G., 2020. Developmentally-related changes in phenolic and L-ascorbic acid content and antioxidant capacity of Chinese cabbage sprouts. *Journal of food science and technology*, *57*, pp.702-712.

Solleiro-Villavicencio, H. and Rivas-Arancibia, S., 2018. Effect of chronic oxidative stress on neuroinflammatory response mediated by CD4+ T cells in neurodegenerative diseases. *Frontiers in cellular neuroscience*, *12*, p.114.

Song, B., Fu, M., He, F., Zhao, H., Wang, Y., Nie, Q. and Wu, B., 2021. Methionine deficiency affects liver and kidney health, oxidative stress, and ileum mucosal immunity in broilers. *Frontiers in veterinary science*, *8*, p.722567.

Spinneker, A., Sola, R., Lemmen, V., Castillo, M.J., Pietrzik, K. and González-Gross, M., 2007. Vitamin B6 status, deficiency and its consequences-an overview. *Nutricion hospitalaria*, *22*(1), pp.7-24.

Spital, A., 1999. Diuretic-induced hyponatremia. *American journal of nephrology*, *19*(4), pp.447-452.

Srilatha, B., Adaikan, P.G. and Moore, P.K., 2006. Possible role for the novel gasotransmitter hydrogen sulphide in erectile dysfunction—A pilot study. *European journal of pharmacology*, *535*(1-3), pp.280-282.

Stach, K., Stach, W. and Augoff, K., 2021. Vitamin B6 in health and disease. *Nutrients*, *13*(9), p.3229.

Stael, S., Miller, L.P., Fernandez-Fernandez, A.D. and Van Breusegem, F., 2022. Detection of Damage-Activated Metacaspase Activity Activities by Western Blot in Plants. In *Plant Proteases and Plant Cell Death: Methods and Protocols* (pp. 127-137). New York, NY: Springer US.

Statistics Canada (2021). Prescription medication use among Canadian adults, 2016 to 2019 https://www150.statcan.gc.ca/n1/daily-quotidien/210628/dq210628e-eng.htm (Accessed April 2023).

Stipanuk, M.H., 2004. Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. *Annu. Rev. Nutr.*, *24*(1), pp.539-577.

Stipanuk, M.H., Londono, M., Lee, J.I., Hu, M. and Anthony, F.Y., 2002. Enzymes and metabolites of cysteine metabolism in nonhepatic tissues of rats show little response to changes in dietary protein or sulfur amino acid levels. *The Journal of nutrition*, *132*(11), pp.3369-3378.

Stover, P.J. and Field, M.S., 2015. Vitamin B-6. *Advances in Nutrition*, *6*(1), pp.132-133.

Struijk, E.A., Lana, A., Guallar-Castillón, P., Rodríguez-Artalejo, F. and Lopez-Garcia, E., 2018. Intake of B vitamins and impairment in physical function in older adults. *Clinical nutrition*, *37*(4), pp.1271-1278.

Su, L.J., Zhang, J.H., Gomez, H., Murugan, R., Hong, X., Xu, D., Jiang, F. and Peng, Z.Y., 2019. Reactive oxygen species-induced lipid peroxidation in apoptosis, autophagy, and ferroptosis. *Oxidative medicine and cellular longevity*, *2019*.

Sun, H.J., Leng, B., Wu, Z.Y. and Bian, J.S., 2020. Polysulfide and hydrogen sulfide ameliorate cisplatin-induced nephrotoxicity and renal inflammation through Persulfidating STAT3 and IKKβ. *International Journal of Molecular Sciences*, *21*(20), p.7805.

Sun, H.J., Wu, Z.Y., Nie, X.W. and Bian, J.S., 2021. Role of hydrogen sulfide and polysulfides in neurological diseases: focus on protein S-persulfidation. *Current Neuropharmacology*, *19*(6), pp.868-884.

Sun, H.J., Xiong, S.P., Cao, X., Cao, L., Zhu, M.Y., Wu, Z.Y. and Bian, J.S., 2021. Polysulfide-mediated sulfhydration of SIRT1 prevents diabetic nephropathy by suppressing phosphorylation and acetylation of p65 NF-κB and STAT3. *Redox biology*, *38*, p.101813.

Sun, Y., Zheng, Y., Wang, C. and Liu, Y., 2018. Glutathione depletion induces ferroptosis, autophagy, and premature cell senescence in retinal pigment epithelial cells. *Cell death & disease*, *9*(7), p.753.

Sutjiati, E., Wirjatmadi, B. and Kalim, H., 2018. Effect of Low Methionine Formula on Levels of IL-1β Serum and IL-1β Gene Expression in Knee Joint Cartilage Tissues of Normal Rabbits and ACL Induction OA Models. *Indian Journal of Public Health Research & Development*, *9*(10).

Switzer, C.H., 2023. How super is supersulfide?: Reconsidering persulfide reactivity in cellular biology. *Redox Biology*, *67*, p.102899.

Sykes, L., Thomson, K.R., Boyce, E.J., Lee, Y.S., Rasheed, Z.B., MacIntyre, D.A., Teoh, T.G. and Bennett, P.R., 2015. Sulfasalazine augments a pro-inflammatory response in interleukin-1β-stimulated amniocytes and myocytes. *Immunology*, *146*(4), pp.630- 644.

Taggart, A.J., McDermott, B.J. and Roberts, S.D., 1992. The effect of age and acetylator phenotype on the pharmacokinetics of sulfasalazine in patients with rheumatoid arthritis. *Clinical pharmacokinetics*, *23*, pp.311-320.

Taguchi, K., Tokuno, M., Yamasaki, K., Kadowaki, D., Seo, H. and Otagiri, M., 2015. Establishment of a model of acetaminophen-induced hepatotoxicity in different weekly-aged ICR mice. *Laboratory animals*, *49*(4), pp.294-301.

Takahashi, S., Hisatsune, A., Kurauchi, Y., Seki, T. and Katsuki, H., 2018. Polysulfide protects midbrain dopaminergic neurons from MPP+-induced degeneration via enhancement of glutathione biosynthesis. *Journal of Pharmacological Sciences*, *137*(1), pp.47-54.

Tang, G., Wu, L., Liang, W. and Wang, R., 2005. Direct stimulation of KATP channels by exogenous and endogenous hydrogen sulfide in vascular smooth muscle cells. *Molecular pharmacology*, *68*(6), pp.1757-1764.

Tang, Y., Xu, M. and Shiu-Ming, K., 2018. Factors Contributing to the High Prevalence of Vitamin B6 Deficiency in US: A System-atic Review. *J. Hum. Nutr.*, *2*(1), pp.58-64.

Tanimizu, N., Ichinohe, N., Suzuki, H. and Mitaka, T., 2020. Prolonged oxidative stress and delayed tissue repair exacerbate acetaminophen-induced liver injury in aged mice. *Aging (Albany NY)*, *12*(19), p.18907.

Tardy, A.L., Pouteau, E., Marquez, D., Yilmaz, C. and Scholey, A., 2020. Vitamins and minerals for energy, fatigue and cognition: a narrative review of the biochemical and clinical evidence. *Nutrients*, *12*(1), p.228.

Tariq, M. and Din, F.U., 2017. Poor knowledge of university students regarding paracetamol; a wakeup call for public healthcare practitioners. *Cogent Medicine*, *4*(1), p.1320848.

Tavares, A.I., Ferreira, P.L. and Cavadas, V., 2022. Factors contributing to self-medication and consumption of non-prescribed drugs in Portugal. *International journal of public health*, *67*, p.1604852.

Tesseraud, S., Coustard, S.M., Collin, A. and Seiliez, I., 2008. Role of sulfur amino acids in controlling nutrient metabolism and cell functions: implications for nutrition. *British Journal of Nutrition*, *101*(8), pp.1132-1139.

The global use of medicines 2024: Outlook to 2028 (2014) *IQVIA*. Available at: https://www.iqvia.com/insights/the-iqvia-institute/reports-and-publications/reports/the-global-use-of-medicines-2024-outlook-to-2028#:~:text=Global%20use%20of%20medicines%20grew,3.8%20trillion%20defined%20daily%20doses (Accessed: 26 June 2022).

The global use of medicines 2024: Outlook to 2028 (2024) *IQVIA*. Available at: https://www.iqvia.com/insights/the-iqvia-institute/reports-and-publications/reports/the-global-use-of-medicines-2024-outlook-to-2028#:~:text=Global%20use%20of%20medicines%20grew,3.8%20trillion%20defined%20daily%20doses (Accessed: 26 June 2022).

Thornberry, N.A. and Lazebnik, Y., 1998. Caspases: enemies within. *Science*, *281*(5381), pp.1312-1316.

Tian, L., Mi, N., Wang, L., Huang, C., Fu, W., Bai, M., Gao, L., Ma, H., Zhang, C., Lu, Y. and Zhao, J., 2024. Regular use of paracetamol and risk of liver cancer: a prospective cohort study. *BMC cancer*, *24*(1), p.33.

Tian, M., Wang, Y., Lu, Y.Q., Yan, M., Jiang, Y.H. and Zhao, D.Y., 2012. Correlation between serum H2S and pulmonary function in children with bronchial asthma. *Molecular medicine reports*, *6*(2), pp.335-338.

Tirmenstein, M.A. and Nelson, S.D., 1990. Acetaminophen-induced oxidation of protein thiols. Contribution of impaired thiol-metabolizing enzymes and the breakdown of adenine nucleotides. *Journal of Biological Chemistry*, *265*(6), pp.3059-3065.

Townsend, D.M., Tew, K.D. and Tapiero, H., 2003. The importance of glutathione in human disease. *Biomedicine & pharmacotherapy*, *57*(3-4), pp.145-155.

Townsend, E., Hawton, K., Harriss, L., Bale, E. and Bond, A., 2001. Substances used in deliberate self-poisoning 1985–1997: trends and associations with age, gender,

repetition and suicide intent. *Social psychiatry and psychiatric epidemiology*, *36*, pp.228-234.

Trenti, T., Bertolotti, M., Castellana, C.N., Ferrari, A., Pini, L.A. and Sternieri, E., 1992. Plasma glutathione level in paracetamol daily abuser patients. Changes in plasma cysteine and thiol groups after reduced glutathione administration. *Toxicology letters*, *64*, pp.757-761.

Tricco, A.C., Lillie, E., Zarin, W., O'Brien, K.K., Colquhoun, H., Levac, D., Moher, D., Peters, M.D., Horsley, T., Weeks, L. and Hempel, S., 2018. PRISMA extension for scoping reviews (PRISMA-ScR): checklist and explanation. *Annals of internal medicine*, *169*(7), pp.467-473.

Tripatara, P., SA Patel, N., Collino, M., Gallicchio, M., Kieswich, J., Castiglia, S., Benetti, E., Stewart, K.N., Brown, P.A., Yaqoob, M.M. and Fantozzi, R., 2008. Generation of endogenous hydrogen sulfide by cystathionine γ-lyase limits renal ischemia/reperfusion injury and dysfunction. *Laboratory investigation*, *88*(10), pp.1038-1048.

Tsai, M.Y., Hanson, N.Q., Bignell, M.K. and Schwichtenberg, K.A., 1996. Simultaneous detection and screening of T833C and G919A mutations of the cystathionine β-synthase gene by single-strand conformational polymorphism. *Clinical biochemistry*, *29*(5), pp.473-477.

Tulchinsky, T.H., 2010. Micronutrient deficiency conditions: global health issues. *Public health reviews*, *32*, pp.243-255.

Tummolo, A., Carella, R., De Giovanni, D., Paterno, G., Simonetti, S., Tolomeo, M., Leone, P. and Barile, M., 2023. Micronutrient Deficiency in Inherited Metabolic Disorders Requiring Diet Regimen: A Brief Critical Review. *International Journal of Molecular Sciences*, *24*(23), p.17024.

Uba, T., Matsuo, Y., Sumi, C., Shoji, T., Nishi, K., Kusunoki, M., Harada, H., Kimura, H., Bono, H. and Hirota, K., 2021. Polysulfide inhibits hypoxia-elicited hypoxia-inducible factor activation in a mitochondria-dependent manner. *Mitochondrion*, *59*, pp.255-266.

Uba, T., Matsuo, Y., Sumi, C., Shoji, T., Nishi, K., Kusunoki, M., Harada, H., Kimura, H., Bono, H. and Hirota, K., 2021. Polysulfide inhibits hypoxia-elicited hypoxia-inducible factor activation in a mitochondria-dependent manner. *Mitochondrion*, *59*, pp.255-266.

Ubbink, J.B., Delport, R., Bissbort, S., Vermaak, W.H. and Becker, P.J., 1990. Relationship between vitamin B-6 status and elevated pyridoxal kinase levels induced by theophylline therapy in humans. *The Journal of nutrition*, *120*(11), pp.1352-1359.

Ueland, P.M., Ulvik, A., Rios-Avila, L., Midttun, Ø. and Gregory, J.F., 2015. Direct and functional biomarkers of vitamin B6 status. *Annual review of nutrition*, *35*, pp.33-70.

Ulvik, A., Ebbing, M., Hustad, S., Midttun, Ø., Nygard, O., Vollset, S.E., Bønaa, K.H., Nordrehaug, J.E., Nilsen, D.W., Schirmer, H. and Ueland, P.M., 2010. Long-and short-term effects of tobacco smoking on circulating concentrations of B vitamins. *Clinical chemistry*, *56*(5), pp.755-763.

Umemura, A., 2023. Impacts of drug-induced oxidative stress. *Frontiers in Medicine*, *10*, p.1191864.

UN 2019. World population prospects 2019: highlights (ST/ESA/SER.A/423). In: DEPARTMENT OF ECONOMIC AND SOCIAL AFFAIRS, P. D. (ed.). New York: United Nations.

UN 2022. *World population ageing 2019: Highlights*. Available at: https://www.un.org/en/development/desa/population/publications/pdf/ageing/WorldPopulationAgeing2019-Highlights.pdf (Accessed: 30 August 2023).

van de Poll, M., Dejong, C. and Soeters, P., 2006. Adequate Range for Sulfur-Containing Amino Acids and Biomarkers for Their Excess: Lessons from Enteral and Parenteral

Nutrition. *The Journal of Nutrition*, 136(6), pp.1694S-1700S.

van den Born, J.C., Frenay, A.R.S., Bakker, S.J., Pasch, A., Hillebrands, J.L., Heerspink, H.J.L. and van Goor, H., 2016. High urinary sulfate concentration is associated with reduced risk of renal disease progression in type 2 diabetes. *Nitric Oxide*, *55*, pp.18-24.

van den Born, J.C., Frenay, A.R.S., Koning, A.M., Bachtler, M., Riphagen, I.J., Minovic, I., Feelisch, M., Dekker, M.M., Bulthuis, M.L., Gansevoort, R.T. and Hillebrands, J.L., 2019. Urinary excretion of sulfur metabolites and risk of cardiovascular events and all-cause mortality in the general population. *Antioxidants & redox signaling*, *30*(17), pp.1999-2010.

Venables, M.C., Roberts, C., Nicholson, S., Bates, B., Jones, K.S., Ashford, R., Hill, S., Farooq, A., Koulman, A., Wareham, N.J. and Page, P., 2022. Data resource profile: United Kingdom National Diet and nutrition survey rolling Programme (2008– 19). *International Journal of Epidemiology*, *51*(4), pp.e143-e155.

Volpi, E., Campbell, W.W., Dwyer, J.T., Johnson, M.A., Jensen, G.L., Morley, J.E. and Wolfe, R.R., 2013. Is the optimal level of protein intake for older adults greater than the recommended dietary allowance?. *Journals of gerontology series A: Biomedical sciences and medical sciences*, *68*(6), pp.677-681.

Wallace, J.L., Caliendo, G., Santagada, V., Cirino, G. and Fiorucci, S., 2007. Gastrointestinal safety and anti-inflammatory effects of a hydrogen sulfide–releasing diclofenac derivative in the rat. *Gastroenterology*, *132*(1), pp.261-271.

Wallace, J.L., Dicay, M., McKnight, W. and Martin, G.R., 2007. Hydrogen sulfide enhances ulcer healing in rats. *The FASEB Journal*, *21*(14), pp.4070-4076.

WALTERS, L., 1988. Vitamin B, Nutritional Status in Asthma: The Effect of Theophylline Therapy on Plasma Pyridoxal-5'-Phosphate and Pyridoxal Levels.

Wang, B., Mo, X., Wu, Z. and Guan, X., 2022. Systematic review and meta-analysis of the correlation between plasma homocysteine levels and coronary heart disease. *Journal of Thoracic Disease*, *14*(3), p.646.

Wang, J. and Hegele, R.A., 2003. Genomic basis of cystathioninuria (MIM 219500) revealed by multiple mutations in cystathionine gamma-lyase (CTH). *Human genetics*, *112*, pp.404-408.

Wang, J., Huff, A.M., Spence, J.D. and Hegele, R.A., 2004. Single nucleotide polymorphism in CTH associated with variation in plasma homocysteine concentration. *Clinical genetics*, *65*(6), pp.483-486.

Wang, K., Ahmad, S., Cai, M., Rennie, J., Fujisawa, T., Crispi, F., Baily, J., Miller, M.R., Cudmore, M., Hadoke, P.W. and Wang, R., 2013. Dysregulation of hydrogen sulfide producing enzyme cystathionine γ-lyase contributes to maternal hypertension and placental abnormalities in preeclampsia. *Circulation*, *127*(25), pp.2514-2522.

Wang, P., Huang, J., Xue, F., Abuduaini, M., Tao, Y. and Liu, H., 2024. Associations of serum vitamin B6 status with the risks of cardiovascular, cancer, and all-cause mortality in the elderly. *Frontiers in Immunology*, *15*, p.1354958.

Wang, X., Lin, T., He, Y., Zhou, Y., Peng, Y., Zhang, W. and Ni, X., 2023. Reduced Renal CSE/CBS/H2S Contributes to the Progress of Lupus Nephritis. *Biology*, *12*(2), p.318.

Wang, X., Liu, K., Shirai, K., Tang, C., Hu, Y., Wang, Y., Hao, Y. and Dong, J.Y., 2023. Prevalence and trends of polypharmacy in US adults, 1999–2018. *Global Health Research and Policy*, *8*(1), p.25.

Wang, Y., Hunt, K., Nazareth, I., Freemantle, N. and Petersen, I., 2013. Do men consult less than women? An analysis of routinely collected UK general practice data. *BMJ open*, *3*(8), p.e003320.

Wang, Y., Wang, S., Xin, Y., Zhang, J., Wang, S., Yang, Z. and Liu, C., 2021. Hydrogen sulfide alleviates the anxiety-like and depressive-like behaviors of type 1 diabetic mice via inhibiting inflammation and ferroptosis. *Life sciences*, *278*, p.119551.

Weber, C.K., Liptay, S., Wirth, T., Adler, G. and Schmid, R.M., 2000. Suppression of NF-κB activity by sulfasalazine is mediated by direct inhibition of IκB kinases α and β. *Gastroenterology*, *119*(5), pp.1209-1218.

Weisshof, R. and Chermesh, I., 2015. Micronutrient deficiencies in inflammatory bowel disease. *Current Opinion in Clinical Nutrition & Metabolic Care*, *18*(6), pp.576-581.

Wendel, A., Feuerstein, S. and Konz, K.H., 1979. Acute paracetamol intoxication of starved mice leads to lipid peroxidation in vivo. *Biochemical pharmacology*, *28*(13), pp.2051-2055.

Wenting, L., Ping, L., Haitao, J., Meng, Q. and Xiaofei, R., 2014. Therapeutic effect of taurine against aluminum-induced impairment on learning, memory and brain neurotransmitters in rats. *Neurological Sciences*, *35*, pp.1579-1584.

Werge, M.P., McCann, A., Galsgaard, E.D., Holst, D., Bugge, A., Albrechtsen, N.J.W. and Gluud, L.L., 2021. The role of the transsulfuration pathway in non-alcoholic fatty liver disease. *Journal of clinical medicine*, *10*(5), p.1081.

Who model lists of essential medicines (2021) *World Health Organization*. Available at: https://www.who.int/groups/expert-committee-on-selection-and-use-of-essential-medicines/essential-medicines-lists (Accessed: 28 March 2022).

Wiliński, B., Wiliński, J., Somogyi, E., Góralska, M. and Piotrowska, J., 2011. Paracetamol (acetaminophen) decreases hydrogen sulfide tissue concentration in brain but increases it in the heart, liver and kidney in mice. *Folia biologica (Kraków)*, *59*(1-2), pp.41-44.

William, J.H. and Danziger, J., 2016. Proton-pump inhibitor-induced hypomagnesemia: Current research and proposed mechanisms. *World journal of nephrology*, *5*(2), p.152.

Wilson, M.P., Plecko, B., Mills, P.B. and Clayton, P.T., 2019. Disorders affecting vitamin B6 metabolism. *Journal of inherited metabolic disease*, *42*(4), pp.629-646.

Wishart, K., 2017. Increased micronutrient requirements during physiologically demanding situations: Review of the current evidence. *Vitam Miner*, *6*(166), pp.2376-1318.

Wójcik, O.P., Koenig, K.L., Zeleniuch-Jacquotte, A., Pearte, C., Costa, M. and Chen, Y., 2013. Serum taurine and risk of coronary heart disease: a prospective, nested case–control study. *European journal of nutrition*, *52*, pp.169-178.

World Health Organization, 2000. Guidelines for the regulatory assessment of medicinal products for use in self-medication. Available at: . https://apps.who.int/iris/bitstream/handle/10665/66154/WHO_EDM_QSM_00.1_eng.pdf (Accessed April 2022).

Wu, B., Cui, H., Peng, X., Fang, J., Cui, W. and Liu, X., 2013. Pathology of bursae of Fabricius in methionine-deficient broiler chickens. *Nutrients*, *5*(3), pp.877-886.

Wu, B., Li, L., Ruan, T. and Peng, X., 2018. Effect of methionine deficiency on duodenal and jejunal IgA+ B cell count and immunoglobulin level of broilers. *Iranian Journal of Veterinary Research*, *19*(3), p.165.

Wu, C., Xu, Z. and Huang, K., 2016. Effects of dietary selenium on inflammation and hydrogen sulfide in the gastrointestinal tract in chickens. *Biological trace element research*, *174*, pp.428-435.

Wu, N., Siow, Y.L. and Karmin, O., 2010. Ischemia/reperfusion reduces transcription factor Sp1-mediated cystathionine β-synthase expression in the kidney. *Journal of Biological Chemistry*, *285*(24), pp.18225-18233.

Wu, Q., Yu, P., Bi, Y., Li, Z., Guo, W., Chen, Y. and Duan, Z., 2022. Naringin regulates mitochondrial dynamics to protect against acetaminophen-induced hepatotoxicity by activating the AMPK/Nrf2 signaling pathway in vitro. *Brazilian Journal of Medical and Biological Research*, *55*, p.e12040.

Wylie, C.E., Daniels, B., Brett, J., Pearson, S.A. and Buckley, N.A., 2020. A national study on prescribed medicine use in Australia on a typical day. *Pharmacoepidemiology and drug safety*, *29*(9), pp.1046-1053.

Xiong, S.P., Sun, H.J., Cao, X., Wu, Z.Y., Zhu, M.Y., Cao, L., Nie, X.W. and Bian, J.S., 2023. Polysulfide Protects Against Diabetic Cardiomyopathy Through Sulfhydration of Peroxisome Proliferator-Activated Receptor-γ and Sirtuin 3. *Antioxidants & Redox Signaling*, *38*(1-3), pp.1-17.

Xu, H., Wang, S., Gao, F. and Li, C., 2022. Vitamin B6, B9, and B12 intakes and cognitive performance in elders: National Health and Nutrition Examination Survey, 2011– 2014. *Neuropsychiatric Disease and Treatment*, *18*, p.537.

Yan, H., Du, J. and Tang, C., 2004. The possible role of hydrogen sulfide on the pathogenesis of spontaneous hypertension in rats. *Biochemical and biophysical research communications*, *313*(1), pp.22-27.

Yan, X., Gao, R., Hu, Y. and Jin, J., 2018. Pernicious anemia associated with cryptogenic cirrhosis: Two case reports and a literature review. *Medicine*, *97*(39), p.e12547.

Yanaka, N., Koyama, T.A., Komatsu, S.I., Nakamura, E., Kanda, M. and Kato, N., 2005. Vitamin B6 suppresses NF-κB activation in LPS-stimulated mouse macrophages. *International journal of molecular medicine*, *16*(6), pp.1071-1075.

Yang, G., Wu, L., Jiang, B., Yang, W., Qi, J., Cao, K., Meng, Q., Mustafa, A.K., Mu, W., Zhang, S. and Snyder, S.H., 2008. H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine γ-lyase. *Science*, *322*(5901), pp.587-590.

Yang, M., Huang, Y., Chen, J., Chen, Y.L., Ma, J.J. and Shi, P.H., 2014. Activation of AMPK participates hydrogen sulfide-induced cyto-protective effect against dexamethasone in osteoblastic MC3T3-E1 cells. *Biochemical and Biophysical Research Communications*, *454*(1), pp.42-47.

Yazdanpanah, N., Zillikens, M.C., Rivadeneira, F., De Jong, R., Lindemans, J., Uitterlinden, A.G., Pols, H.A. and Van Meurs, J.B., 2007. Effect of dietary B vitamins on BMD and risk of fracture in elderly men and women: the Rotterdam study. *Bone*, *41*(6), pp.987-994.

Ye, X., Maras, J.E., Bakun, P.J. and Tucker, K.L., 2010. Dietary intake of vitamin B-6, plasma pyridoxal 5′-phosphate, and homocysteine in Puerto Rican adults. *Journal of the American Dietetic Association*, *110*(11), pp.1660-1668.

Yiang, G.T., Yu, Y.L., Lin, K.T., Chen, J.N., Chang, W.J. and Wei, C.W., 2015. Acetaminophen induces JNK/p38 signaling and activates the caspase-9-3-dependent cell death pathway in human mesenchymal stem cells. *International journal of molecular medicine*, *36*(2), pp.485-492.

Yin, L.B., Li, Z.W., Wang, J.L., Wang, L., Hou, L., Hu, S.Y., Chen, H., Luo, P., Cui, X.B. and Zhu, J.L., 2023. Sulfasalazine inhibits esophageal cancer cell proliferation by mediating ferroptosis. *Chemical Biology & Drug Design*, *102*(4), pp.730-737.

Young, E.H., Pan, S., Yap, A.G., Reveles, K.R. and Bhakta, K., 2021. Polypharmacy prevalence in older adults seen in United States physician offices from 2009 to 2016. *PLoS One*, *16*(8), p.e0255642.

Yun, L.I., Qi, Z.H.A.O., Liu, X.L., Wang, L.Y., Lu, X.F., Li, H.F., Chen, S.F., Huang, J.F. and Gu, D.F., 2008. Relationship between cystathionine γ-lyase gene polymorphism and essential hypertension in Northern Chinese Han population. *Chinese medical journal*, *121*(8), pp.716-720.

Zainol Abidin, Q.H., Ida, T., Morita, M., Matsunaga, T., Nishimura, A., Jung, M., Hassan, N., Takata, T., Ishii, I., Kruger, W. and Wang, R., 2023. Synthesis of sulfides and persulfides is not impeded by disruption of three canonical enzymes in sulfur metabolism. *Antioxidants*, *12*(4), p.868.

Zanetti, M., Veronese, N., Riso, S., Boccardi, V., Bolli, C., Cintoni, M., Di Francesco, V., Mazza, L., Onfiani, G., Zenaro, D. and Pilotto, A., 2023. Polypharmacy and malnutrition in older people: A narrative review. *Nutrition*, *115*, p.112134.

Zhang, D., Wen, X., Wu, W., Guo, Y. and Cui, W., 2015. Elevated homocysteine level and folate deficiency associated with increased overall risk of carcinogenesis: meta-analysis of 83 case-control studies involving 35,758 individuals. *PloS one*, *10*(5), p.e0123423.

Zhang, F., Mamtani, R., Scott, F.I., Goldberg, D.S., Haynes, K. and Lewis, J.D., 2016. Increasing use of prescription drugs in the United Kingdom. *Pharmacoepidemiology and drug safety*, *25*(6), pp.628-636.

Zhang, H.F., Geltink, R.I.K., Parker, S.J. and Sorensen, P.H., 2022. Transsulfuration, minor player or crucial for cysteine homeostasis in cancer. *Trends in cell biology*, *32*(9), pp.800-814.

Zhang, J., Shi, C., Wang, H., Gao, C., Chang, P., Chen, X., Shan, H., Zhang, M. and Tao, L., 2019. Hydrogen sulfide protects against cell damage through modulation of PI3K/Akt/Nrf2 signaling. *The international journal of biochemistry & cell biology*, *117*, p.105636.

Zhang, L.M., Jiang, C.X. and Liu, D.W., 2009. Hydrogen sulfide attenuates neuronal injury induced by vascular dementia via inhibiting apoptosis in rats. *Neurochemical Research*, *34*, pp.1984-1992.

Zhang, N., Sundquist, J., Sundquist, K. and Ji, J., 2020. An increasing trend in the prevalence of polypharmacy in Sweden: a nationwide register-based study. *Frontiers in pharmacology*, *11*, p.326.

Zhang, T., Ono, K., Tsutsuki, H., Ihara, H., Islam, W., Akaike, T. and Sawa, T., 2019. Enhanced cellular polysulfides negatively regulate TLR4 signaling and mitigate lethal endotoxin shock. *Cell Chemical Biology*, *26*(5), pp.686-698.

Zhang, Z., Kebreab, E., Jing, M., Rodriguez-Lecompte, J.C., Kuehn, R., Flintoft, M. and House, J.D., 2009. Impairments in pyridoxine-dependent sulphur amino acid metabolism are highly sensitive to the degree of vitamin B6 deficiency and repletion in the pig. *Animal*, *3*(6), pp.826-837.

Zhang, Z., Zhang, X., Fang, X., Niimi, M., Huang, Y., Piao, H., Gao, S., Fan, J. and Yao, J., 2017. Glutathione inhibits antibody and complement-mediated immunologic cell injury via multiple mechanisms. *Redox Biology*, *12*, pp.571-581.

Zhao, L.G., Shu, X.O., Li, H.L., Gao, J., Han, L.H., Wang, J., Fang, J., Gao, Y.T., Zheng, W. and Xiang, Y.B., 2019. Prospective cohort studies of dietary vitamin B6 intake and risk of cause-specific mortality. *Clinical nutrition*, *38*(3), pp.1180-1187.

Zhao, W. and Wang, R., 2002. H2S-induced vasorelaxation and underlying cellular and molecular mechanisms. *American Journal of Physiology-Heart and Circulatory*

Physiology, *283*(2), pp.H474-H480.

Zheng, D., Bao, Y., Gu, J., Lv, T. and Yang, Y., 2023. Effects of New Antiepileptic Drugs on Homocysteine in Epileptic Patients: A Systematic Review and Meta-Analysis. *Journal of Clinical Pharmacy and Therapeutics*, *2023*.

Zheng, Y.W., Miao, X.Y., Xiong, L., Chen, B., Kong, F.H., Zhou, J.J., Liu, Z.T., Wen, Y., Zhang, Z.J. and Zou, H., 2021. Sulfasalazine sensitizes polyhematoporphyrin-mediated photodynamic therapy in cholangiocarcinoma by targeting xCT. *Frontiers in Pharmacology*, *12*, p.723488.

Zhou, L. and Wang, Q., 2023. Advances of H2S in regulating neurodegenerative diseases by preserving mitochondria function. *Antioxidants*, *12*(3), p.652.

Zhou, W., He, H., Wei, Q., Che, L., Zhao, X., Liu, W., Yan, Y., Hu, L., Du, Y., Yin, Z. and Shuai, Y., 2023. Puerarin protects against acetaminophen-induced oxidative damage in liver through activation of the Keap1/Nrf2 signaling pathway. *Food Science & Nutrition*, *11*(10), pp.6604-6615.

Zhou, X., Cheung, C.M., Yang, J.M., Or, P.M., Lee, W.Y. and Yeung, J.H., 2015. Danshen (S alvia miltiorrhiza) water extract inhibits paracetamol-induced toxicity in primary rat hepatocytes via reducing CYP2E1 activity and oxidative stress. *Journal of Pharmacy and Pharmacology*, *67*(7), pp.980-989.

Zhu, J., Chen, C., Lu, L., Shikany, J.M., D'Alton, M.E. and Kahe, K., 2023. Folate, vitamin B6, and vitamin B12 status in association with metabolic syndrome incidence. *JAMA Network Open*, *6*(1), pp.e2250621-e2250621.

Zinellu, A., Zinellu, E., Sotgiu, E., Fois, A.G., Paliogiannis, P., Scano, V., Piras, B., Sotgia, S., Mangoni, A.A., Carru, C. and Pirina, P., 2020. Systemic transsulfuration pathway thiol concentrations in chronic obstructive pulmonary disease patients. *European Journal of Clinical Investigation*, *50*(8), p.e13267.

Zou, L., Gao, Z., Zeng, F., Xiao, J., Chen, J., Feng, X., Chen, D., Fang, Y., Cui, J., Liu, Y. and Li, Z., 2019. Sulfasalazine suppresses thyroid cancer cell proliferation and metastasis through T-cell originated protein kinase. *Oncology Letters*, *18*(4), pp.3517-3526.

Zulli, A., Lau, E., Wijaya, B.P., Jin, X., Sutarga, K., Schwartz, G.D., Learmont, J., Wookey, P.J., Zinellu, A., Carru, C. and Hare, D.L., 2009. High dietary taurine reduces apoptosis and atherosclerosis in the left main coronary artery: association with reduced CCAAT/enhancer binding protein homologous protein and total plasma homocysteine but not lipidemia. *Hypertension*, *53*(6), pp.1017-1022.

Appendices

Appendix 1.1 Effect of representative therapeutic drugs on micronutrients depletion.

Micronutrient	Drug class	Mechanism	Reference
Vitamin B_1 (Thiamine)	Diuretic	↑ Urinary loss of vitamin B_1	(Rieck et al.,1999)
Vitamin B_6 (Pyridoxine)	Diuretic	↑ Vitamin B_6 excretion	(Mydlík, Derzsiová and Žemberová, 1999)
	Antiepileptic	↑ Catabolism rate of vitamin B_6	(NIH, 2023)
	Xanthine (for asthma)	↑ Pyridoxal (a form of vitamin B_6) oxidation, lead to ↓ the availability of PLP **(*)**	(Ubbink et al., 1990)
	Antidepression	↓ PLP synthesis	(Heller and Friedman, 1983)
Vitamin B_9 (Folate)	Anti-inflammatory	↓ Folate absorption, competitively inhibits the enzyme dihydrofolate reductase (DHFR) **(**)**	(Lechner, 2015)
	Oral contraceptive	Impair folate metabolism	(Shere et al., 2015; Shojania, 1982)
	Antibiotic	Inhibit the DHFR **(**)** enzyme	(Lechner, 2015)
Vitamin B_{12} (Cobalamin)	Antidiabetic	Calcium-dependent endocytosis **(***)** in the gut is disrupted by metformin. The absorption of Intrinsic Factor-B_{12} complex is impaired as a result, which lowers the absorption of vitamin B_{12}	(Damião et al., 2016)
	Acid suppressing drug	↓ Absorption of Intrinsic Factor-B_{12} complex ↓ Gastric acid secretion	(Valuck and Ruscin, 2004)

(*) PLP (pyridoxal-5′-phosphate) is the active form of vitamin B_6.

(**) DHFR is an enzyme needed for converting folate to its active form (tetrahydrofolate).

(***) Calcium plays a crucial role in the uptake of the B_{12}-intrinsic factor complex in the terminal ileum.

Appendix 1.1 (continued) Effect of representative therapeutic drugs on micronutrients depletion.

Micronutrient	Drug class	Mechanism	Reference
Calcium (Ca)	Diuretics	↑ Ca excretion	(Friedman and Bushinsky, 1999)
	Corticosteroids	↓ Absorption and ↑ excretion	(Lechner, 2015)
	Acid suppressing drugs	↓ Ca absorption	(Sipponen and Härkönen, 2010)
Iron (Fe)	Acid suppressing drugs	↓ The release of iron from food proteins, ↓ its bioavailability and absorption	(McColl, 2009)
Magnesium (Mg)	Diuretics	↓ Mg reabsorption in the kidney	(Davies and Fraser, 1993)
	Antidiabetic	↓ Mg uptake in intestinal and renal cells through downregulation of TRPM6 mRNA (**) expression leading to ↓ absorption	(Bouras et al., 2020; Kurstjens et al., 2017)
	Acid suppressing drugs	↓Solubility of Mg in the intestinal lumen, ↓activity and expression of Mg transporters like TRPM6 and TRPM7 (§) in the intestine and colon, ↓ Mg absorption	(William and Danziger, 2016)
Potassium (K)	Diuretics	↑ K excretion	(Lin, Wong and Cheung, 2022)
Selenium (Se)	Lipid lowering drugs	↓ Selenoprotein (¥) syntheses	(Moosmann and Behl, 2004)
Sodium (Na)	Diuretics	↓ Na reabsorption in nephron lead to increase Na excretion	(Spital, 1999)
Zinc (Zn)	ACE Inhibitors	↑ Zn excretion	(Mohn et al., 2018)
	Diuretics	↑ Zn excretion	(Reyes et al., 1983)
	Antibiotics	↓ Zn absorption	(Lechner, 2015)
	Acid suppressing drugs	↓ Zn absorption	(Ozutemiz et al., 2002)

(**) TRPM6 is a channel in the intestine and kidney required for Mg homeostasis.

(§) TRPM7 is a channel in the intestine, kidney and other cells required for Mg and Ca homeostasis and also possesses kinase activity.

(¥) Selenoprotein is protein that require Se for their synthesis and function.

Appendix 4.1 Associations of plasma PLP concentration and dietary vitamin B_6 with medication usage using linear regression analysis.

Variable	Plasma PLP concentration		Dietary vitamin B_6 intake	
	***P* value**	**R^2**	***P* value**	**R^2**
Age	<0.01	0.12	<0.01	0.12
Sex	0.2	0.12	<0.01	0.12
Male: +age	<0.01	0.12	<0.01	0.02
Female: +age	<0.01	0.01	<0.01	0.003
Type of diet	0.3	0.06	0.2	0.12
Smoking	<0.01	0.11	0.3	0.13
Alcohol	<0.01	0.08	0.9	0.14
Analgesics	<0.01	0.18	0.9	0.16
Antibacterial	0.05	0.08	0.6	0.15
Antidiabetic	<0.01	0.08	0.7	0.15
Antidepressant	0.03	0.08	<0.01	0.15
ACE inhibitor	0.7	0.06	0.3	0.13
Beta blocker	0.3	0.07	0.1	0.14
Ca^{2+} blocker	0.03	0.07	0.3	0.15
Diuretic	0.09	0.07	0.1	0.13
Antiplatelet	0.2	0.07	0.3	0.15
Asthma prescribed	<0.01	0.07	0.3	0.14
Lipid lowering	0.01	0.06	0.4	0.14
Proton pump inhibitor	0.4	0.07	0.9	0.15

Abbreviations: PLP, pyridoxal 5'-phosphate

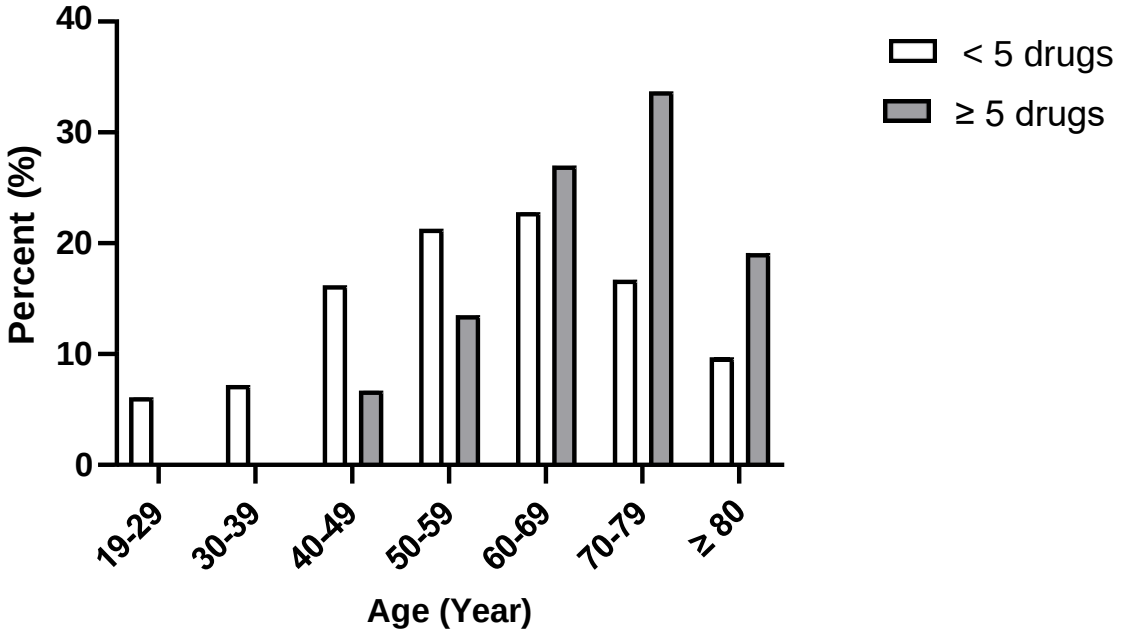
Appendix 4.2 Percentage of participants having vitamin B_6 deficiency (plasma PLP concentration < 20 nmol L-1) among UK adults aged ≥ 19 years based on sex and age group, 2008 2017.

Plasma PLP concentration (nmol L^{-1})	All participants	Between sex		*P* value	Among age group (year) in each sex						
					19 - 29	**30 - 39**	**40 - 49**	**50 - 59**	**60 - 69**	**70 - 79**	**≥ 80**
Above the cut-off of vitamin B_6 deficiency	2937 (90%)	**M**	1247 (91%)	< 0.05	138 (11%)	211 (17%)	274 (22%)	257 (20%)	234 (19%)	100 (8%)	33 (3%)
		F	1690 (88%)		209 (12%)	277 (16%)	381 (23%)	318 (19%)	274 (16%)	168 (10%)	63 (4%)
Marginal vitamin B6 deficiency *	558 (17%)	**M**	196 (14%)	< 0.05	10 (5%)	21 (11%)	36 (18%)	34 (17%)	60 (31%)	29 (15%)	6 (3%)
		F	362 (19%)		45 (12%)	55 (15%)	86 (24%)	51 (14%)	72 (20%)	33 (9%)	20 (6%)
Vitamin B6 deficiency**	344 (10%)	**M**	116 (9%)	< 0.05	1 (1%)	7 (6%)	11 (9%)	22 (19%)	21 (18%)	31 (27%)	23 (20%)
		F	228 (12%)		18 (8%)	35 (15%)	53 (23%)	33 (15%)	45 (20%)	23 (10%)	21 (9%)

Appendix 4.3 Number and percentage of participants depending on the cut-off value of plasma PLP concentration among UK adults aged ≥19 years based on therapeutic drug, 2008-2017.

Medication	Above the cut-off of vitamin B_6 deficiency	Marginal vitamin B_6 deficiency *	Vitamin B_6 deficiency**
Analgesic (user) (n = 194)	85 (76%)	26 (23%)	27 (24%)
Analgesic (non-user) (n = 1260)	751 (89%)	139 (17%)	96 (11%)
Antibacterial (user) (n = 26)	13 (81%)	4 (25%)	3 (19%)
Antibacterial (non-user) (n = 1428)	823 (87%)	161 (17%)	120 (13%)
Antidiabetic (user) (n = 89)	39 (72%)	17 (31%)	15 (28%)
Antidiabetic (non-user) (n = 1365)	797 (88%)	148 (16%)	108 (12%)
Antidepressant (user) (n = 185)	88 (77%)	26 (23%)	26 (23%)
Antidepressant (non-user) (n = 1269)	748 (88%)	139 (16%)	97 (12%)
ACE inhibitor (user) (n = 309)	162 (83%)	31 (16%)	33 (17%)
ACE inhibitor (non-user) (n = 2149)	1280 (88%)	243 (17%)	180 (12%)
Beta blocker (user) (n = 173)	70 (79%)	23 (26%)	18 (21%)
Beta blocker (non-user) (n = 2285)	1372 (88%)	251 (16%)	195 (12%)
Ca^{2+} blocker (user) (n = 199)	97 (78%)	31 (25%)	27 (22%)
Ca^{2+} blocker (non-user) (n = 2259)	1345 (88%)	243 (16%)	186 (12%)
Diuretic (user) (n = 188)	88 (77%)	26 (23%)	27 (23%)
Diuretic (non-user) (n = 2270)	1354 (88%)	248 (16%)	186 (12%)
Antiplatelet (user) (n = 107)	45 (70%)	14 (22%)	19 (30%)
Antiplatelet (non-user) (n = 1347)	791 (88%)	151 (17%)	104 (12%)
Asthma prescribed (user) (n = 144)	52 (75%)	16 (23%)	17 (25%)
Asthma prescribed (non-user) (n = 1340)	784 (88%)	149 (17%)	106 (12%)
Lipid lowering (user) (n = 405)	192 (78%)	47 (19%)	53 (22%)
Lipid lowering (non-user) (n = 2053)	1250 (89%)	227 (16%)	160 (11%)
Proton pump inhibitor (user) (n = 184)	89 (83%)	27 (25%)	18 (17%)
Proton pump inhibitor (non-user) (n = 1270)	747 (88%)	138 (16%)	105 (12%)

Appendix 4.4 Frequency of polypharmacy in the NDNS data set among UK adults aged ≥19 years based on age groups, 2008 – 2017.



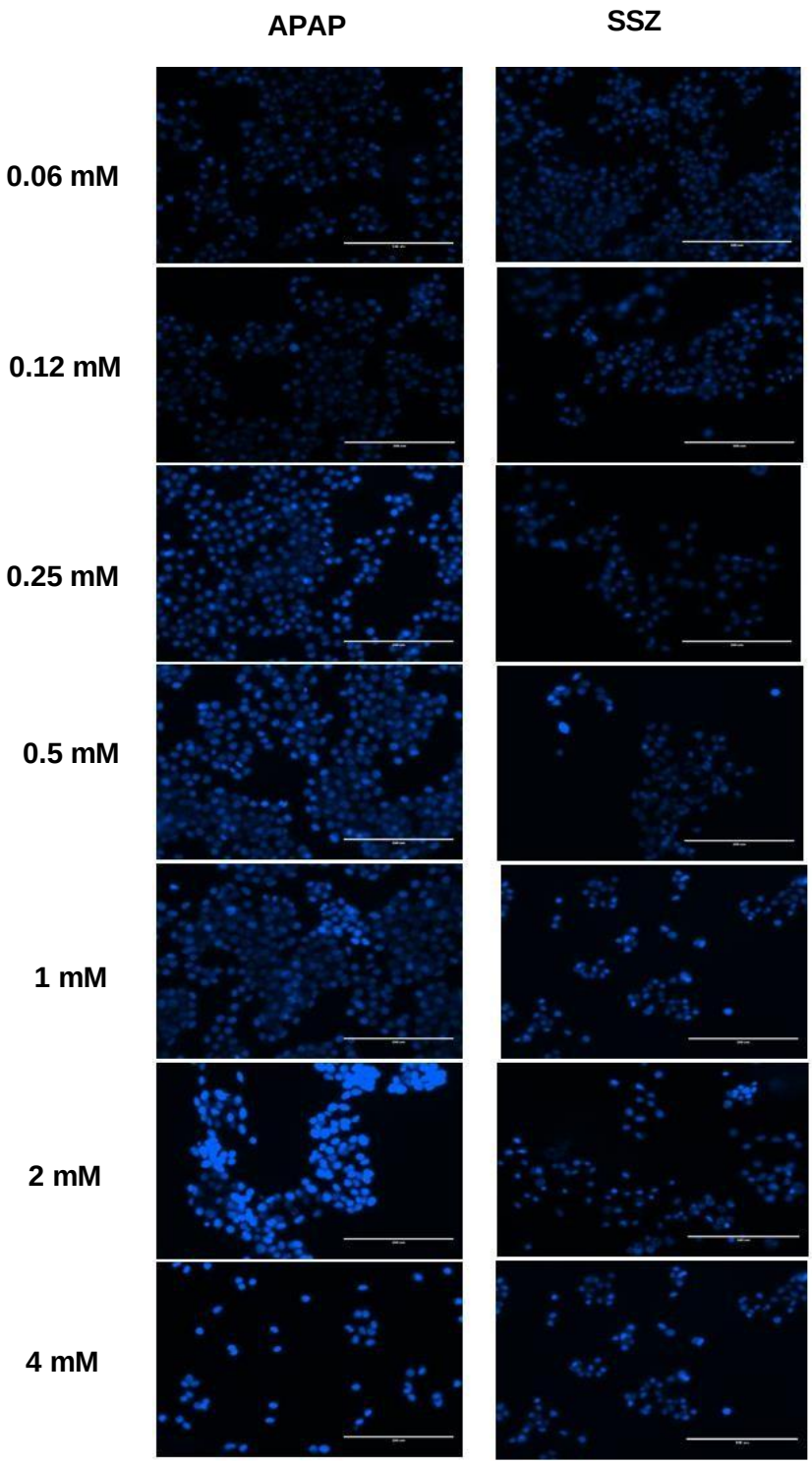
Appendix 4.5 (A) Number and percentage of prescribed medications in the NDNS data set among UK adults aged ≥19 years based on age group, 2008 – 2017.

Medication	**Age (years)**						
	19 - 29	**30 - 39**	**40 - 49**	**50 - 59**	**60 - 69**	**70 - 79**	**≥ 80**
Analgesic **(n = 194)**	5 (3%)	16 (8%)	23 (12%)	42 (22%)	47 (24%)	39 (20%)	22 (11%)
Antibacterial **(n = 26)**	2 (8%)	2 (8%)	3 (12%)	6 (22%)	5 (19%)	5 (19%)	3 (12%)
Antidiabetic **(n = 89)**	5 (6%)	3 (3%)	15 (17%)	14 (16%)	23 (26%)	17 (19%)	12 (13%)
Antidepressant **(n = 185)**	16 (9%)	15 (8%)	42 (23%)	38 (20%)	42 (23%)	24 (13%)	8 (4%)
ACE inhibitor **(n = 309)**	1 (0.3%)	7 (2%)	19 (6%)	67 (21.7%)	92 (30%)	82 (27%)	41 (13%)
Beta blocker **(n = 173)**	3 (2%)	2 (1%)	10 (6%)	29 (16%)	49 (28%)	46 (27%)	34 (20%)
Ca^{2+} blocker **(n = 199)**	0	3 (1%)	16 (8%)	32 (16%)	63 (32%)	53 (27%)	32 (16%)
Diuretic **(n = 188)**	0	1 (1%)	4 (2%)	27 (14%)	48 (26%)	64 (34%)	44 (23%)
Antiplatelet **(n = 107)**	0	0	5 (5%)	9 (8%)	33 (31%)	34 (32%)	26 (24%)
Asthma prescribed **(n = 114)**	9 (8%)	8 (7%)	18 (16%)	25 (22%)	29 (25%)	14 (12%)	11 (10%)
Lipid lowering **(n = 405)**	0	3 (1%)	19 (4%)	73 (18%)	144 (36%)	113 (28%)	53 (13%)
Proton pump inhibitor **(n = 184)**	2 (1%)	9 (5%)	17 (9%)	38 (21%)	37 (20%)	48 (26%)	33 (18%)

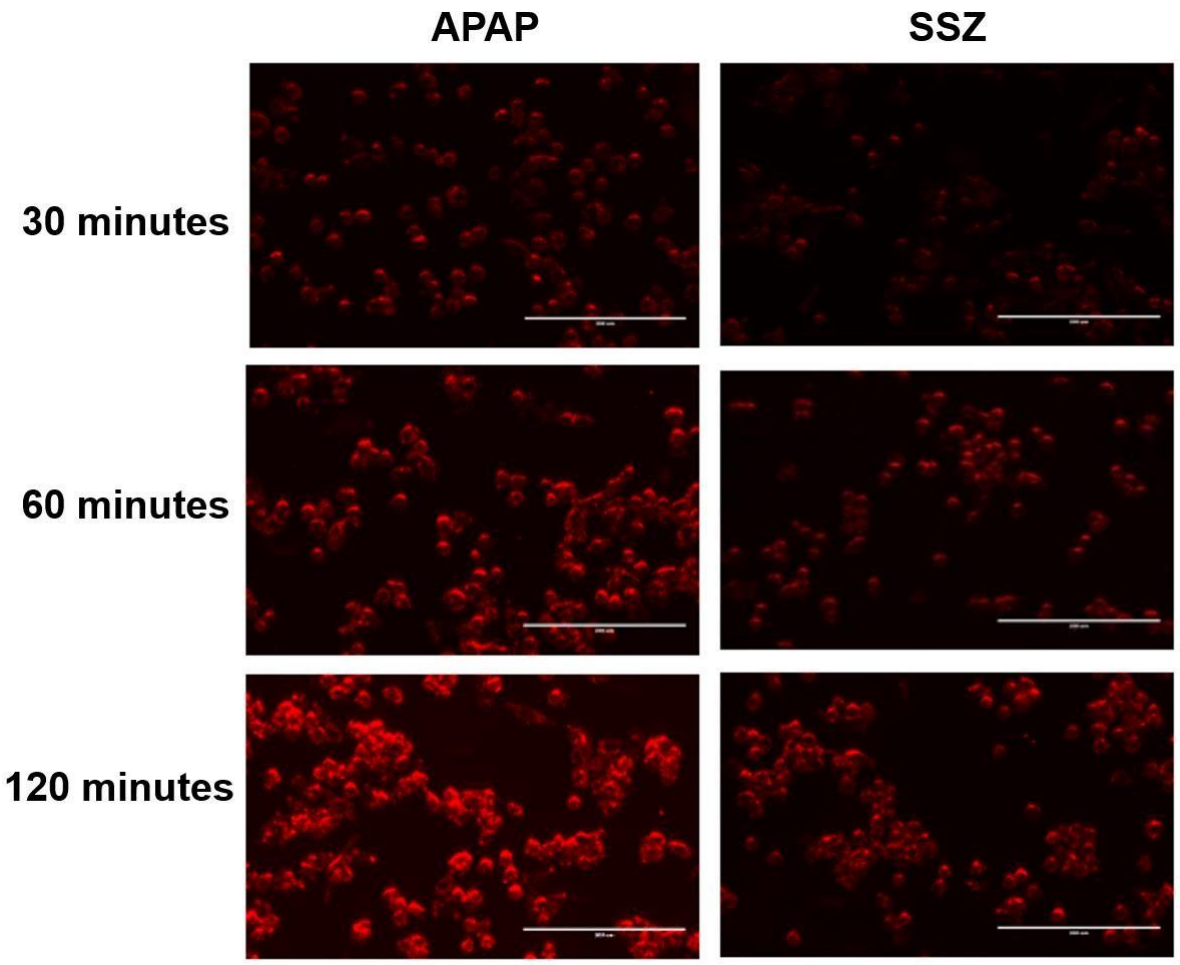
Appendix 4.5 (B) Number and percentage of prescribed medications in the NDNS data set among UK adults aged ≥19 years based on age (< 65 vs ≥ 65 years), 2008 – 2017.

Drug	< 65 years, n (%)	≥ 65 years, n (%)
Analgesic (n = 194)	105 (54%)	89 (46 %)
Antibacterial (n = 26)	15 (58 %)	11 (42 %)
Antidiabetic (n = 89)	49 (55 %)	40 (45 %)
Antidepressant (n = 185)	130 (70 %)	55 (30 %)
ACE inhibitor (n = 309)	137 (44 %)	172 (56 %)
Beta blocker (n = 173)	70 (40 %)	103 (60 %)
Ca^{2+} blocker (n = 199)	78 (39 %)	121 (61 %)
Diuretic (n = 188)	53 (28 %)	135 (72 %)
Antiplatelet (n = 107)	29 (27 %)	78 (73 %)
Asthma prescribed (n = 114)	69 (61 %)	45 (39 %)
Lipid lowering (n = 405)	158 (39 %)	247 (61 %)
Proton pump inhibitor (n = 184)	84 (46 %)	100 (54 %)

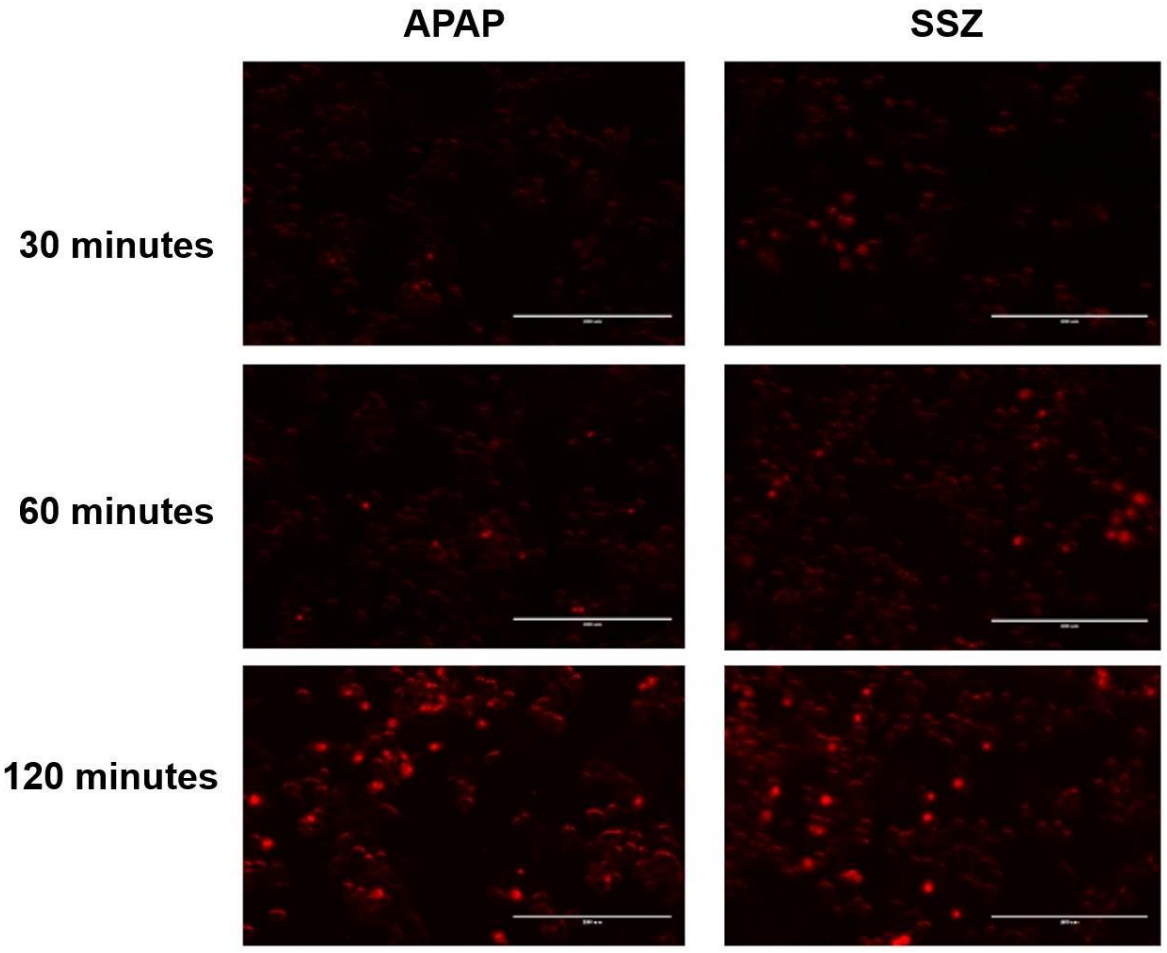
Appendix 5.4 Concentration-dependent effects of the drugs on HepG2 DNA damage and nuclear changes for 24 hours treatment stained by DAPI. Arrows indicate apoptotic bodies of nuclear condensation observed at 10× magnification under an EVOS microscope.



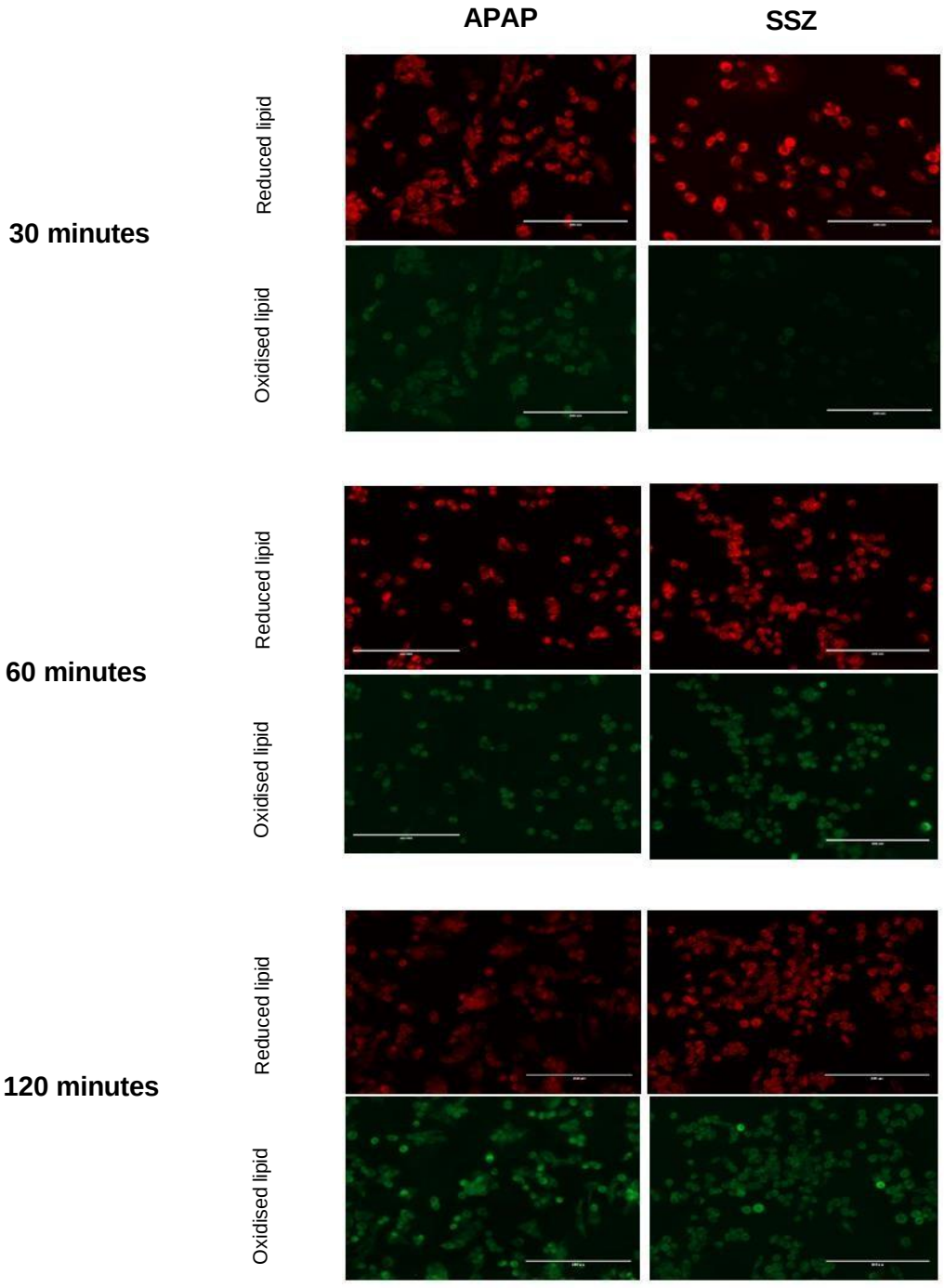
Appendix 5.1 Time-dependent effects of the drugs on cytoplasmic ROS levels measured at 30, 60 and 120 minutes after exposure to 4mM drug concentrations, determined with CellROX probe.



Appendix 5.2 Time-dependent effects of the drugs on mitochondrial ROS levels measured at 30, 60 and 120 minutes after exposure to 4mM drug concentrations, determined with MitoSOX™ probe.



Appendix 5.3 Time-dependent effects of the drugs on lipid peroxidation levels measured at 30, 60 and 120 minutes after exposure to 4mM drug concentrations, determined with Image-iT probe.



Appendix 6.1 Effects of potential interfering of APAP (**A** and **C**) and SSZ (**B** and **D**) on AzMC and SSP4 fluorescence in buffer