Osteocalcin and the vasculature

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Statement of authenticity

I declare that this thesis has been completed by myself under supervision of Professor Saoirse O'Sullivan and Professor Susan Anderson at the Division of Medical Sciences & Graduate Entry Medicine, School of Medicine, University of Nottingham, Royal Derby Hospital. This thesis is a record of work that has not been submitted previously for a higher degree.

Sophie Millar September 2019

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Conferences

Presenting author:

Sept 2019 'A lack of role for osteocalcin in human vascular cell calcification'. Poster presentation. British Atherosclerosis Society Annual Meeting. Oxford, United Kingdom.

Sept 2019 'The anti-inflammatory effects of the novel oestrogen receptor GPR30 on human endothelial cells'. Poster presentation. British Atherosclerosis Society Annual Meeting. Oxford, United Kingdom.

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July 2018 **'**A systematic review on the pharmacokinetic profile of cannabidiol in humans'. Poster presentation. International Cannabinoid Research Society. Leiden, The Netherlands.

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July 2018 'A review of cannabidiol dosing in patient studies'. Poster presentation. International Cannabinoid Research Society. Leiden, The Netherlands.

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An investigation into the role of osteocalcin in high phosphate induced human arterial smooth muscle cell calcification. S. Millar, S. John, C. McIntyre, V. Ralevic, S. Anderson, and S. O'Sullivan. *Under review.*

Osteocalcin does not influence acute or chronic inflammation in human vascular cells. S. Millar, I. Zala, S. Anderson and S. O'Sullivan. *Journal of Cellular Physiology (2019:1-11).*

Human vascular cell responses to the circulating bone hormone osteocalcin. S. Millar, S. Anderson and S. O'Sullivan. *Journal of Cellular Physiology (2019;11:234).*

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A systematic review and meta-analysis of the haemodynamic effects of cannabidiol. S. Sultan, S. Millar, T. England, and S. O'Sullivan. *Frontiers in Pharmacology (2017;8:81).*

Manuscripts in preparation:

The anti-inflammatory effects of the novel oestrogen receptor GPR30 on human endothelial cells. S. Millar, I. Gaskell, E. Kavanagh, S. O'Sullivan.

Effect of sodium-glucose cotransporter-2 inhibitors on endothelial function: A systematic review of pre-clinical studies. A. Alshnbari, S. Millar, S. O'Sullivan, I. Idris.

Evaluation of the *in vivo* effects of cannabidiol in brain tumours: A systematic review. M. Mabrouk, S. Millar, S. O'Sullivan, L. Storer, R. Grundy.

1. Abstract

Osteocalcin (OCN) is an intriguing hormone having established roles throughout the body beyond its origin of the bone extra cellular matrix. Uncarboxylated osteocalcin (ucOCN) is the major form found within the circulation. However, knowledge surrounding the effects of ucOCN within the vasculature is very limited. Therefore, the aim of this thesis was to create a novel portfolio of work examining the effects of ucOCN within the vasculature.

A systematic review was performed to examine the relationship between osteocalcin and atherosclerosis or vascular calcification. *In vitro* experiments were conducted to establish a broad range of biological responses of human aortic endothelial cells (HAECs) and smooth muscle cells (HASMCs) to ucOCN. These included intracellular signalling, protein secretion, migration, proliferation, angiogenesis, and permeability investigations. We further examined whether ucOCN affected inflammation in HAECs and HASMCs, and vascular calcification in HASMCs.

From the literature, no definitive association was determined between OCN and vascular calcification or atherosclerosis. In *in vitro* investigations, we found ucOCN has direct biological activity in vascular cells and increases proliferation. Cell permeability, migration and angiogenesis were not affected by ucOCN. ucOCN did not affect inflammation in either cell type and is unlikely to have importance in the process of atherosclerosis. In calcification experiments, ucOCN did not increase or speed up the extent of calcification, nor did it have any inhibitory effects. Thus, ucOCN is unlikely to have a contributing role to the progression of vascular calcification.

In conclusion, ucOCN does not appear to have a direct physiologically relevant role in the vasculature. It may be relevant to assess the effects of osteocalcin on cardiovascular health and disease at a whole body level, and to explore effects in the context of diabetes.

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2. General introduction

Osteocalcin is a circulating hormone originating from bone that has far reaching extra-skeletal effects (Han et al., 2018). For example, it increases insulin sensitivity and insulin secretion in the pancreas via a feed-forward loop in mice and in humans (Lee et al., 2007, Ferron et al., 2008, Ferron et al., 2012). However, its role in the vasculature has largely been under explored despite epidemiological evidence linking it with cardiovascular health and disease (this is covered in detail in my published systematic review, Chapter 5). The aim of this thesis was to undertake a comprehensive investigation into the biological responses of human vascular cells to osteocalcin.

Bone is continuously being broken down and rebuilt in a process known as bone remodelling. Bone remodelling is a homeostatic balance between osteoclasts, the bone resorbing cells, and osteoblasts, the bone forming cells, under regulation by local and systemic factors in a complex co-ordinated communication system (Phan et al., 2004, Chen et al., 2018). These processes are required for bone growth, repair, and calcium homeostasis, and a disruption in this delicate balance can lead to bone diseases and bone loss (Phan et al., 2004). Osteoblasts differentiate from mesenchymal stem cells and secrete the osteoid for the bone matrix, an organic material including collagen proteins, non-collagenous proteins such as osteocalcin, and proteoglycan (Florencio-Silva et al., 2015).

Despite being the most abundant non-collagenous protein found in bone (accounting for approximately 10%), the exact role and function of osteocalcin (OCN) in bone reformation is still unknown, although it has been suggested to potentially regulate bone resorption through osteoclasts (Chenu, 1994). An increase in bone mass was observed in OCN knock out ($OCN^{-/-}$) mice, suggesting a limiting role of OCN on bone formation (Ducy, 1996). In other rodent studies, OCN has been reported to enhance the mechanical properties of bone, and to have osteoclast-maturing and recruiting properties (Li et al., 2016). OCN (see Figure 1) can be gamma-carboxylated intracellularly followed by secretion and incorporation into the organic bone matrix during matrix mineralization (Lacombe and Ferron, 2015). This gives rise to two main forms, carboxylated and uncarboxylated (cOCN and ucOCN; discussed in further detail in my published literature review, Chapter 4). The majority of newly synthesized OCN from osteoblasts is incorporated into the bone matrix, but about 10% to 30% is released

into the circulation and may be used as a marker of bone turnover (Bellows et al., 1999, Cremers et al., 2008).

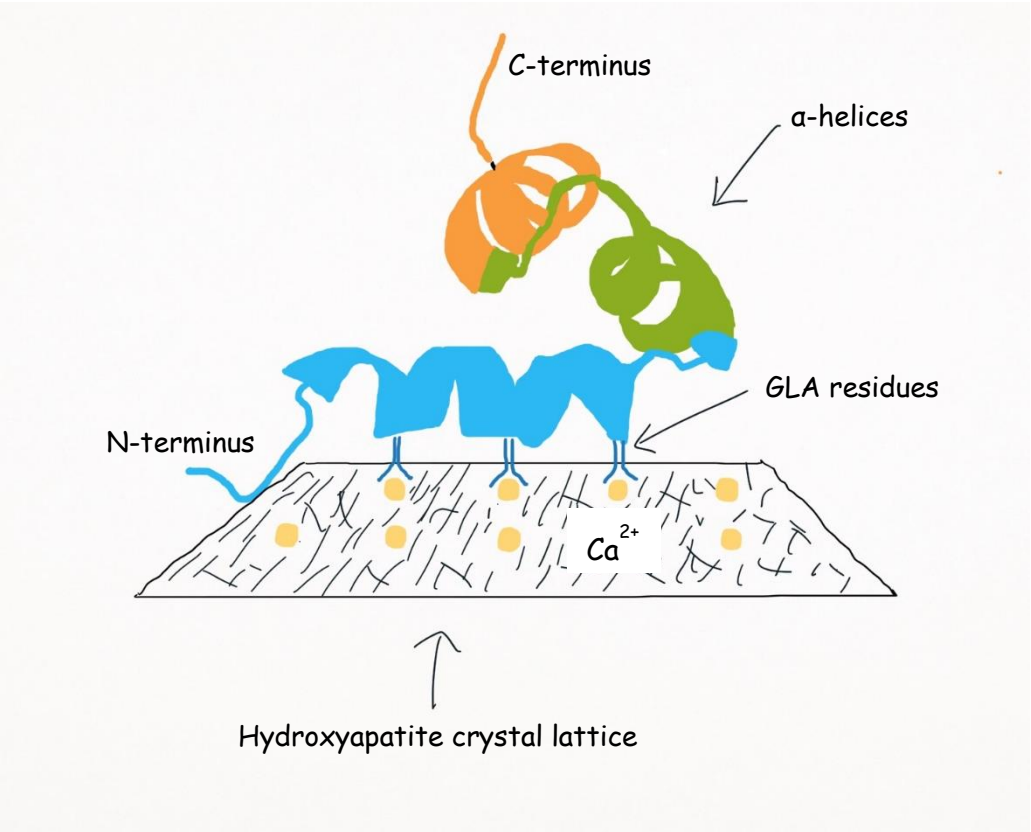

Figure 1. Structural representation of osteocalcin.

Core-binding factor subunit alpha-1 (cbfα1) is a bone-specific transcription factor encoded by the runt-related transcription factor 2 (RUNX2) gene, which regulates the expression of the OCN gene and is essential for bone formation (Viereck et al., 2002). Regulation of the gene that codes for OCN has also been shown to involve the vitamin D receptor-vitamin D response element pathway, with vitamin D (1,25-dihydroxyvitamin D3) increasing OCN expression in human osteoblasts (Zhang et al., 1997). Diseases characterized by increased concentrations of circulating OCN include Paget's disease (although this is variable), hyperparathyroidism, hyperthyroidism, osteomalacia, renal osteodystrophy, and acromegaly (Fraser et al., 2013, Cundy et al., 2014). Conversely, alcohol intake and glucocorticoids decrease concentrations of OCN by about 10 and 50 % respectively (Cundy et al., 2014).

The heightened interest in OCN within the last decade has stemmed from the realisation of its function as a hormone, extending the role of the skeleton into one of an endocrine organ (this is discussed in further detail in my published literature review,

Chapter 4). Obesity protects from bone loss and osteoporosis in mammals and leptin, an adipocyte hormone, affects bone remodelling through osteoblasts (Ducy et al., 2000, Karsenty, 2006). Thus, it was logical for researchers to hypothesise that the skeleton in return may affect energy metabolism (Karsenty, 2006). OCN, in particular the uncarboxylated form (ucOCN), has been reported to play an active role in the regulation of energy metabolism by favouring glucose tolerance, insulin sensitivity and production (Oury et al., 2013, Oury et al., 2011, Lee et al., 2007, Ferron et al., 2015). Studies have shown ucOCN can regulate pancreatic beta cells and glucose metabolism and has been implicated in diabetes (Lee et al., 2007). Studies in humans have indicated a negative correlation between OCN and fasting blood glucose, HOMO-IR, and body mass index (BMI) (Kindblom et al., 2009, Yeap et al., 2010). It has been suggested that insulin may regulate OCN and favour its bioavailability by encouraging decarboxylation (Fulzele et al., 2010, Ferron et al., 2010).

Another area of interest with osteocalcin has been fertility (see Figure 2). There have been clinical observations of the negative effects of menopause in women, and gonadal failure in both men and women on bone health. This led to investigations into the regulation of fertility by bone. $OCN^{-/-}$ male mice were observed to be poor breeders due to decreased testosterone production, however interestingly no changes in oestrogen concentrations were observed (Oury et al., 2011). It has been demonstrated that OCN-stimulated testosterone production is positively regulated by insulin signalling in osteoblasts (Oury et al., 2013). However, in a study in young male adults, no associations were found between OCN, testosterone and sperm count (Schwetz et al., 2013). Elsewhere, serum OCN and testosterone levels were positively associated in the general population, as well as in those with bone disorders and type 2 diabetes patients (Hannemann et al., 2013, Kanazawa et al., 2013). Interestingly, $OCN^{-/-}$ knockout mice also displayed behaviours associated with anxiety and depression (Oury et al., 2013). It was also demonstrated that OCN can cross the blood-brain-barrier and affect monoamine neurotransmitter synthesis (Oury et al., 2013).

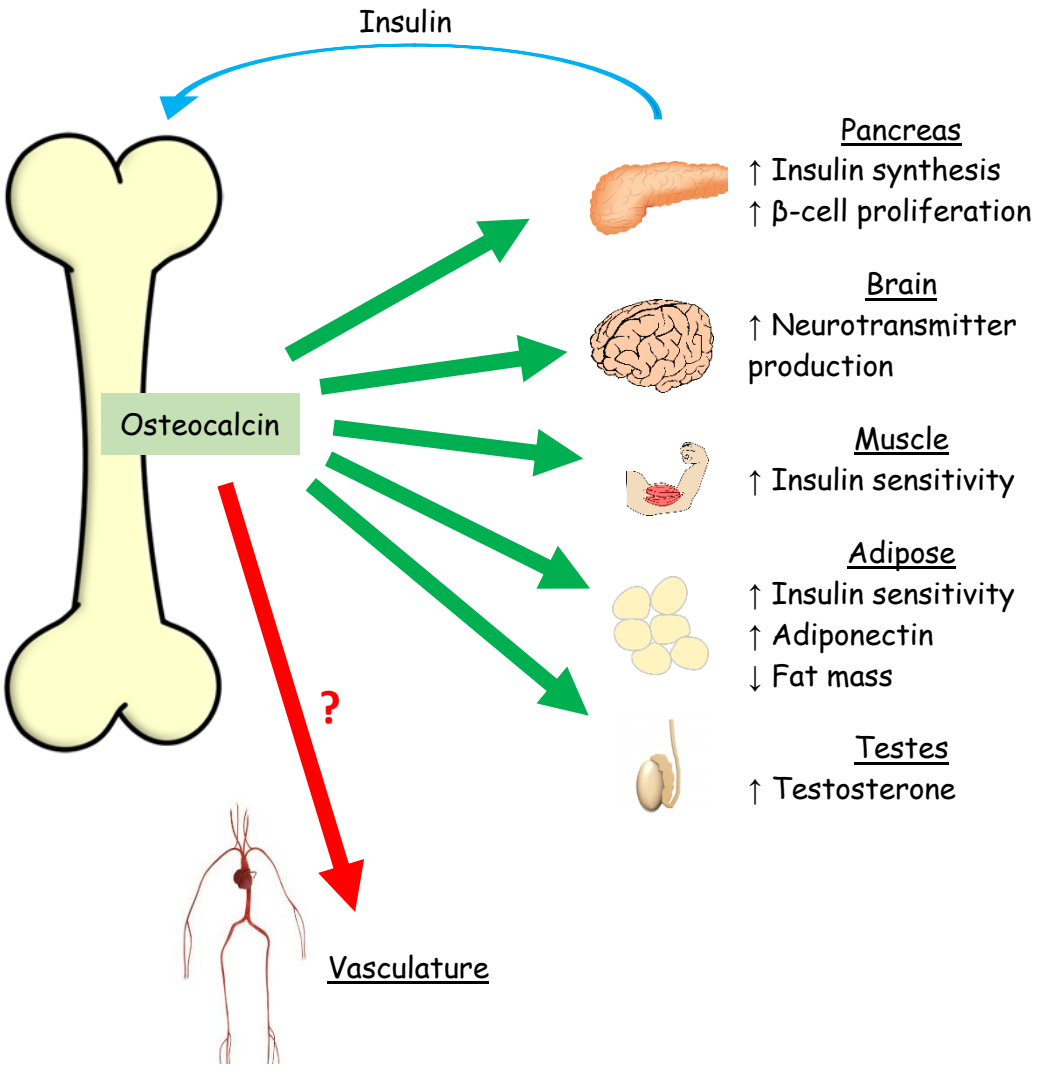

Figure 2. A summary depiction of the extra-skeletal endocrine functions of osteocalcin.

Cardiovascular disease

Despite the advances in acknowledging the functions of OCN in a range of organs and systems, its influence in the vasculature itself has largely remained unstudied. Blood vessels are lined by a thin monolayer of endothelial cells which quickly respond and adapt to their environment. They play an integral role in maintaining vessel function and structure and can signal to surrounding cells to adapt blood vessel diameter and regulate blood flow (Alberts et al., 2002). Endothelial cells repair and renew the endothelium and establish new blood vessels. Chronic inflammation or high glucose levels can lead to endothelial dysfunction and a pro-thrombotic phenotype associated with cardiovascular disease (CVD). Vascular smooth muscle cells (VSMCs) provide the structural integrity of blood vessels and contract or relax to change their vessel diameter. VSMCs can present as contractile or synthetic phenotypes, as well as

intermediate phenotypes, which are synergistic to morphological changes, proliferation and migration rates, and protein marker expression (Thyberg, 1996, Rensen et al., 2007). Their differentiation from a quiescent, contractile state to a proliferative, secretory state can be a result of genetic or environmental cues, such as biochemical factors, shear stress, or cell to cell interaction with endothelial cells (Rensen et al., 2007). The latter synthetic state is indicted in atherosclerosis (Thyberg, 1996, Hao et al., 2003).

Cardiovascular diseases, such as ischaemic heart disease, coronary heart disease or stroke, are the leading cause of death worldwide (WHO, 2011). Atherosclerosis is the underlying pathological process in blood vessels that develops over many years (WHO, 2011). There are many documented risk factors for atherosclerosis ranging from genetics to lifestyle factors such as smoking, exercise, diet or weight (WHO, 2011). The complex process involves deposition of fatty material and cholesterol, inflammatory conditions and membrane disruptions leading to plaque formation and narrowing of vessel lumens (WHO, 2011). As such, atherosclerosis can be described as a progressive inflammatory disease, involving revolving phases of inflammation leading to macrophage infiltration and micro calcifications (Ross, 1999, Hutcheson and Aikawa, 2014). Inflammatory cytokines, chemoattractant proteins, and adhesion molecules are present throughout the atherosclerotic process, of which the endothelium and smooth muscle cell layers are both pivotal. The process of atherosclerotic calcification is also crucially triggered by inflammatory-related pathways (Mazzini and Schulze, 2006, Bessueille and Magne, 2015). A paradox vascular-bone axis exists as while bone formation decreases with age, bio-mineralisation of the vasculature is increased, which is suggested to be due to the shared aetiology of inflammation (Bessueille and Magne, 2015, Sage et al., 2010).

The most dangerous complication of atherosclerosis is the rupture of a vulnerable plaque but it is not yet understood what characterises the unstable plaque. An atherosclerotic plaque consists of the following characteristics: eccentric, lumen deforming, outward remodelling lesion possessing a fibrous cap, cholesterol-laden macrophages, and lipoprotein deposits, intensive focal inflammatory cell infiltration and localised elastinolysis. It is widely reported that it is the type and location of calcifications that contribute greatly to plaque stability rather than the extent of calcifications. It is also reported that micro calcifications are also important particularly when present within the fibrous cap (Fakhry et al., 2017). These form before trans-

differentiation and are hardly detectable by von kossa or alizarin red staining. Plaque rupture can trigger blood clots which can cause heart attacks or stroke (WHO, 2011).

Disruption to the vascular endothelium is a primary altercation in vascular disease. Endothelial dysfunction is crucial to atherosclerosis and thus is a well-studied platform in vascular pathological investigations (Onat et al., 2011). Inflammation is pivotal to the atherosclerotic process from conception to end. Inflammation induced for example by oxidised low-density lipoprotein (LPL) causes expression of adhesion molecules such as VCAM-1 and ICAM-1 (Son, 2007). These, as well as selectins, bind leukocytes. Chemokine release, such as IL-8, causes further attraction and proliferation of leukocytes. Monocyte chemoattractant protein-1 (MCP-1) secretion is also increased in hyperglycaemia and atherosclerosis by endothelial cells (Takaishi et al., 2003). Macrophages become lipid-laden foam cells and drive atherosclerosis progression by releasing further inflammatory mediators and cytokines alongside leukocytes and endothelial cells such as pro-inflammatory cytokines like IL-6 and IL-17. Altogether these promote proliferation and migration of vascular smooth muscle cells (VSMCs). Smooth muscle cells also express receptors for a number of cytokines and chemokines and actively respond to inflammation which can impact their function (Shea-Donohue et al., 2012). Matrix metalloproteinase-3 (MMP-3) is involved in the breakdown of extracellular matrix proteins and thought to be involved in the progression of atherosclerosis.

Endothelin is a potent vasoconstrictor and elevated levels are most likely due to a stress response, activated through the MAPK/ERK (mitogen-activated protein kinase) branch. Endothelin is released by human aortic endothelial cells and is one way in which the cells communicate with smooth muscle cells, causing them to constrict. This action is often balanced and counteracted by nitric oxide release from endothelial cells, which acts as a potent vasodilator. Nitric oxide production is catalysed by endothelial nitric-oxide synthase (eNOS) whose activity is regulated by phosphorylation at multiple sites including the activation site Ser1177 which can be activated by protein kinases such as Akt in response to various stimuli. Endothelin and eNOS are important regulators in the cardiovascular system, linked to blood pressure, angiogenesis and vascular remodelling.

The link between diabetes-associated hyperglycaemia and vascular disease is well established (Ebong et al., 2013). Those with diabetes are at an increased risk of

cardiovascular disease, and likewise hyperglycaemia is an independent risk factor for developing diabetes and associated atherosclerosis (Tabit et al., 2010, Kim et al., 2006). Exposure of endothelial cells to high glucose levels perturbs cell homeostasis, and an imbalance of biochemical pathways contributes ultimately to endothelial dysfunction (Bakker et al., 2009). High glucose exposure can induce endothelial cell apoptosis, increase adhesion molecule expression and monocyte adhesion, increase inflammation, enhance cell permeability, increase reactive oxygen species (ROS) generation, and decrease nitric oxide (NO) bioavailability, amongst others (Gerrity, 1981, Kim et al., 1994, Morigi et al., 1998, Graier et al., 1999, Duffy et al., 2006, Hattori et al., 2000, Popov, 2010).

Insulin, which regulates glucose homeostasis, is also linked to cardiovascular disease. Insulin resistance is a pathological feature of diabetes mellitus and is often also present in obesity, coronary artery disease and metabolic syndrome (Petersen et al., 2007, DeFronzo and Ferrannini, 1991). Within the endothelium, a balance of the two main insulin signalling pathways are needed; the phosphatidylinositol 3-kinase (PI3K) and mitogen activated protein kinase (MAPK)-insulin dependent pathways. These insulin-dependent pathways balance NO-dependent vasodilation and endothelin-1 (ET-1)-dependent vasoconstriction, thus regulating vascular tone and vasoreactivity (Muniyappa and Sowers, 2013). Decreased sensitivity or responsiveness to insulin within the endothelium reduces the PI3K-NO pathway and enhances the MAPK-ET-1 pathway, leading to a dysfunctional vascular phenotype (Muniyappa and Sowers, 2013). Insulin resistance may arise from glucotoxicity, lipotoxicity, inflammation or other dysmetabolic states (Muniyappa and Sowers, 2013).

Vascular calcification is a known major risk factor for mortality and morbidity, particularly within chronic kidney disease patients, and is an independent risk factor for cardiovascular disease (Wayhs et al., 2002, Arad et al., 2000, Bowman and McNally, 2012). Low bone turnover has been widely reported to have an inverse relationship with calcification in chronic kidney disease patients (Adragao et al., 2009, London et al., 2008, Braun et al., 1996). CKD-mineral bone disorder (CKD-MBD) entails derangements in mineral metabolism, bone remodelling abnormalities, and accelerated medial and intimal calcification, which worsens under haemodialysis (Nakamura et al., 2009). In stage 5 CKD patients, calcification in particular is driven by vascular apoptosis and osteochondrogenic differentiation triggered by increased phosphate levels (Nakamura et al., 2009).

Calcification involves the progressive deposition of calcium within vessels, reducing elasticity and impairing cardiovascular function by promoting mechanical failure (Demer and Tintut, 2008). As the human population continues to age and increase longevity, the consequences of such diseases are further pronounced. Long believed to be a passive part of ageing 'wear and tear', vascular calcification is now considered an active, cell-mediated complex process that is a regulated form of extracellular matrix bio-mineralisation but is not yet fully understood (Demer and Tintut, 2008, Evrard et al., 2015). Convoluted interconnected networks underlie the physiopathological process that continues to be unravelled. Under normal conditions, inhibitors (e.g. matrix GLA protein) and promoters (e.g. inflammation and oxidative stress) of vascular calcification exist.

In bone, bio-mineralisation occurs via endochondral ossification or membranous ossification programmed by chondrocytes and osteoblasts, initiated by matrix vesicles whose function is nucleation and growth of calcium crystals. Vascular smooth muscle cells (VSMCs) can trans-differentiate into osteoblast or chondrocyte-like cells displaying osteogenic fingerprints generally characterised by a decrease in smooth muscle cell markers (e.g. α-SMA, SM-MHC) and an increase in osteochondrogenic markers such as alkaline phosphatase (ALP), Runx2, SOX9, and osteocalcin (see Figure 3) (Evrard et al., 2015, Steitz et al., 2001). In a remarkably similar way to bone, differentiated VSMCs demonstrate hydroxyapatite production and mineralisation (Evrard et al., 2015). Hydroxyapatite crystals form within matrix vesicles secreted from the membranes of osteoblasts, odontoblasts, and chondrocytes (Leopold, 2015). These buds provide a nidus for calcium, phosphate and mineral nucleation which is then deposited in the extracellular matrix between collagen fibrils (Leopold, 2015). This active osteogenic process can be triggered by oxidative stress, oxylipids, phosphate, inflammatory oxylipids and oxLDL (Evrard et al., 2015).

Within the calcified wall, osteoclast-like cells can also develop from macrophages or monocytes, indicating presence of a remodelling balance, although this has not yet been confirmed (Doherty et al., 2002). On the other hand, calciotropic hormones have direct and indirect effects on the vasculature integrity, both excesses and insufficiencies in hormones such as PTH can contribute to vascular disease. Matrix gla protein (MGP) acts as a vascular calcification inhibitor and has a high affinity for binding to calcium ions. A model by Chatrou *et al*. depicts loss of functional MGP might trigger calcification,

along with TNAP activation by inflammation and subsequent hydrolysis of Pi (Chatrou et al., 2015).

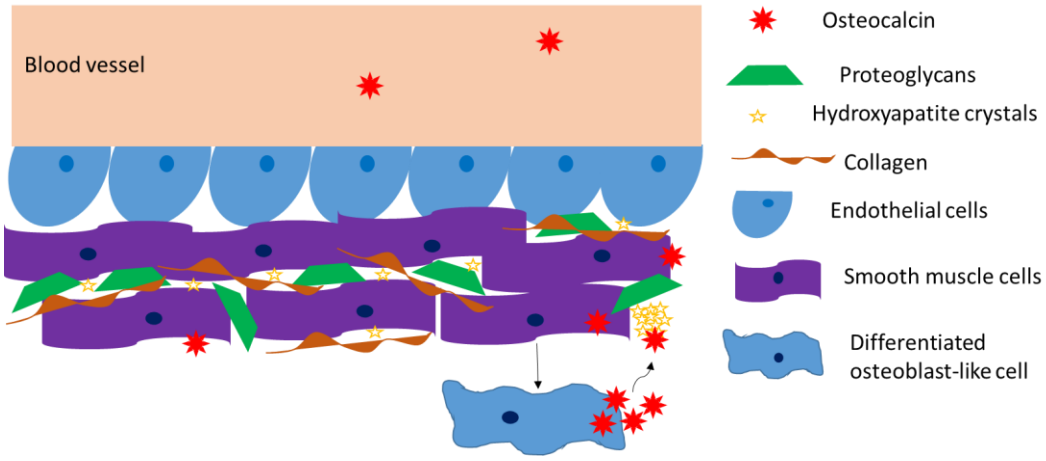

Figure 3. A depiction of components involved in vascular calcification.

OCN is used as a marker of vascular calcification, however whether the origin of this embedded OCN is from the circulation, or produced uniquely by vascular osteoblast-like cells, or both, is not known (see Figure 4). OCN produced by differentiated VSMCs could lead to localised areas of concentrated OCN and may be present in its various forms. It could be hypothesised that it is the OCN present in the calcified regions and plaques that is more clinically relevant, synthesised independently from the circulating OCN originating in bone. This would explain in part why when OCN is measured in serum, results are conflicting and not consistently in agreement with markers of calcification and atherosclerosis in humans, while when OCN is measured *in situ* in calcified tissue, it correlates with the extent and severity of calcification (Millar et al., 2017).

Osteocalcin has been proposed to have a role in atherosclerosis (Gossl et al., 2008; Kim et al., 2016). A small number of *in vivo* studies in rodents have been conducted, reviewed elsewhere (Tacey et al., 2018). Briefly, mice and rats treated with 30 ng/g of OCN over 8-12 weeks demonstrated vascular improvements including normalised diastolic blood pressure following a high fat diet, normalised pulse wave velocity in diabetic rats, increased nitric oxide concentration and attenuation of autophagy and ER stress (Dou et al., 2014, Zhou et al., 2013, Kondo et al., 2016, Huang et al., 2017).

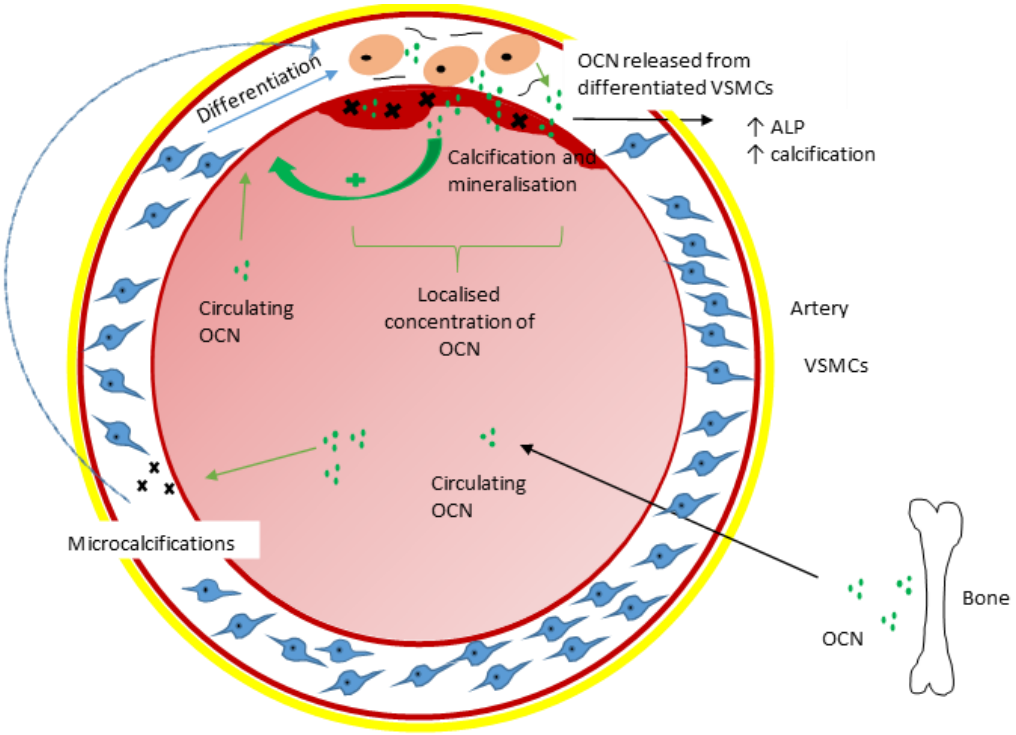

Figure 4. A depiction of how concentrations of osteocalcin may accumulate in the circulation, particularly at areas of vascular calcification and mineralisation where it may contribute to the differentiation of VSMCs or calcification process. *OCN, osteocalcin; ALP, alkaline phosphatase; VSMCs, vascular smooth muscle cells.*

A number of cross-sectional studies have also highlighted associations between OCN concentrations and atherosclerosis and calcification, however, we found that a meta-analysis of these data could not provide a conclusive role as study results were mixed and conflicting with a number of limitations (Millar et al., 2017, Chapter 5). Cross-sectional evidence also exists regarding a role for OCN in inflammation, with some population data highlighting an inverse association between OCN concentrations and systemic inflammatory makers such as C-reactive protein and interleukin-6 (Pittas et al., 2009; Chen et al., 2013; Lucey et al., 2013; Sarkar & Choudhury, 2013; Liao et al., 2015). Overall, studies support a hypothesis that OCN, ucOCN particularly, is directly vasoactive, and can increase nitric oxide via the PI3K/Akt/eNOS signalling pathway, which ultimately influences vasodilation. Additionally, further atheroprotective effects are demonstrated through protecting against high fatty acid induced apoptosis and improving insulin signalling. Although Jung et al. (2013) who used relatively physiological concentrations demonstrated a potential positive role of ucOCN in HAECs (increasing NO production and thus improving endothelial function), Idelevich et al. (2011) in contrast propose that OCN stimulates mineralisation and differentiation of mice VSMCs. It remains to be seen whether these disparities are due to differing effects of forms of OCN within different cell types and species, incubation timings or origin and

concentrations of OCN, and whether OCN displays various functionalities that cannot be simply categorised into a good or bad role within the vasculature. Analysing the direct interaction of OCN and its forms with vascular cells may elucidate whether OCN is a mediator or marker of vascular calcification or atherosclerosis, and whether it functions to affect vascular cells independently of its previously noted metabolic influences.

Thesis aims and hypotheses

The aim of this thesis was to create a novel portfolio of work examining the effects of the circulating bone hormone osteocalcin within the vasculature. We aimed to examine a wide variety of responses of human vascular endothelial and smooth muscle cells to osteocalcin in physiological and pathophysiological conditions.

Specifically, our objectives were:

1. To investigate cross-sectional and observational human data correlating a link between osteocalcin and vascular calcification or atherosclerosis, or lack thereof;
2. To investigate direct effects of uncarboxylated osteocalcin (ucOCN) on human vascular endothelial and smooth muscle cells, including angiogenesis and processes therein, and related intracellular pathways and mechanisms of action;
3. To investigate effects of ucOCN within acute or chronic inflammation in vascular endothelial and smooth muscle cells;
4. To investigate any direct role of ucOCN within vascular calcification of smooth muscle cells;

We aimed to do this in order to ultimately reflect on the potential relevance of osteocalcin in cardiovascular health including atherosclerosis and vascular calcification, diagnostics, and therapeutics, and to contextualise conflicting epidemiological human data. Based on review of the literature (chapters 4 and 5), it was hypothesised that ucOCN may be protective against atherosclerosis and inflammation but promote calcification.

3. General methods & principles

Cell culture

General

Cell culture work was conducted in a sterile environment using aseptic technique within biological safety cabinets (Class II HEPA filtered) and personal protective equipment was worn. Surfaces were disinfected with 1% distel followed by 70% ethanol. All other items were disinfected with 70% ethanol before being placed in the safety cabinet. All cell cultures were maintained in a humid, CO_2 incubator at 37°C and with 5% CO_2 and 95% air which allows for a controlled appropriate atmosphere of high humidity and increased CO_2 tension.

Cell types

All cells used were adherent cultures. Primary cell cultures included human aortic endothelial cells (HAECs; C-12271) and human aortic smooth muscle cells (HASMCs; C-12532) which were purchased from PromoCell (UK) and supplied cryofrozen in vials containing approximately 500,000 cells at passage 2. Cells were not used beyond passage 6 as per manufacturer's recommendations in order to prevent phenotypic and characteristic loss with greater passaging.

Human osteoblasts (HOBs) were also used in some experiments. These were isolated previously from bone by a modified method described elsewhere (Henstock et al., 2014, Anderson et al., 1998, Huang et al., 1997) and stored in liquid nitrogen until use.

Media

Cells were maintained in commercial proprietary media with supplements provided as a bullet kit purchased from PromoCell, specific to each cell type used (C-22120 for HAECs; C-22162 for HASMCs; C-27015 for HOBs). Basic compositions of cell media includes essential nutrients and components such as amino acids, carbohydrates, vitamins and minerals, growth factors, hormones and gases. This regulates the physio-chemical environment (pH, osmotic pressure, temperature). Serum is a vitally important source of growth and adhesion factors, hormones, lipids and minerals. Media was aspirated and replaced every 2-3 days. Reagents were pre-warmed to 37◦C unless otherwise stated. 1% penicillin/streptomycin solution (Sigma-Aldrich P4333; 10,000 units penicillin and 10 mg/mL streptomycin) was added to help prevent bacterial contamination.

Revival

Prior to use, cells were stored in liquid nitrogen in media containing 5% dimethyl sulfoxide (DMSO) for cryopreservation. To revive cells from storage, the cryovials containing the cells were removed from liquid nitrogen and immediately thawed with gentle swirling in a 37°C water bath for approximately two minutes. The cells were then transferred from the vial into a T75 cell culture flask (75 cm^2) containing 10-12 mL of appropriate pre-warmed media. The cells were left to adhere for approximately 6 hours or overnight before replacing the media.

Sub-culturing/passaging

When the cells reached approximately 80% confluency in T75 flasks (still in growth phase), cells were harvested and either divided into further flasks (1:3 ratio), cryopreserved (see following section), or divided into cell culture plates for experiments. To passage the cells, the media was removed and the cell layer rinsed gently with warmed phosphate buffered saline (PBS, Gibco) to remove any residual media containing serum, calcium or magnesium traces which would prevent Trypsin-EDTA from working. Trypsin-EDTA (0.25%) solution was used to enzymatically detach cells from the cell culture flasks and left for about 1 minute at room temperature whilst observing cellular changes with an inverted microscope. Cell detachment was encouraged by a firm tap of the flask by hand. Once the cells had detached, about 10 mL of complete media containing serum was added to the flask as serum neutralises trypsin and thus prevents any further cellular membrane damage. The cell suspension was then collected and placed into a falcon tube and centrifuged at 32 x g for 5 minutes to collect a cell pellet. The supernatant was carefully aspirated without disturbing the cell pellet and the remaining pellet was re-suspended with 1 mL of media. The cells were then diluted further in media and seeded into plates, flasks, or frozen for later use.

Cryopreservation

Cells were collected into a pellet as described above. Cells were then resuspended in room temperature media containing 5% DMSO which acts as a cryoprotective agent and placed into cryovials on ice. The vials were left on ice for 10-15 minutes and then transferred to a cryofreezing container and placed into a -80°C freezer overnight. The cryofreezing container allows for a cooling rate of -1°C per minute. The following day the cryovials were transferred to liquid nitrogen storage.

Collecting media and lysate samples

When required, media samples from cell cultures were collected in eppendorfs and analysed immediately or frozen at -80°C prior to further analysis. Frozen samples were defrosted on ice when used in order to minimise protein degradation. To collect cell lysates, media was removed and the cell monolayer was washed gently with ice cold PBS. RIPA buffer (Sigma-Aldrich R0278), containing a protease and phosphatase inhibitor cocktail (A32959, Thermo Fisher), was then added. The inhibitor cocktail contains aprotinin, bestatin, E-64, EDTA, and leupeptin which protect against degradation by serine proteases, cysteine proteases, and aspartic acid proteases. Additionally, sodium fluoride, sodium orthovanadate, sodium pyrophosphate, and beta-glycerophosphate aid to prevent the dephosphorylation activity of serine/threonine and tyrosine phosphatases. The plates were then left on a plate shaker for one hour in the cold room (4-8°C). The cell layers were then scraped with the wider end of a 1 mL pipette tip and collected into eppendorfs on ice. Depending on future analysis required, samples were either analysed immediately, frozen at -80°C, or spun at 4°C in a centrifuge at 12,850 x g for ten minutes and supernatants removed into fresh eppendorfs and then frozen at -80°C. For western blotting purposes to extract membranous or cytoplasmic bound fractions, the whole cell lysates were disrupted by sheer force by passing them through a 21 gauge needle (0.8mm bore, 40mm length) 25 times, followed by sonication on ice for 15 minutes, and repeated disruption through a needle 25 times. The cells were then frozen as whole cell lysates or as supernatants after centrifuging as described above depending on cellular location of target protein to be analysed.

Limitations of cell culture

It is recognised and acknowledged that although the cells purchased from PromoCell are primary cells, the sub-culturing and passaging allows for phenotypic drift and intrinsic alterations and may not be completely representative of P1 or P2 primary cells. This was minimised by not using cells later than passage 6. The cells obtained were donated from donors under consent from approved medical centres, and were from either thoracic or abdominal origin. In the case of 'thoracic' origin, tissue from the aorta ascending up to the aortic arch were used; in the case of 'abdominal' origin, tissue from the descending portion starting at the diaphragm, i.e. from the aorta abdominalis, were used. Tissue was obtained during either heart surgery where small amounts of aortic

tissue is removed, or during forensic medicine where aortic tissue is removed during the post-mortem examination. However, information on donor characteristics such as sex, age or health status were unfortunately not collected for the cells obtained throughout the thesis period. This may have proved beneficial in determining differences in responses of cells from different batches.

It is also acknowledged that the cell culture experiments performed do not represent the *in vivo* vascular environment. *Ex vivo*, *in vivo*, and clinical studies in the future will continue to answer questions on the role of osteocalcin in the vascular system.

Osteocalcin protein

Human uncarboxylated osteocalcin (ucOCN; amino acids 1-49, [Glu17,21,24]) was purchased from US Biological, MA (O8060-09C-USB) and AnaSpec Inc. CA (AS-65307). The amino acid sequence of purchased osteocalcin was as follows: Tyr-Leu-Tyr-Gln-Trp-Leu-Gly-Ala-Pro-Val-Pro-Tyr-Pro-Asp-Pro-Leu-Glu-Pro-Arg-Arg-Glu-Val-Cys-Glu-Leu-Asn-Pro-Asp-Cys-Asp-Glu-Leu-Ala-Asp-His-Ile-Gly-Phe-Gln-Glu-Ala-Tyr-Arg-Arg-Phe-Tyr-Gly-Pro-Val. Osteocalcin was reconstituted in 4:1 dH_2O:Acetonitrile and stored as stock concentrations of 1 µg/µL and then intermediate stocks at 1 ng/mL. An osteocalcin duoset ELISA (DY1419, R&D systems) was performed periodically to ensure stability and to check for degradation (see ELISA section below).

Microplate spectrophotometer

A Multiskan Spectrum plate reader with a built-in monochromator and SkanIt Software 2.2 (Thermo Scientific) was used to measure colorimetric optical density, achieved by measuring light absorbance at a specific wavelength. A detector quantified how much of an initial light at a specific wavelength is transmitted through the samples, which usually directly related to the concentration of the analyte in question. This was then used to interpolate unknowns with corresponding standard curves of known concentrations.

Enzyme-linked immunosorbent assays

Kits

Sandwich enzyme-linked immunosorbent assays (ELISAs) were used to measure multiple intracellular and secreted proteins. Kits were purchased from R&D systems and performed as per manufacturer's guidelines. Human ELISA duoset kits used included:

osteocalcin (DY1419), interleukin-6 (DY206), matrix metalloproteinase-3 (DY513), endothelin pan specific (DY1160), vascular cell adhesion molecule-1 (DY809), human intracellular adhesion molecule-1 (DY720), interleukin-8 (DY208), interleukin-10 (DY217B), monocyte chemoattractant protein-1 (DY279), and interleukin-17 (DY177).

Procedure

Clear, flat-bottomed, high-binding 96-well microplates (Greiner Bio-One, 655061) were coated with a primary capture antibody (diluted in PBS) overnight. The following day the plates were washed and then coated with a blocking solution/reagent diluent (varied per kit but generally 1% bovine serum albumin [BSA] in PBS), to prevent any non-specific binding of samples to plate wells. Standards, quality controls, and samples (diluted in reagent diluent) were then incubated on the plate in duplicate for two hours to allow for binding between the capture antibody and the antigens present. A biotinylated secondary detection antibody (diluted in reagent diluent) was then incubated on the plate to allow for binding to the antibody-antigen complexes. Streptavidin-horseradish peroxidase enzyme (HRP) was then added which binds to the detection body. Biotinylation allows for streptavidin biding which increases the number of binding sites and therefore the sensitivity of the assay by having more HRP attachment that can then interact with the substrate to increase the following colour reaction. Washing steps in between stages is essential to remove excess antibody or samples and occurred by filling and emptying the wells sequentially with 0.05% Tween20 in PBS three times. Substrate solution was added which is a 1:1 solution of colour reagent A (H_2O_2) and colour reagent B (3,3',5,5'-tetramethylbenzidine, TMB) which forms a colorimetric product relative to the amount of HRP present per well. The reaction was stopped by adding stop solution/sulphuric acid (2N H_2SO_4) to prevent over development. Absorbance was read at 450 nm on the plate reader, with reference wavelength readings taken at 570 nm to account for optical imperfections in the plate. Sample analyte concentrations were analysed by interpolating from a standard curve of known concentrations of the analyte in question.

Controls

Quality controls containing known concentrations of the analyte of interest were included in every ELISA performed to check consistency in performance of the kit and user performance. Intraplate and interplate variability of quality controls was assessed and deemed acceptable with a variance <15%.

Multiplex immunoassays

Kits

The MILLIPLEX MAP 9-plex Multi-Pathway Magnetic Bead Signalling Kit (48-680MAG), MILLIPLEX MAP Human Angiogenesis Assay (HAGP1MAG-12K), and the MILLIPLEX MAP 2-plex Phospho/Total mTOR Cell Signalling Multiplex Assay (48-625MAG) were used.

Procedure

Multiplex assays were performed which allow the simultaneous measurement of more than one analyte in one well/sample. Luminex xMAP® technology and a MAGPIX™ analysing system from Merck Millipore were used. 6.45 µm magnetic bead microspheres are fluorescently colour coded to up to 80 different concentrations and are used as identifiers and as the basis to build the assay. Capture antibodies are coated on the beads for specific analytes. The samples were incubated in 96-well microplates (Greiner Bio-one, 655096) with the coated magnetic beads. Biotinylated detection antibody was then added which attach to the analytes resulting in an antibody-antigen sandwich. The beads were then incubated with streptavidin-phycoerythrin conjugate which acts as the reporter molecule. The MAGPIX™ analysing system is a fluorescent based instrument that uses two LEDs – one to identify the bead based on the internal fluorescent dye concentration, and the other to detect the quantity of the analyte in question. A charge-coupled device (CCD) camera was used to image each well.

Protein quantification

The bicinchoninic acid (BCA) assay was used to quantify total amount of protein present in cell lysate samples, usually to normalise secreted protein quantifications to total cell protein content per well. The BCA assay works on the principle that the peptide bonds in protein reduce Cu^{2+} ions from cupric sulphate to Cu^{+}, and the amount reduced is proportional to the amount of protein present in the sample (Smith et al., 1985). BCA forms a purple colour with Cu^{+} whose absorbance can be read at 562 nm using a spectrophotometer plate reader. A standard curve with known quantities of protein using BSA was freshly prepared in RIPA buffer. Sample and standards were then incubated with BCA and copper II sulfate pentahydrate 4% solution (50:1 ratio) at 37°C for 30 minutes in a 96-well microplate (Greiner Bio-One, 655101). Absorbance was read at 562 nm on the plate reader and sample protein concentrations were analysed by interpolating from the standard curve.

Transepithelial electrical resistance

Cells were grown until confluent on 12 mm diameter, 0.4 µM pore polycarbonate membrane inserts (Corning® Costar®; Sigma-Aldrich, UK). Transepithelial electrical resistance (TEER) was measured using an EVOM™ voltohmmeter (World Precision Instruments) to evaluate paracellular permeability of cell monolayers. The EVOM™ produces an AC current and the electrical resistance (Ohms) across a cell monolayer grown on a cell culture well insert/membrane was measured using STX3 electrodes connected to the voltohmmeter (World Precision Instruments). These are a pair of double electrodes (4 mm wide, 1 mm thick) whose ends contain a silver/silver-chloride pellet which measures voltage, and a silver electrode which passes the current through the insert sample. The two electrodes are unequal in length to allow the longer one outside the insert to touch the cell culture plate bottom while submerged in media, and the shorter one to be submerged in media inside the insert but not touching or disturbing the cell layer. The confluence or decrease in permeability of a cell monolayer can be determined by an increase in TEER readings. TEER was recorded 3 times per well and averaged. The blank resistance (cell culture insert with media but without cells) was subtracted from sample readings. The resistance is inversely proportional to the surface area thus the unit area resistance was reported and calculated by multiplying the readings by 1.12 to account for the surface area of the insert.

Cell proliferation assay

A CellTiter 96® Aqueous One Solution Cell Proliferation Assay (G3580, Promega) was performed to measure cell proliferation according to manufacturer's instructions. This assay uses a tetrazolium salt compound (MTS) and an electron coupling reagent (phenazine ethosulfate, PES) which helps stabilise MTS. MTS is reduced by metabolically active cells by NADPH or NADH produced by dehydrogenase enzymes into a coloured formazan product. The MTS reagent was added directly into cell culture wells, incubated for 4 hours in the cell culture incubator and then absorbance was recorded at 490 nm which directly related to the quantity of formazan product produced. Black, clear-bottomed, opaque-walled 96 well microplates were used to minimise light scattering (Corning, #3916). The MTS assay is a robust, sensitive and simple assay to perform, however there are many available assays to assess cell proliferation including perhaps more sensitive fluorescent protocols which could have been employed. For example, a resazurin based assay in which resazurin is reduced to resorufin (Zhang et

al., 2004). Both these methods assess cellular metabolism as an indication of cell proliferation. Alternatively, assays are also available to assess rate of DNA replication/synthesis as an indicator of cell proliferation which may be deemed more accurate as there is only one nucleus per cell compared to measuring mitochondrial function which may vary from cell to cell (Muir et al., 1990).

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a soluble cytosolic oxireductase enzyme that is released from cells when they lose their plasma membrane integrity. Thus, measuring LDH is an indicator of necrosis and apoptosis. LDH colorimetric assay kits were purchased from Abcam (ab102526) and performed according to the manufacturer's instructions. LDH reduces NAD to NADH which interacts with a probe to produce a colour which can then be measured at 450 nm on the plate reader. Samples were incubated in 96-well microplates (Greiner Bio-One, 655101) with the probe and absorbance was quantified by interpolation of a standard curve.

Western blotting

Antibodies

Antibody	Host species	Primary or secondary	Manufacturer	Catalogue number	Dilution
Smooth muscle α-actin	Rabbit	Primary	Abcam	ab32575	1:2500
RUNX2/CBFA1	Goat	Primary	R&D systems	AF2006	1:2000
GAPDH	Mouse	Primary	Proteintech	60004-1	1:10000
SOX9	Goat	Primary	R&D systems	AF3075	1:400
GPRC6A	Rabbit	Primary	Abbexa	abx015319	1:1000
Beta-actin	Rabbit	Primary	Abcam	8227	1:5000
Anti-rabbit	Goat	Secondary	Sigma	A3937	1:25000
Anti-mouse	Goat	Secondary	Sigma	A3562	1:30000
Anti-goat	Rabbit	Secondary	Abcam	ab97097	1:5000

All antibodies used were anti-human.

Procedure

Cell lysate samples were diluted with Laemmli 2X buffer (Bio-Rad, #1610737) with β-mercaptoethanol (BME) to achieve a loading protein concentration of 5-10 µg/lane in 15-well 10% Mini-protean TGX precast gels (Bio-Rad, #4561036). The BME encourages reduction of the protein by disrupting polypeptide-polypeptide interactions between disulphide bonds to enable better separation of proteins by size during electrophoresis

migration. Once diluted, samples were boiled for 5 minutes at 100°C to further denature the sample proteins to enable access of the antibody to the recognition epitope. Equal amounts of ice-cooled samples were loaded onto the gels and proteins were resolved by electrophoresis with Tris-Glycine/SDS running buffer (Bio-Rad, #1610772; 25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3) at 40 mA in constant current mode. The SDS (sodium dodecyl sulphate) is an ionising detergent that further prevents protein folding and coats proteins with a net negative charge in a relatively uniform manner to obtain net charge vs molecular weight proportionality. Electrophoresis separates out the proteins according to molecular weight as they migrate towards the positive anode.

The proteins separated in the gel were then transferred by electroblotting onto a high protein binding nitrocellulose membrane (0.45 µM pore size) using a wet transfer in transfer buffer (Bio-Rad, #1610771; 25mM Tris, 192mM glycine, pH 8.3) containing 20% methanol over two hours at 100V. In order to do this, a 'sandwich' of sponges, filter paper, gel, and membrane was made. This was then immersed in a transfer apparatus and filled with transfer buffer cooled to 4°C. Ice packs containing transfer buffer frozen to -80°C were placed in the transfer tank and the entire tank was then immersed in wet ice to counter effects of solution heating. When transfer time was 2 hours or more, the ice pack was changed at 1 hour. To visualise transferred proteins, the membrane proteins was reversibly stained with Ponceau S solution (Sigma, 0.1%) for 5 minutes. The membrane was then rinsed with distilled water (dH_2O) to remove excess staining of the proteins and an image captured using ChemiDoc™ MP Imaging system. Total lane protein was quantified colorimetrically using Image Lab™ software (Bio-Rad) and the membrane was then de-stained with Tris Buffered Saline (TBS; Fisher Bioreagents, Tris/Tris-HCL 25mM, NaCl 0.13M, KCL 0.0027M, pH 7.4) containing 1% Tween 20 (Merck #655204).

The membrane was then incubated in blocking buffer (5% Marvel or BSA in TBS with Tween 20 [TBST]) for two hours at room temperature to reduce non-specific binding and background interference. The membrane was then incubated with primary antibody overnight at 4◦C (diluted in 3% Marvel or BSA in TBST). Following washing steps (10 minutes x 6 in TBST) the membrane was incubated for 1.5 hours with an alkaline phosphatase conjugated secondary antibody diluted to required concentration in 3% Marvel or BSA in TBST. The membrane was washed again as described. Immunoreactive bands were visualised by chemiluminescence (Bio-Rad Immun-Star™

AP Substrate Pack #1705012) using the ChemiDoc™ MP Imaging system with Image Lab™ software (Bio-Rad) against a molecular weight marker (Precision Plus Protein™ Standards Kaleidoscope™, Bio-Rad #1610375).

When required, the membrane was then stripped for reblotting using ReBlot Plus Mild Antibody Stripping Solution (Sigma-Aldrich, #2502) for 10 minutes at room temperature which removes primary and secondary antibodies and then blocked with 5% marvel in TBST for two hours at room temperature. The membrane was then incubated with the next primary antibody (usually a loading control) overnight at 4°C. Finally, the membrane was washed and incubated with secondary antibody in 3% Marvel or BSA in TBST and visualised and quantified as described previously.

Controls

In general, control gels and blots were run wherein to assess the non-specific binding of the secondary antibody, the primary was not added to the TBST, otherwise the protocol described above was followed exactly. Positive and negative controls were also run alongside samples during western blotting, i.e. cell lysates from a source where the target protein was known to be present (positive control), and from a source where the target protein is known not to be present (negative control), in order to correctly identify the protein of interest in test samples.

Sample loading was corrected for by either dividing the protein band densitometry quantification by total lane protein as measured by Ponceau stain for that sample lane, or by dividing by the protein band densitometry quantification by the densitometry quantification of a housekeeping protein such as GAPDH or β-actin (Moritz, 2017).

Limitations

Western blotting is a powerful, relatively inexpensive technique that is widely applied, but it is acknowledged that it is semi-quantitative. A lack of antibody specificity, poor validation, experimental reporting, and reproducibility can be problematic (Bass et al., 2017). In this thesis, western blotting was employed when quantitative ELISAs were not possible or available to detect the presence/absence of a protein, and relative abundance between different samples.

Alizarin Red staining

Alizarin Red, or 1,2-dihydroxyanthraquinone, was used to stain hydroxyapatite mineralized matrixes in cell monolayers producing a red-orange colour (Gregory et al., 2004). Alizarin Red powder (Sigma Aldrich) was dissolved in dH_2O to make a 40 mM solution, and pH was adjusted to 4.1-4.3 with 0.5% ammonium hydroxide. Media from cell culture plates was removed and the cell layer was rinsed with PBS. The cells were then fixed with 10% (v/v) formaldehyde (Sigma Aldrich) at room temperature for 15 minutes which allowed formation of covalent chemical bonds (cross-links) between proteins and their surroundings. The monolayers were then washed twice with excess dH_2O. Alizarin Red solution was then added to each well and incubated at room temperature for 20 minutes. The unincorporated dye was then removed and the plates were washed 4 times with excess dH_2O. The stained wells were then visualised under light microscopy and imaged. To extract and quantify the incorporated dye, 10% (v/v) acetic acid was added to each well. The cell layer mixture in acetic acid was then collected into eppendorfs, vortexed, and overlaid with mineral oil. The eppendorfs were heated to 85°C for 10 minutes and then transferred to ice to cool. The samples were centrifuged at 25,200 x g for 15 minutes and the supernatants were removed and neutralised with ammonium hydroxide (10 % v/v). Aliquots were transferred to black, opaque-walled, clear-bottom 96-well microplates to minimise light scattering (Corning, #3650) and colorimetric detection was carried out at 405 nm on the plate reader. This method gave a moderately sensitive measurement of calcification, but required fixation of cells which limited further measurements in these samples. Alternative stains have been suggested in the literature such as Calcein Green staining. Calcein Green is a non-cell-permanent fluorescent dye that fluoresces when bound to calcium crystals (Serguienko et al., 2018). However this technique is only appropriate to measure calcification in living cells in real time scenarios.

Calcium quantification

Calcium content of monolayers was measured using a calcium detection assay kit (Abcam, ab102505) according to manufacturer's instructions in 96-well microplates (Greiner Bio-One, 655101) which gave more sensitive results compared with Alizarin Red Staining. Briefly, cells were decalcified overnight with 0.6N hydrochloric acid (HCL). The calcium contents of the supernatants were then quantified using the 0-cresolphthalein method in which a chromogenic complex is formed between calcium

ions and 0-cresolphthalein and then measured at 575 nm on the plate reader (Gitelman, 1967). Sample calcium content was calculated by interpolating against a standard curve prepared by diluting a 5 mM calcium standard.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured in 96-well microplates (Greiner Bio-One, 655101) using an ALP detection assay (Abcam, ab83369) according to manufacturer's instructions. Briefly, p-nitrophenyl phosphate (pNPP) was used as a phosphatase substrate which turns yellow when dephosphorylated by ALP. The absorbance was then measured at 405 nm on the plate reader and the activity of samples was interpolated and calculated against a standard curve prepared by diluting a 1 mM pNPP standard.

Haemotoxylin & Eosin staining

Before staining cells they need to be fixed and permeabilized. Fixation halts the cells decomposition and denatures proteins leaving subcellular structures intact, while permeabilisation allows perforation of the cell membranes to allow substances to enter the cell. An organic solvent method was used to fix cells which removes lipids, dehydrates the sample, and denatures and precipitates the proteins. Cell monolayers were washed three times with PBS and then incubated with ice cold acetone:methanol (50:50) for 10 minutes at room temperature which fixes and permeabilizes at the same time. The monolayers were then washed again in PBS. Haematoxylin and eosin staining was used to assess visual morphology of cell monolayers. Haematoxylin stains nuclei blue, specifically the chromatin and nuclear membrane. Eosin Y was used as a counterstain as it stains the cytoplasm and most other structures red, pink or orange variants. Cells were stained in Mayer's Haematoxylin solution for 15 minutes, washed with tap water five times, and then counterstained with Eosin Y solution for 30 seconds, washed in distilled water, visualised and then imaged.

Scratch wound healing assay

A scratch wound assay was conducted based on a previously published protocol (Liang et al., 2007). Briefly, HASMCs and HAECs were seeded in 6-well cell culture plates and left to adhere until confluent in usual cell growth media. Cells were then maintained with basal medium without growth or supplement factors and the minimum amount of serum to allow cell survival and discourage proliferation (pre-determined using an MTS

assay and various serum concentrations to measure cell survival over 24-72 hours). After 24 hours, a scratch was marked on each well with a P200 pipette tip, washed with PBS to remove any debris, and replaced with basal media. Images of the scratch were captured at baseline and at various time points (0-12 hrs for HAECs; 0-48 hrs for HASMCs) to monitor cell migration. A reference template plastic sheet was designed that could be attached to the bottom of the cell culture plate in order to locate the exact imaging position across the time period (a minimum of 3 imaging positions per well were used). The area of the scratch wound at baseline and at each time point was measured using ImageJ software. This method was a simple and feasible way to assess cell migration following a 'wound', however other methods could equally have been employed such as a trans-well migration assay or Boyden chamber assay. The migrated cells at the bottom of the cell culture plate can then be stained and quantified. This may have provided more sensitive information on cell motility as the cells migrate through a cell culture insert with a permeable membrane towards a chemoattractant or angiogenic inducer.

Statistical analysis

All statistical analyses were performed using Prism for Windows (GraphPad Software Inc, versions 7-8). Data were checked for normality using the D'Agostini & Pearson test and for statistical outliers using the ROUT method. Normal data were assessed using parametric analysis while non-parametric tests were used for non-normal data. In general, one-way analysis of variance (ANOVA) tests were used to detect mean differences between three or more groups. Multiple comparisons were adjusted for by Dunnett's or Sidak's statistical hypothesis test. When only two groups were being compared, a parametric (Student's) or non-parametric (Mann-Whitney) *t*-test was performed, with or without Welch's correction depending on distribution and variance as appropriate. Pearson's or Spearman's correlations were performed to assess correlations between two variables. Two-way ANOVAs were performed to assess mean differences between groups when two independent factors were present, e.g. treatment and time. In general, data are presented as mean ± standard error of the mean (SEM). P-values were considered significant at $p<0.05$.

4. Osteokines and the vasculature: a review of the *in vitro* effects of osteocalcin, fibroblast growth factor-23 and lipocalin-2

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Osteokines and the vasculature: a review of the *in vitro* effects of osteocalcin, fibroblast growth factor-23 and lipocalin-2

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ABSTRACT

Bone-derived factors that demonstrate extra-skeletal functions, also termed osteokines, are fast becoming a highly interesting and focused area of cross-disciplinary endocrine research. Osteocalcin (OCN), fibroblast growth factor-23 (FGF23) and lipocalin-2 (LCN-2), produced in bone, comprise an important endocrine system that is finely tuned with other organs to ensure homeostatic balance and health. This review aims to evaluate *in vitro* evidence of the direct involvement of these proteins in vascular cells and whether any causal roles in cardiovascular disease or inflammation can be supported. PubMed, Medline, Embase and Google Scholar were searched for relevant research articles investigating the exogenous addition of OCN, FGF23 or LCN-2 to vascular smooth muscle or endothelial cells. Overall, these osteokines are directly vasoactive across a range of human and animal vascular cells. Both OCN and FGF23 have anti-apoptotic properties and increase eNOS phosphorylation and nitric oxide production through Akt signalling in human endothelial cells. OCN improves intracellular insulin signalling and demonstrates protective effects against endoplasmic reticulum stress in murine and human endothelial cells. OCN may be involved in calcification but further research is warranted, while there is no evidence for a pro-calcific effect of FGF23 *in vitro*. FGF23 and LCN-2 increase proliferation in some cell types and increase and decrease reactive oxygen species generation, respectively. LCN-2 also has anti-apoptotic effects but may increase endoplasmic reticulum stress as well as have pro-inflammatory and pro-angiogenic properties in human vascular endothelial and smooth muscle cells. There is no strong evidence to support a pathological role of OCN or FGF23 in the vasculature based on these findings. In contrast, they may in fact support normal endothelial functioning, vascular homeostasis and vasodilation. No studies examined whether OCN or FGF23 may have a role in vascular inflammation. Limited studies with LCN-2 indicate a pro-inflammatory and possible pathological role in the vasculature but further mechanistic data is required. Overall, these osteokines pose intriguing functions which should be investigated comprehensively to assess their relevance to cardiovascular disease and health in humans.

Subjects Cell Biology, Anatomy and Physiology, Cardiology, Diabetes and Endocrinology

Keywords Osteocalcin, Fibroblast growth factor 23, Lipocalin-2, Endothelial cells, Vasculature, Vascular smooth muscle cells, Osteokines

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INTRODUCTION

The role of the skeleton has greatly evolved from its premise of being a functional tissue providing primary protection, support, and haematopoietic maintenance. It is now recognised as an endocrine organ providing exciting opportunities for cross-disciplinary research. One candidate thread of enquiry is the skeleton's interaction with the vascular system. Bone is a highly vascularised tissue, which is important for growth, remodelling and repair. The endothelial cells lining blood vessels have an important influence on bone cells, often in a paracrine manner, establishing a 'bone-vascular axis' (*Brandi & Collin-Osdoby, 2006*). This traditionally viewed axis is also evolving to reflect that on the other hand, secreted, circulating bone-derived factors have direct effects on vascular cells themselves.

Recent research has demonstrated that three 'osteokines', namely osteocalcin (OCN), fibroblast growth factor-23 (FGF23) and lipocalin-2 (LCN-2), comprise an important endocrine system that is finely tuned with other organs to ensure homeostatic balance and health (*Han et al., 2018*). The endocrine functions of OCN have been extensively reviewed elsewhere (*Ducy, 2011*; *Li et al., 2016*; *Karsenty, 2017*; *Han et al., 2018*). Briefly, OCN has been reported to play an active role in the regulation of energy metabolism by improving glucose tolerance and insulin sensitivity and insulin production (*Lee et al., 2007*; *Oury et al., 2011*; *Oury et al., 2013*). Elsewhere, $OCN^{-/-}$ male mice were observed to be poor breeders due to decreased testosterone production and thus has been linked to fertility (*Oury et al., 2011*). Interestingly, these $OCN^{-/-}$ mice also displayed behaviours associated with anxiety and depression and it was demonstrated that OCN can cross the blood–brain-barrier and enhance monoamine neurotransmitter synthesis, drawing a link between OCN and cognition (*Oury et al., 2013*). FGF23 is well characterised as the regulator of phosphate, parathyroid hormone (PTH), and vitamin D in the body which has been recently reviewed elsewhere (*Richter & Faul, 2018*). Briefly, in the kidneys, FGF23 increases phosphate excretion in urine, thus lowering serum concentrations. FGF23 is secreted by bone following stimulation by increased circulating vitamin D and extracellular phosphate. FGF23 in turn also supresses $1,25(OH)_2D_3$ production. Thus, a regulatory feedback loop exists between the kidneys and bone through FGF23. In the kidneys, FGF23 also increases reabsorption of calcium and sodium (*Andrukhova et al., 2014a*; *Andrukhova et al., 2014b*), while in the parathyroid gland FGF23 supresses PTH production. The endocrine role of LCN-2 was an interesting discovery through the use of mice lacking LCN-2 in osteoblasts ($Lcn2^{osb-/-}$). These mice displayed a decrease in insulin sensitivity and secretion, through direct activity on pancreatic β-cells, and decreased glucose tolerance (*Mosialou et al., 2017*). It has also been shown that LCN-2 crosses the blood brain barrier and activates melanocortin 4 receptor-dependent appetite signalling in the hypothalamus (*Mosialou et al., 2017*).

While these investigations have drawn evidence for the skeletal control of energy metabolism, mineral homeostasis and much more, the effects of these hormones on the vasculature itself has also gathered interest. Conflicting observational data exists between OCN, FGF23 and LCN-2 and markers of cardiovascular disease in humans (*Dalal et al., 2011*; *Wang, 2012*; *Millar et al., 2017*), which are discussed further within this review.

In order to assess the relevance of these osteokines to cardiovascular health, we aimed to provide an analysis of the direct effects of OCN, FGF23 and LCN-2 on micro- and macro-vascular cells *in vitro*, and to highlight gaps in the literature which need addressing, for use by clinicians and researchers.

SURVEY METHODOLOGY

To retrieve articles concerning the exogenous administration of OCN, FGF23 and LCN-2 to micro- or macro-vascular cells, the following search terms were used for database searching (PubMed, Embase, Medline, Google Scholar): fibroblast growth factor 23, bone gamma-carboxyglutamic acid protein, osteocalcin, lipocalin 2, LCN-2, neutrophil gelatinase-associated lipocalin, NGAL, vascular cell, vascular endothelial cell, and vascular smooth muscle cell. Additionally, the reference lists of relevant review articles and included articles were hand-searched for further studies. Searches were carried out up until 20/09/2018. Retrieved articles were included if they described the exogenous addition of OCN, FGF23 or LCN-2 to vascular cells *in vitro* i.e., studies that examined the endogenous over-expression of these osteokines were excluded, as the results would not be deemed to be bone-derived. Similarly, passive reporting of osteokines e.g., expression studies, were not evaluated as this approach excludes the possibility of bone-derived effects and we were interested in clarifying any direct, causal effects of 'circulating' osteokines. Only peer-reviewed, full-text articles were included, not conference abstracts or articles not in English.

OSTEOCALCIN

Osteocalcin (OCN) has been pioneered as a skeletal hormone for over a decade now, solidifying a role in influencing energy metabolism (*Lee et al., 2007*). The mature OCN is a 46 or 49 amino acid protein in mice and humans respectively, predominantly produced by osteoblasts, and is the most abundant, non-collagenous protein in the mineralised matrix of bone (*Hauschka, Lian & Gallop, 1975*; *Price et al., 1976*; *Celeste et al., 1986*; *Hauschka et al., 1989*). Due to the presence of 3 glutamic acid residues which may undergo carboxylation, OCN is present in various forms. These include undercarboxylated OCN with 1 or 2 glutamic acid residues (unOCN), carboxylated OCN with no glutamic acid residues (cOCN), or uncarboxylated OCN (ucOCN) with 3 glutamic acid residues. Carboxylation induces a conformational change resulting in a high affinity for calcium ions. Thus, OCN is also referred to as bone γ-carboxyglutamic acid-containing protein (BGLAP) and binds to hydroxyapatite crystal lattices present in bone extra-cellular matrix (*Hauschka et al., 1989*). unOCN and ucOCN have less affinity to hydroxyapatite and are more readily released into the circulation. These forms have been the most widely studied and are considered to be the 'active' forms of OCN (*Plantalech et al., 1991*; *Cairns & Price, 1994*; *Ferron et al., 2010*). Despite the abundance of OCN in bone, its exact role and function in bone reformation has not been clearly defined. However, serum concentrations of OCN may be used as a marker of bone turnover as it is widely accepted to be released from mature osteoblasts during bone formation (*Bellows, Reimers & Heersche, 1999*).

Circulating concentrations of OCN

There is currently no consensus on which OCN fragments to measure, and there are no international or national standards (*Lee, Hodges & Eastell, 2000*). Total OCN concentrations have been reported by Hannemann et al. by age and gender (*Hannemann et al., 2013*). However, reported serum ucOCN and unOCN concentrations in studies have largely varied, for example between 0.1 and 0.3 ng/mL, and up to 21.4 ng/mL or 33.0 ng/mL (*Plantalech et al., 1991*; *Binkley et al., 2000*; *Iki et al., 2012*). OCN concentrations increase with age but can be reduced by supplementation with vitamin K (*Knapen, Hamulyak & Vermeer, 1989*; *Plantalech et al., 1991*; *Knapen et al., 1993*). cOCN concentrations have also not been defined and have been reported to lie around 8–10 ng/mL or higher e.g., 23.9 ng/mL (*Luukinen et al., 2000*; *Tsukamoto et al., 2000*; *Sugiyama & Kawai, 2001*). To add to the complexity of measuring circulating OCN, only 25% is found intact, and further additional forms are also present including N-terminal, mid-region, mid-region-C-terminal, and C-terminal fragments released by proteolytic cleavage (*Garnero et al., 1994*).

OCN and the vasculature

A number of cross-sectional studies have highlighted associations between OCN concentrations and atherosclerosis and calcification, however, we found that a meta-analysis of these data could not provide a conclusive role as study results were mixed and conflicting with a number of limitations (*Millar et al., 2017*). Analysing the direct interaction of OCN and its forms with vascular cells may elucidate whether OCN is a mediator or marker of vascular calcification or atherosclerosis, and whether it functions to affect vascular cells independently of its previously noted metabolic influences. Cross-sectional evidence also exists regarding a role for OCN in inflammation, with some population data highlighting an inverse association between OCN concentrations and systemic inflammatory makers such as C-reactive protein and interleukin-6 (*Pittas et al., 2009*; *Chen et al., 2013*; *Lucey et al., 2013*; *Sarkar & Choudhury, 2013*; *Liao et al., 2015*). A recent summary of *in vivo* ucOCN treatments in mice and rats concluded that ucOCN protects vascular function and protects against markers that are commonly associated with the development of atherosclerosis (*Tacey et al., 2018*). However, these findings are concomitant with improved metabolic status, and as such, the establishment of direct effects of OCN and its forms on vascular cells needs confirmation. The following section aims to summarise *in vitro* investigations on the exogenous addition of OCN to vascular endothelial or smooth muscle cells (see Table 1).

OCN in human and animal cells

Jung et al. (2013) reported an increase in Akt and eNOS phosphorylation and nitric oxide (NO) production in a PI3-kinase dependent manner in human aortic endothelial cells (HAECs) when treated with ucOCN (0.3–30.0 ng/mL) (*Jung et al., 2013*). They also demonstrated an anti-apoptotic effect of ucOCN, in which 30 ng/mL pre-treatment prevented linoleic acid induced apoptosis, also via the PI3K/Akt pathway. Similarly, another study showed an increase in eNOS phosphorylation in HAECs after 30 min incubation with ucOCN (25 and 100 ng/mL) (*Kondo et al., 2016*). Interestingly, eNOS phosphorylation

Table 1 Summary of studies investigating *in vitro* effects of osteocalcin on human and animal vascular cells.

Study	Cell type	Type of OCN	Concentration	Results	Conclusions
Idelevich, Rais & Monsonego-Ornan (2011)	Murine VSMCs	Total OCN	Unknown	OCN was shown to be a glucose metabolism-modulating factor, through HIF-1α.	OCN is involved in glucose-metabolism
Zhou et al. (2013)	Murine VECs and VSMCs	ucOCN	5 ng/mL	ucOCN protected against tunicamycin induced ER stress and autophagy, and improved insulin signalling in an Akt/mTOR/NFκB pathway.	Protective effect of ucOCN and improved insulin signalling
Jung et al. (2013)	Human AECs	ucOCN	0.3–30 ng/mL	Increased eNOS and NO, and prevented linoleic acid induced apoptosis in PI3-K/Akt dependent manner.	Protective effect of ucOCN
Dou et al. (2014)	HUVECs	Total OCN	10–150 ng/mL	>30 ng/mL OCN increased eNOS and Akt phosphorylation in a time- and dose-dependent manner.	Potential protective effect of total OCN
Kondo et al. (2016)	Human AECs	ucOCN cOCN	25 and 100 ng/mL	ucOCN increased eNOS phosphorylation after 30 min but cOCN had no effect.	Potential protective effect of ucOCN
Guo et al. (2017)	HUVECs	ucOCN	5 ng/mL	In insulin-resistant cells (induced by tunicamycin) ucOCN improved insulin signal transduction via PI3-K/Akt/NFκB pathways.	ucOCN is involved in insulin signalling

Notes.

Abbreviations: OCN, osteocalcin; VECs, vascular endothelial cells; VSMCs, vascular smooth muscle cells; AECs, aortic endothelial cells; HUVECs, human umbilical vein endothelial cells; ucOCN, uncarboxylated OCN; cOCN, carboxylated OCN; ER, endoplasmic reticulum; mTOR, mechanistic target of rapamycin; NFκB, Nuclear factor-κB; HIF-1α, hypoxia inducible factor 1 – α; PI3-K, phosphoinositide 3-kinase; eNOS, endothelial nitric oxide synthase.

however was not affected by the same concentrations of cOCN (*Kondo et al., 2016*). Total OCN (>30.0 ng/mL) was reported to increase Akt and eNOS phosphorylation in human umbilical vein endothelial cells (HUVECs) in a time- and dose-dependent manner (*Dou et al., 2014*). Another study in HUVECs, in which insulin resistance was induced by tunicamycin, ucOCN (5 ng/mL) improved insulin signal transduction via PI3K/Akt/NF-κB pathways (*Guo et al., 2017*). Similarly, mouse vascular endothelial cells (VECs) and smooth muscle cells (VSMCs) were treated with 5 ng/mL ucOCN which protected against tunicamycin induced endoplasmic reticulum (ER) stress and autophagy, and improved insulin signalling in an Akt/mTOR/NF-κB pathway (*Zhou et al., 2013*).

Idelevich, Rais & Monsonego-Ornan (2011) importantly identified that OCN may indeed be an active player in vascular calcification, and not merely a product of differentiated VSMCs to an osteogenic phenotype. They demonstrated that overexpression of OCN stimulated glucose utilisation through activation of HIF-1α and promoted mineralisation and osteogenic differentiation in mouse vascular smooth muscle cells (MOVAS). Silencing of OCN RNA suppressed these effects. However, this does not distinguish an effect of osteoblast derived, circulating OCN, but rather locally produced effects from overexpression. However, when total OCN was exogenously added to wild-type MOVAS, OCN was shown to be a glucose metabolism-modulating factor, through HIF-1α. No

investigations have been conducted in human vascular smooth muscle cells to date. Ultimately, further investigation is needed into the potential direct influence of OCN on vascular calcification.

Overall, these studies support a hypothesis that OCN, ucOCN particularly, is directly vasoactive, and can increase nitric oxide via the PI3K/Akt/eNOS signalling pathway, which ultimately influences vasodilation. Additionally, further atheroprotective effects are demonstrated through protecting against high fatty acid induced apoptosis and improving insulin signalling. Although *Jung et al. (2013)* who used relatively physiological concentrations demonstrated a potential positive role of ucOCN in HAECs (increasing NO production and thus improving endothelial function), Idelevich et al. in contrast propose that OCN stimulates mineralisation and differentiation of mice VSMCs. It remains to be seen whether these disparities are due to differing effects of forms of OCN within different cell types and species, incubation timings or origin and concentrations of OCN, and whether OCN displays various functionalities that cannot be simply categorised into a good or bad role within the vasculature. The direct role of OCN within inflammation in the vasculature has also not been investigated. These limited studies and abundant questions signal there is need to clarify the role of physiologically relevant concentrations of OCN in human vascular cells. Moreover, the effects of both unOCN and cOCN, and potentially other OCN fragments, must be distinguished and deserves greater focus. Particularly as unOCN is more likely to be in circulation than fully non carboxylated OCN.

FIBROBLAST GROWTH FACTOR 23

Osteocytes, which are the most abundant bone cell type, are the predominant secretors along with osteoblasts of fibroblast growth factor 23 (FGF23). FGF23 is the first described bone hormone and original phosphotonin (*Bonewald & Wacker, 2013*; *Bonnet, 2017*). FGF23 is a 251 amino acid protein in humans regulated at gene and protein level (*Bonewald & Wacker, 2013*). FGF23 is proteolytically cleaved upon secretion. Susceptibility to cleavage is reduced by O-glycosylation by polypeptide N-acetylgalactosaminyltransferase 3 which allows more intact, biologically active FGF23 to be secreted (approximately 32 kDa) (*Erben, 2017*). On the other hand, phosphorylation of FGF23 promotes cleavage resulting in C-terminus and N-terminus fragments, whose biological activity is unclear. Soluble or transmembrane α-Klotho is believed to be the cofactor required to allow for activation of FGF receptors by FGF23, though in some tissues FGF23 can have klotho independent effects (*Urakawa et al., 2006*; *Chen et al., 2018*; *Richter & Faul, 2018*). Within bone, the actions of locally produced FGF23 by osteocytes, as well as osteoblasts, have not been fully elucidated. Within mice, FGF23 may be a regulator of bone mineralisation in an autocrine/paracrine manner through inhibiting tissue non-specific alkaline phosphatase (TNAP). This results in increased pyrophosphate concentrations, likely in a klotho independent manner due to low concentrations of klotho within bone (*Murali et al., 2016*; *Erben, 2017*).

Circulating concentrations of FGF23

FGF23 reaches its target organs through the circulation. Similar to OCN, there is complexity in measuring FGF23 as there are various presenting forms. Intact FGF23 (iFGF23) is the

most commonly used measurement, while C-terminus FGF23 (cFGF23) assays can also be used which measure both iFGF23 and cFGF23 (*Smith, 2014*). iFGF23 is also subject to *ex vivo* degradation and exhibits diurnal variation, while cFGF2 concentrations can be influenced by iron levels, fibrous dysplasia or familial tumoral calcinosis (*Wolf, Koch & Bregman, 2013*; *Smith, 2014*). In adults, mean cFGF23 has been reported at 49.0 RU/mL, and iFGF23 at 26.1 pg/mL, but high intra-individual variability exists which limits its application as a diagnostic or management tool (*Smith et al., 2012*). Paediatric reference values have been published for cFGF23 only (22.0–91.0 RU/ml) (*Fischer et al., 2012*). In patients with chronic kidney disease (CKD), circulating FGF23 is increased with decreasing renal function, and can reach up to 1000 times higher than normal levels (*Larsson et al., 2003*; *Fliser et al., 2007*; *Gutierrez et al., 2008*; *Isakova et al., 2011*).

FGF23 and the vasculature

A number of epidemiological studies in humans have reported associations between increased circulating FGF23 concentrations and vascular dysfunction (*Mirza et al., 2009a*), vascular calcification (*Roos et al., 2008*; *Desjardins et al., 2012*) and increased risk of cardiovascular disease (*Dalal et al., 2011*). FGF23 concentrations have been positively correlated with left ventricular hypertrophy (LVH), which is particularly apparent in CKD patient cohorts (*Hsu & Wu, 2009*; *Mirza et al., 2009b*). Other studies have not shown an association between FGF23 and vascular calcification or atherosclerotic events in CKD patients (*Seiler et al., 2014*; *Sarmento-Dias et al., 2016*). A recent meta-analysis of prospective studies concluded that the relationship between FGF23 and cardiovascular disease risk is unlikely to be causal (*Marthi et al., 2018*). An established line of thought is that increased FGF23 may follow, as opposed to initiate, cardiovascular dysregulation (*Stohr et al., 2018*). It is likely that negative effects of high FGF23 are due to dysregulated phosphate metabolism. However, the research surrounding FGF23 is constantly evolving as the investigation into α-klotho dependent and independent effects continues, as well as the potential effects of locally produced FGF23 within the cardiovascular system. FGF23 levels have also been associated with inflammation in CKD patients and in other inflammatory disorders (*David, Francis & Babitt, 2017*). The following section reviews the direct effects of exogenously added FGF23 on vascular cells in order to provide insight into the possible bone-derived effects as FGF23 travels through the circulation to reach its canonical target organs (see Table 2).

FGF23 in human and animal cells

In recent years, literature has emerged directed at elucidating the direct effects of FGF23 in the vasculature. In human aortic smooth muscle cells (HASMCs), 10 ng/mL of exogenous FGF23 has been shown to increase phosphorylation of ERK (extracellular signal-regulated kinase) after 10 min in serum-starved cells, which in turn increased production of Early Growth Response Protein-1 (EGR-1) (*Nakahara et al., 2016*). Also in human VSMCs, 10 ng/mL of FGF23 increased hydrogen peroxide production and did not induce nitric oxide (NO) production on its own, however when soluble klotho and phosphate were also added, NO was increased (*Six et al., 2014*). In the context of calcification, 5 ng/mL of

Table 2 Summary of studies investigating *in vitro* effects of FGF23 on human and animal vascular cells.

Study	Cell type	Context	Concentration	Results	Conclusions
Lim et al. (2012)	Human ASMCs	Calcification	5 ng/mL	FGF23 stimulated proliferation and phosphorylation of ERK and AKT in a klotho dependent fashion. Pre-treatment with FGF23 and calcitriol followed by treatment with calcification media inhibited development of calcification, in a klotho dependent fashion.	Vitamin D receptor activators can restore Klotho expression and unmask FGF23 anti-calcific effects. Klotho is required for vascular FGF23 signalling.
Zhu et al. (2013)	Murine VSMCs	Calcification	10 and 50 ng/mL	FGF23 reduced calcium deposition and increased phosphorylation of ERK1/2 (but not AKT). Concomitant exposure to FGF-23 and an ERK1/2 inhibitor (PD98059; 10 μM) increased calcification.	FGF23 signalling is likely to be a protective mechanism in calcification.
Lindberg et al. (2013)	Bovine VSMCs	Calcification	0.125–2 ng/mL	FGF23 did not modify calcification.	No support for a role of FGF23 in vascular calcification.
Scialla et al. (2013)	Human VSMCs, murine VSMCs	Calcification	1, 2, 10, 20 and 50 ng/mL	FGF23 had no effect on phosphate uptake or phosphate-induced calcification even in the presence of soluble klotho. FGF23 did not induce phosphorylation of ERK or FRS2α.	No support for a role of FGF23 in vascular calcification
Silswal et al. (2014)	MurineAECs	Normal	9 ng/mL	FGF23 increased superoxide levels compared with vehicle which was inhibited by pre-treatment with tiron.	FGF23 may reduce vasorelaxation by decreasing NO.
Six et al. (2014)	Human VSMCs, HUVECs	Normal	10 ng/mL	H_2O_2 concentrations increased with FGF23 and klotho in HVSMCs but not in HUVECs. FGF-23 augmented H_20_2 concentration induced by phosphate. FGF23 did not increase NO production in HUVECs, but FGF23, klotho and phosphate together, did.	Klotho deficiency may be deleterious as appears protective against increased ROS production from FGF23/phosphate.
Nakahara et al. (2016)	Human ASMCs	Normal	10 ng/mL	FGF23 increased phosphorylated ERK, which subsequently increased EGR-1 expression.	FGF23 increased phosphorylated ERK expression and EGR-1 in HASMCs.

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Table 2 (*continued*)

Study	Cell type	Context	Concentration	Results	Conclusions
Richter et al. (2016)	Human CAECs	Normal	10 ng/mL	FGF23 increased activation of FGFR1. Klotho levels were unaffected. FGF23 enhanced Klotho release and increased levels of ADAM17. FGF23 increased NO through Akt and eNOS. Cytosolic ROS increased via Nox2 and ROS detoxification via SOD2 and CAT. Blocking Klotho resulted in enhanced ROS formation and reduced NO availability.	Excess FGF23 may promote oxidative stress and endothelial dysfunction.
Chung et al. (2017)	Human AECs and Human BMECs	Normal	0.5–100 ng/mL	FGF23 stimulated cell proliferation, eNOS, and NO production in AECs. High phosphate mitigated these effects which could not be rescued by soluble Klotho. Adding soluble α-Klotho following α-Klotho knockdown also did not rescue EC resistance to FGF23. None of these effects were observed in HBMECs.	FGF23 promoted proliferation and phosphorylation of eNOS. AECs were only responsive to FGF23 in the presence of α-Klotho synthesized by ECs. HBMECs did not respond to FGF23.
Verkaik et al. (2018)	Human MVECs	Normal	Approx. 3.2 ng/mL	30 min treatment with FGF23 did not increase ERK phosphorylation.	FGF23 did not induce signal transduction through ERK.

Notes.

Abbreviations: FGF-23, fibroblast growth factor 23; VSMCs, vascular smooth muscle cells; AECs, aortic endothelial cells; ASMCs, aortic smooth muscle cells; HUVECs, human umbilical vein endothelial cells; OPG, osteoprotogerin; MVECs, microvascular endothelial cells; BMECs, brain microvascular endothelial cells; CAECs, coronary artery endothelial cells; FRS2α, FGF receptor substrate 2α; EGR-1, early growth response protein-1; NO, nitric oxide; ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; ROS, reactive oxygen species; Nox2, NADPH oxidase 2; SOD2, superoxide dismutase 2; CAT, catalase; FGFR1, fibroblast growth factor receptor 1.

FGF23 increased HASMC proliferation after 24 h in cells cultured in calcification media, and increased phosphorylation of ERK and AKT in a klotho-dependent manner (*Lim et al., 2012*). 24 h pre-treatment with FGF23 and calcitriol, followed by culture in calcification media, deterred calcification in HASMCs in a klotho dependent fashion, but this effect was not seen with FGF23 alone (without calcitriol) (*Lim et al., 2012*). *Scialla et al. (2013)* did not find any evidence for a pro-calcific effect of FGF23 in HASMCs (1–50 ng/mL), nor an increase in ERK phosphorylation (*Scialla et al., 2013*). Reflecting results shown in human VSMCs, FGF23 alone (10 and 50 ng/mL) reduced calcium deposition in the context of calcification in murine VSMCs through ERK signalling (*Zhu et al., 2013*), whereas in bovine VSMCs 0.2–2.0 ng/mL FGF23 did not modify calcification (*Lindberg et al., 2013*). In a study with murine VSMCs no effect of FGF23 on calcification was observed (1–50 ng/mL) (*Scialla et al., 2013*).

In microvascular endothelial cells, FGF23 did not increase ERK phosphorylation, nor in human umbilical vein endothelial cells did FGF23 increase hydrogen peroxide production (*Six et al., 2014*; *Verkaik et al., 2018*). In HAECs, FGF23 (50 and 100 ng/mL) increased proliferation after 48 h, while 10 ng/mL stimulated phosphorylated eNOS and NO production (*Chung et al., 2017*). These results were no longer observed following α-klotho knockdown and could not be rescued by addition of soluble klotho, indicating

that these effects were dependent on localised endogenous α-klotho production in HAECs. Interestingly, no increase in proliferation, nor eNOS phosphorylation or NO production were observed in the same conditions in human brain microvascular cells (*Chung et al., 2017*). In coronary artery endothelial cells (HCAECs), *Richter et al. (2016)* demonstrated that 10 ng/mL of FGF23 increased expression of the FGF receptor-1, increased eNOS phosphorylation and NO in an Akt-dependent manner, increased ROS production and ROS detoxification, and additionally increased secretion of soluble klotho via upregulation of ADAM17 (*Richter et al., 2016*). In blocking klotho, the authors further demonstrated an upregulation of ROS and decrease in NO. In murine aortic endothelial cells, 9 ng/mL FGF23 significantly increased superoxide levels (*Silswal et al., 2014*).

These collated results begin to untie whether FGF23 may have any causal effects within the context of cardiovascular pathologies. A few conclusions can be made including: FGF23 initiates direct effects on vascular cells including SMCs and ECs but actions may be tissue specific; FGF23 appears to increase eNOS phosphorylation and NO production likely in an Akt dependent manner, and predominantly in a klotho-dependent manner (perhaps restricted to locally produced klotho and not systemic circulating klotho); however, FGF23 also appears to increase ROS and oxidative stress, which is counteracted by klotho; within calcification models, FGF23 increased proliferation of SMCs, but does not appear to increase calcification and indeed may be anti-calcific. Bringing these results together paints a conflicting but potential positive role of FGF23 in vascular function. The results presented encourages the hypothesis that the associations and correlations between FGF23 and CVD are most likely due to deleterious effects of altered phosphate and mineral metabolism and indeed that FGF23 levels likely follow, as oppose to direct, cardiovascular disturbances. It is a complex area, with further discrepancies such as variable physiological and disease states, deficiency of klotho, origin of secretion of FGF23 and klotho, and many more factors leaving the role of FGF23 in the vasculature not a simple question to be answered. Further confirmative examinations are necessary and investigations into whether N- and C-terminal fragments of FGF23 are additionally biologically active and if so, how they differ from intact FGF23. Interestingly, any direct pro- or anti-inflammatory properties of FGF23 on vascular cells have not yet been explored despite accumulating evidence in other tissues for a role of FGF23 in inflammation (*David, Francis & Babitt, 2017*).

LIPOCALIN-2

Lipocalin-2 (LCN-2), also referred to as neutrophil gelatinase-associated lipocalin (NGAL) or 24p3, is the most recently identified osteokine. However, it is debatable whether LCN-2 can be strictly described as such as it is secreted by a wide variety of cell types, and has indeed been more widely described as an adipokine to date. Despite this, it has recently been reported to be produced in bone ten-fold more than in white fat, at least in mice, and it remains to be reported whether this is also the case in humans (*Mosialou et al., 2017*). As such, LCN-2 is predominantly an osteoblast and adipocyte derived 25 kDa secreted glycoprotein which acts as a lipid chaperone positioning itself as a key pro-inflammatory link between obesity and associated metabolic disorders and vascular disease (*Kjeldsen*

et al., 1993; *Wang, 2012*). It exists *in vivo* as a monomer, homodimer, or heterodimer by forming a complex with matrix metalloproteinase-9 (MMP-9) which stabilizes MMP-9 by preventing autodegradation (*Kjeldsen et al., 1993*; *Yan et al., 2001*; *Chakraborty et al., 2012*). LCN-2 has been identified to mediate an innate immune response to bacteria by sequestering iron and induces apoptosis in many cell types (*Devireddy et al., 2001*; *Flo et al., 2004*).

Circulating concentrations of LCN-2

LCN-2 is increased in obese individuals, which is presumed derived from increased adipose tissue and also increased expression from the liver, but may also be due to changes in skeletal homeostasis which is also altered in obesity (*Wang et al., 2007*; *Luo et al., 2016*). It has been suggested that LCN-2 can be used as a biomarker for early renal injury (*Mishra et al., 2004*; *Mishra et al., 2005*). For example, in children undergoing surgery, those who did not subsequently develop acute renal injury had LCN-2 serum concentrations <50 ng/mL, but 50% of those who did develop acute renal injury has concentrations >50 ng/mL (*Mishra et al., 2005*). In a study of 1,203 Chinese obese and non-obese men, LCN-2 serum concentrations were in the range 29.3–53.5 ng/mL (*Luo et al., 2016*). In patients with Type 2 Diabetes, serum levels were significantly higher in those with subclinical atherosclerosis than those without (112.9 ng/mL versus 77.2 ng/mL) (*Xiao et al., 2013*). LCN-2 serum concentrations are also higher in patients with metabolic syndrome compared to those without (83.2 ng/mL versus 67.5 ng/mL) (*Wang et al., 2007*).

Lipocalcin-2 and the vasculature

LCN-2 is a novel, debatable osteokine. Examining its effects on vascular cells when originating from osteoblasts presents a difficult task, requiring for example tissue specific knock-down studies. Further validation to characterise LCN-2 as an osteokine with more convincing human data is primarily needed. As LCN-2 has been associated with a stage-dependent contribution to atherosclerosis, promoting lipotoxicity within the vasculature in obese states, and causing endothelial dysfunction and cardiovascular complications, it is important to establish whether bone-derived LCN-2 is a key mediator that could be therapeutically targeted (*Wang, 2012*; *Amersfoort et al., 2018*). LCN-2 is also proposed as a sensitive marker for cardio-renal disease in patients with acute heart failure (*Mishra et al., 2005*; *Alvelos et al., 2011*).

Most studies to date have focused on observational and epidemiological data on the involvement of LCN-2 in obesity and diabetic associated vascular complications. It is currently unfounded to speculate whether these effects are influenced by bone derived LCN-2. The limited investigations on the direct exogenous addition of this interesting protein in vascular cells are summarised below and in Table 3.

LCN-2 in human and animal cells

Wang and colleagues, interested in investigating pulmonary hypertension, demonstrated that in human pulmonary artery smooth muscle cells (HPASMCs), LCN-2 at 10 and 20 ng/mL increased proliferation after 24 hrs (*Wang et al., 2015*). LCN-2 promoted the activity of the PI3-K pathway by increasing Akt phosphorylation (3–30 ng/mL) after 24

Table 3 Summary of studies investigating *in vitro* effects of LCN-2 on human and animal vascular cells.

Study	Cell type	Concentration	Results	Conclusions
Lee et al. (2011)	Rat BMVECs	10,000 ng/mL	LCN-2 increased expression of chemokine CXCL10	LCN-2 may promote chemoattractants and neuroinflammation
Wang et al. (2014)	Human PASMCs	10 ng/mL3–100 ng/mL	Decreased serum deprivation induced apoptosis and H_2O_2 induced apoptosis with LCN-2. LCN-2 decreased the cleavage and activity of caspase-3, and expression of Bax (apoptotic protein). LCN-2 increased expression of SOD1/2 and decreased intracellular ROS.	LCN-2 appears protective against apoptosis and decreased ROS
Wu et al. (2015)	Rat BECs	500–2,000 ng/mL	Enhanced Matrigel tube formation with LCN-2 and migration of cells via iron and ROS related pathways	LCN-2 may contribute to neurovascular recovery
Wang et al. (2015)	Human PASMCs	3–30 ng/mL	LCN-2 increased proliferation, at least in part via PI3-K signalling pathway	LCN-2 increased HPASMC proliferation
Eilenberg et al. (2016)	HUVECs, Human CASMCs	200 ng/mL, 500 ng/mL or 1,000 ng/mL	Increased secretion of inflammatory markers IL-8, IL-6 and MCP-1 dose-dependently with LCN-2	LCN-2 may be pro-inflammatory
Wang et al. (2017)	Human PASMCs	10 ng/mL	LCN-2 increased ER stress and proliferation via increased intracellular iron levels	LCN-2 increased HPASMC proliferation and ER stress

Notes.

Abbreviations: LCN-2, lipocalin-2; BMVECs, brain microvascular endothelial cells; BECs, brain endothelial cells; HUVECs, human umbilical vein endothelial cells; CASMCs, coronary artery smooth muscle cells; PASMCs, pulmonary artery smooth muscle cells; ROS, reactive oxygen species; IL-8, interleukin-8; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; SOD1/2, superoxide dismutase 1/2; ER, endoplasmic reticulum.

hrs, which was abrogated by an Akt inhibitor (*Wang et al., 2015*). This inhibitor also partly prevented the LCN-2 induced increase in proliferation. Interestingly, this group previously showed in the same cell type that 10 ng/mL LCN-2 decreased serum deprivation induced apoptosis after 24 hrs compared to control, and furthermore decreased the susceptibility of HPASMCs to H_2O_2 induced apoptosis (*Wang et al., 2014*). They further showed that LCN-2 was decreasing the cleavage and activity of caspase-3, and decreasing expression of Bax, an important pro-apoptotic factor. Finally, they confirmed LCN-2 (3–100 ng/mL for 24 hrs) increased the expression of superoxide dismutase 1 and 2 (SOD1 and SOD2) dose-dependently and 10 ng/mL decreased intracellular reactive oxygen species (ROS) both with and without H_2O_2. This group went on to show that LCN-2 (10 ng/mL) in fact promoted endoplasmic reticulum (ER) stress and proliferation within HPASMCs via increased intracellular iron levels (*Wang et al., 2017*).

HUVECs and human coronary artery smooth muscle cells (HCASMCs) treated with LCN-2 (200 ng/mL, 500 ng/mL or 1μg/mL) significantly increased secretion of inflammatory markers interleukin-8 (IL-8), IL-6 and monocyte chemoattractant protein-1 (MCP-1) in a dose-dependent manner (*Eilenberg et al., 2016*). Within the central nervous

system, LCN-2 (10 μg/mL) was shown to increase mRNA expression of C-X-C motif chemokine 10 in mice brain microvascular endothelial cells (BMVECs), providing preliminary evidence that LCN-2 may act as a chemoattractant inducer and promote neuroinflammation (*Lee et al., 2011*). In another study in rat brain endothelial cells, LCN-2 (0.5–2 μg/mL) enhanced angiogenesis, shown through Matrigel tube formation and scratch migration, via iron and ROS related pathways (*Wu et al., 2015*).

Despite the clinical associations between LCN-2 and vascular disturbances, the mechanisms at a cellular level by physiological concentrations of exogenous LCN-2 have been largely disregarded and research is warranted. The investigations to date have largely focused on LCN-2 in the context of pulmonary hypertension and the use of HPASMCs. Within this cell type LCN-2 is shown to have anti-apoptotic and pro-proliferative effects, while also decreasing ROS but promoting ER stress. It has also been demonstrated that LCN-2 may be pro-inflammatory in a number of cells types, which may be viewed as beneficial in the acute inflammatory response (*Castellheim et al., 2009*). Concentrations of LCN-2 used in the studies reviewed have been quite varied (3 ng/mL –10 μg/mL). It is clear there is a large scope of research yet to be completed in this area to inform researchers and clinicians about any potential contribution of LCN-2 in vascular physiological and pathophysiology.

SUMMARY AND CONCLUSIONS

The effects of bone-derived factors on systemic health is an emerging, interesting branch of endocrinology research. As conflicting epidemiological reports have linked these bone-derived factors with multiple vascular pathologies such as atherosclerosis and calcification, there is interest in their therapeutic target potential. In order to derive any causal effects, it is important to review the basic *in vitro* science that has been carried out to date. Thus, this review aimed to collate studies investigating the *in vitro* effects of OCN, FGF23 and LCN-2 to clarify whether there is support for a basic biological or pathological role within the vasculature. Overall, the evidence base points to direct vasoactive properties of the osteokines OCN, FGF23 and LCN-2 across human and animal cells. Both OCN and FGF23 have been demonstrated to be anti-apoptotic and increase eNOS phosphorylation and NO production through Akt signalling. Taken together, these findings suggest a role for these osteokines in supporting normal vascular functionality. OCN has been implicated in improving insulin signalling, and ensuing protectiveness against ER stress. OCN may be involved in calcification but further research is warranted, while there is no evidence at least of a pro-calcific effect of FGF23 *in vitro*. FGF23 and LCN-2 have been shown to increase proliferation in some cell types and increase and decrease ROS respectively. LCN-2 has been shown to have anti-apoptotic effects and may increase ER stress as well as have pro-inflammatory properties. LCN-2 has also been demonstrated to promote angiogenesis. There is no strong evidence to suggest a pathological role of OCN or FGF23 in the vasculature, however further research with LCN-2 needs to be completed in order to better understand its role. Despite the topical area, a number of preliminary questions remain to be answered. For example, the role of OCN and FGF-23 in inflammation, the

role of OCN and LCN-2 in calcification, as well as their cellular receptors and related downstream signalling pathways in vascular cells. Various cleaved forms of the circulating proteins should also be investigated to assess their biological activity. It is clear that further, human data should be generated to understand the roles and mechanisms of actions of these osteokines in physiological and pathophysiological states.

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The authors declare there are no competing interests.

Author Contributions

- Sophie A. Millar conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Susan I. Anderson and Saoirse E. O'Sullivan authored or reviewed drafts of the paper, approved the final draft.

Data Availability

The following information was supplied regarding data availability:
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5. Osteocalcin, vascular calcification, and atherosclerosis: a systematic review and meta-analysis

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SYSTEMATIC REVIEW
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Osteocalcin, Vascular Calcification, and Atherosclerosis: A Systematic Review and Meta-analysis

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Background: Osteocalcin (OC) is an intriguing hormone, concomitantly being the most abundant non-collagenous peptide found in the mineralized matrix of bone, and expanding the endocrine function of the skeleton with far-reaching extra-osseous effects. A new line of enquiry between OC and vascular calcification has emerged in response to observations that the mechanism of vascular calcification resembles that of bone mineralisation. To date, studies have reported mixed results. This systematic review and meta-analysis aimed to identify any association between OC and vascular calcification and atherosclerosis.

Methods and results: Databases were searched for original, peer reviewed human studies. A total of 1,453 articles were retrieved, of which 46 met the eligibility criteria. Overall 26 positive, 17 negative, and 29 neutral relationships were reported for assessments between OC (either concentration in blood, presence of OC-positive cells, or histological staining for OC) and extent of calcification or atherosclerosis. Studies that measured OC-positive cells or histological staining for OC reported positive relationships (11 studies). A higher percentage of Asian studies found a negative relationship (36%) in contrast to European studies (6%). Studies examining carboxylated and undercarboxylated forms of OC in the blood failed to report consistent results. The meta-analysis found no significant difference between OC concentration in the blood between patients with "atherosclerosis" and control ($p = 0.13$, $n = 1{,}197$).

Conclusion: No definitive association was determined between OC and vascular calcification or atherosclerosis; however, the presence of OC-positive cells and histological staining had a consistent positive correlation with calcification or atherosclerosis. The review highlighted several themes, which may influence OC within differing populations leading to inconclusive results. Large, longitudinal studies are required to further current understanding of the clinical relevance of OC in vascular calcification and atherosclerosis.

Keywords: osteocalcin, calcification, atherosclerosis, bone hormone, vascular disease, bone glutamic acid protein

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INTRODUCTION

Vascular calcification is a known major risk factor for mortality and morbidity and is an independent risk factor for cardiovascular disease (1–3). Vascular calcification, long believed to be a passive part of aging and "wear and tear," is now considered an active, cell-mediated complex process that is regulated but not yet fully understood. Osteocalcin (OC) [also known as bone glutamic acid protein (BGLAP)] is an intriguing hormone produced by osteoblasts in bone that has been recently linked with an increasing number of extra-osseous biological roles and effects (4–8). One candidate thread of enquiry is its interaction with the vascular system, and its putative role in the process of vascular calcification or atherosclerosis. OC is not only produced by bone but is expressed by vascular smooth muscle cells (VSMCs) displaying an osteoblast-like phenotype (9).

Osteocalcin is the most abundant, non-collagenous component in the mineralized matrix of bone (10). The presence of three glutamic acid (Gla) residues allows for posttranslational γ-carboxylation at positions 17, 21, and 24. Between 60 and 90% of carboxylated OC (cOC) is deposited in the bone matrix; however, it can also be released into the circulation (11). OC can be undercarboxylated (ucOC) to differing degrees (from 0 to 2 carboxyl groups) due to decarboxylation, low activity of the vitamin K-dependent carboxylase enzyme, or vitamin K deficiency. ucOC has less affinity to hydroxyapatite and is more readily released into the circulation than cOC (12, 13).

ucOC has recently been appointed a predictor and potential therapeutic target of a number of diseases including diabetes and is believed to be the active form of OC (14). Studies have shown ucOC to be a regulator of pancreatic β cell and adipocyte gene expression, glucose metabolism and to increase insulin sensitivity in humans (14). Structural inconsistencies between ucOC and cOC have been explored, but discrepancies in reports are numerous and it is unknown the extent to which structural differences may play in their biological functions (15). It is hypothesized that ucOC may be the active form of OC involved in vascular calcification, but this has yet to be investigated.

Having established roles of other Gla containing proteins, such as Matrix Gla protein, in vascular calcification, many researchers have begun to explore the role of OC. Idelevich et al. investigated the effects of OC overexpressing mice cell lines (chondrocytes and VSMCs) (16). They showed that OC stimulates VSMC mineralization and differentiation, in particular through HIF-1α activation, surmising that OC fuels glucose utilization in VSMCs and promotes osteochondrogenic differentiation resulting in calcification. However, further studies are greatly lacking.

The aim of this systematic review and meta-analysis was to investigate and critically appraise the available literature linking OC to calcification and atherosclerosis in humans.

METHODS

Search Strategy

The systematic review was carried out in accordance with the Meta-analysis Of Observational Studies in Epidemiology group proposal for reporting (17). A systematic and comprehensive search of PubMed and EMBASE (including Medline) was conducted to extract all articles examining an association between OC and vascular calcification or atherosclerosis. Identical search terms were used for both databases and included: "Osteocalcin AND Vascular Calcification," "Osteocalcin AND Atherosclerosis," "Osteocalcin AND Arterial Stiffness," "Bone Gla Protein AND Vascular Calcification," "Bone Gla Protein AND Atherosclerosis," "Bone Gla Protein AND Arterial Stiffness," "BGLAP AND Vascular Calcification," "BGLAP AND Atherosclerosis," "BGLAP AND Arterial Stiffness," "Bone gamma-carboxyglutamic acid protein AND Vascular Calcification," "Bone gamma-carboxyglutamic acid protein AND Atherosclerosis," "Bone gamma-carboxyglutamic acid protein AND Arterial Stiffness," "BGP AND Vascular Calcification," "BGP AND Atherosclerosis," "BGP AND Arterial Stiffness." The searches were limited to include only human studies; with no restrictions on publication year, language, population or article type. Articles were subsequently excluded if the full text could not be found in English ($n = 4$). The searches were carried out by the 25/05/2017 with no year restrictions.

Eligibility Criteria

The titles and abstracts for returned items were examined, and inappropriate articles were rejected. The criteria for inclusion was such that the article was an original, peer reviewed paper involving either longitudinal or cross-sectional human studies that investigated the relationship between OC and calcification or atherosclerosis. A further requirement of each study was that a form of OC must have been measured within their sample population. An endpoint relating specifically to the degree or severity of calcification or atherosclerosis was required to have been measured and reported. Studies that used assumptions of an increased risk of cardiovascular disease (CVD), e.g., by age and weight within their sample populations were excluded. All searches were conducted independently by two reviewers and compared. Where differing opinions on study eligibility existed ($n = 6$), they were discussed with the study principle investigator.

Data Extraction and Analysis

The included articles were analyzed, and data were collated using an extraction form. The extracted data included the following: the population characteristics (age, sample size, ethnicity, and health status); type and method of OC measured; endpoint measurements; results of outcome and exposure measures; and the overall conclusions of the article and any key limitations or bias. A risk of bias assessment was performed according to the Cochrane Collaboration's tool for assessing risk of bias (18).

Statistical Analysis

A meta-analysis was performed on those studies that provided OC concentration in blood samples from an "increased vascular calcification/atherosclerosis group" and from a "control/healthy group." Data were analyzed as forest plots using the Cochrane Review Manager software (Version 5.3. Copenhagen: The Nordic

Cochrane Centre, The Cochrane Collaboration, 2014) and as funnel plots using Stata (StataCorp., 2009. Stat Statistical Software: Release 11. College Station, TX, USA). Funnel plot asymmetry (publication bias) was tested by Egger's test (19). Since heterogeneity was expected between study protocols (different population characteristics, different methods of defining calcification or atherosclerosis, different specificity of assessment methods of OC) random-effect models were used. The results of continuous data of OC concentration are expressed as mean and SD. Where studies did not provide mean and SD, authors were contacted for data. In cases where no response from authors was obtained ($n = 8$), they could not be included in the statistical analysis (20–27). One author declined to supply requested information (28). Studies were weighted by sample size, and statistical significance was set at $p < 0.05$. The results are expressed as mean difference as all studies reported OC concentrations in the same units. It was not possible to perform further analyses due to large heterogeneity between studies.

RESULTS

The initial search yielded 1,453 records from which 374 abstracts were reviewed and 46 articles met the inclusion criteria (**Figure 1**). A description of each study is provided in **Table 1**. Of the 46 studies included in this review, 26 (56%) were designed specifically to examine the relationship between OC and markers of calcification or atherosclerosis (20, 22, 24–27, 29–47). The other 20 studies evaluated OC among a number of measurements, as a covariate, or in secondary analyses (21, 23, 28, 48–61). Forty-four out of the 46 studies were cross-sectional in design. Twenty-four studies did not make adjustments for any potential confounding variables, and 22 conducted multivariate analyses (**Table 1**). Ten studies had a sample size greater than 300 (20, 24, 25, 27, 30, 33, 35, 43, 45, 62).

Thirty-three studies (72%) measured OC by enzyme-linked immunosorbent assay, electrogenerated chemiluminescence, or radioimmunoassay, while the remaining studies used flow

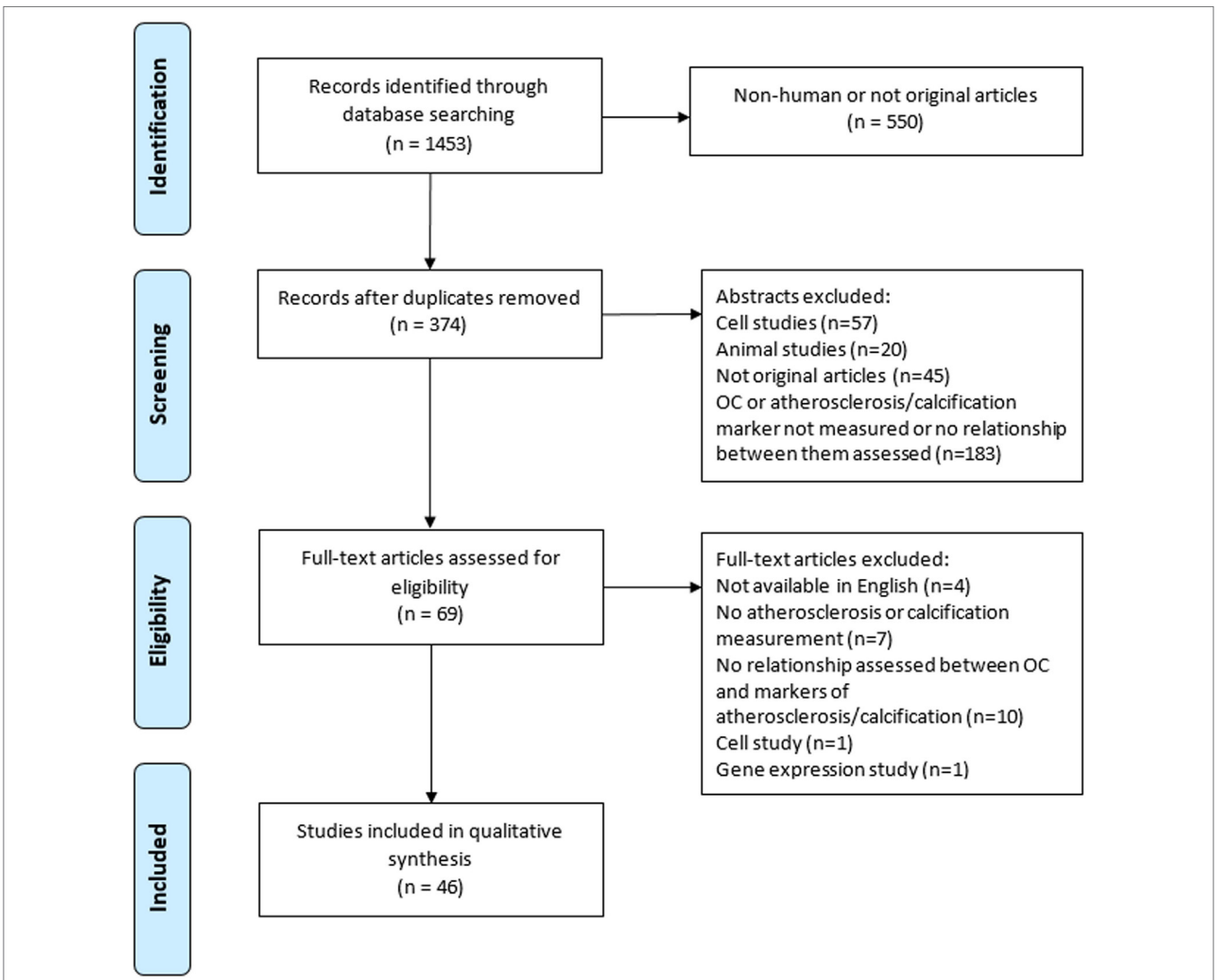

FIGURE 1 | Flow chart for study retrieval and selection. Abbreviation: OC, osteocalcin.

TABLE 1 | Summary of included studies.

Reference	*n*	Sex	Location	Inclusion criteria	OC measurement	OC type	Calcification or atherosclerosis measurement	Study outcomes	Adjustments
Levy et al. (46)	38	M, F	USA	Autopsy samples	RIA	Total	CS	OC was present in all calcified aortic tissue and heart valves and was either not detectable or present at very low levels in non-mineralized lesions and normal tissue	None
Jie et al. (36)	256	F	Netherlands	>55 years old	RIA	Total, free, and bound	CS	None	Age
Watson et al. (49)	173	M, F	USA	High and moderate risk for CHD	RIA	Total	CS	None	None
Bini et al. (57)	22	Not specified	USA	Human carotid endarterectomy specimens	Immunostaining	Total	CS	OC positively associated with calcification, progressing from type V to type VI lesions	None
Montalcini et al. (50)	157	F	Italy	Postmenopausal women aged 45–75 years	RIA	Total	C-IMT	Positive relationship between OC and carotid atherosclerosis prevalence in those with low BMD	Age, systemic hypertension, hyperlipidemia, DM, obesity, smoking
Iba et al. (56)	135	F	Japan	Postmenopausal osteoporotic women	ELISA	Total	ACS	None	None
Rajamannan et al. (59)	58	M, F	USA	T2DM, >18 years old, cardiac valve surgery	Immunostaining	Total	CS	OC expression was upregulated in the calcified rheumatic valves and was present at low levels in the degenerative mitral valves	None
Göossl et al. (37)	72	M, F	USA	Coronary atherosclerosis patients	Flow cytometry	OCN+ EPCs	CA and endothelial function	Positive relationship between OC+ cells and stage of coronary atherosclerosis	None
Kanazawa et al. (35)	328	M, F	Japan	T2DM	RIA	Total	PWV and C-IMT	Negative correlation between OC and PWV and C-IMT in men only	None
Pal et al. (40)	23	M, F	Australia	Peripheral artery disease	Flow cytometry	OC + MNCs	ACS	Positive relationship between OC+ cells and aortic calcification	None
Foresta et al. (38)	35	M	Italy	Erectile dysfunction patients	Flow cytometry	OC + EPCs	C-IMT	Positive relationship between OC+ cells and IMT	None
Zhang et al. (25)	461	M, F	China	Chest pain, heaviness, periodic discomfort, and palpitations	ELISA	Total	CA	OC was significantly higher in those with 0 diseased vessel than in those with 1,2, or ≥3 diseased vessels	None
Parker et al. (27)	363	F	USA	≥65 years old	ELISA	Total	ACS	None	Age, CVD risk factors, BMD, mineral metabolism, estrogen use, kidney function, vitamin D, PTH, and BSAP
Okura et al. (31)	92	M, F	Japan	Essential hypertension	ELISA	ucOC	C-IMT	Positive relationship between ucOC levels and calcification	None
Awan et al. (55)	19	M, F	Canada	Familial hypercholesterolemia	ELISA	Total	CACS	Negative correlations between ACS and OC	None

(*Continued*)

TABLE 1 | Continued

Reference	*n*	Sex	Location	Inclusion criteria	OC measurement	OC type	Calcification or atherosclerosis measurement	Study outcomes	Adjustments
Bao et al. (26)	181	M	China	MS, CA	ELISA	Total	CA	Negative relationship between OC and number of stenotic vessels in subgroup analysis with NGTnormal glucose tolerance (n = 60)	None
Pirro et al. (61)	120	F	Italy	Newly diagnosed, never-treated postmenopausal osteoporosis	FACS	OCN+ OPCs	PWV	Moderate positive correlation between AoPWV and OC+ cells	Age, smoking status, waist circumference, SBP (or alternatively MABP), heart rate, glucose, cholesterol, TG, PTH, osteoporotic status, and the log-transformed count of CD34+/AP+ cells
Kanazawa et al. (47)	50	M, F	Japan	T2DM	RIA	Total	C-IMT	Positive correlation of baseline plaque score with changes in OC,[a] and negative correlation of changes in OC with changes in plaque score[b]	[a,b]
Reyes-Garcia et al. (34)	78	M, F	Spain	T2DM	RIA	Total	C-IMT	Positive association between OC and IMT, carotid plaques, and aortic calcifications, in women only	None
Kim et al. (30)	769	F	Korea	Women	ELISA	Total	ACS	Negative relationship between OC and ACS	Age
Ogawa-FuruyaOgawa et al. (29)	218	M, F	Japan	T2DM	RIA	Total, ucOC	ACS	Negative association between both serum OC and ucOC concentrations and an ACS of 3 and greater, in men only	Age, BMI, serum creatinine and LDL-c, radial BMD, smoking, duration of DM, HbA1c, and HOMA-IR
Janda et al. (48)	67	M, F	Poland	>18 years old, PD ≥2 months, negative history of neoplastic diseases	ELISA	Total	C-IMT	OC positively associated with C-IMT	Age and major CVD risk factors
Sheng et al. (33)	817	M, F	China	>50 years old, T2DM	RIA	Total	C-IMT and plaques	Negative association between OC, carotid plaques,[c] and C-IMT[d]	[c,d]
Janda et al. (23)	59	M, F	Poland	ESRD	ELISA	Total	CS	None	Low HDL-c (<1.0 mmol/L in men, <1.3 mmol/L in women), high TG (>1.7 mmol/L), and high BMI (≥25 kg/m^2), as well as hypertension, CRP, gender, dialysis status of patients, and Ca × Pi
Foresta et al. (39)	3	M	Italy	Carotid endarterectomy	Immunostaining	Total	CS	Positive relationship observed between OC and extent of calcification in lesions/ necrotic core (no OC detected in corresponding healthy portions of carotid wall specimens)	None

(*Continued*)

TABLE 1 | Continued

Reference	*n*	Sex	Location	Inclusion criteria	OC measurement	OC type	Calcification or atherosclerosis measurement	Study outcomes	Adjustments
Yang et al. (20)	1,319	F	China	Postmenopausal women	ELISA	Total	C-IMT	Negative correlation between OC and C-IMT	Age, years since menopause, BMI, waist circumference, SBP, DBP, homeostasis model assessment, insulin resistance, TG, HDL-c, CRP, smoking, antidiabetic therapy, antihypertensive therapy, lipid lowering therapy, and family history of CVD
O'Neill and Adams (58)	19	F	USA	Mastectomy, partial mastectomy, or lumpectomy patients and a diagnosis of ESRD or CKD	Immunostaining	Total	CS	OC positively related with more heavily calcified arteries and appeared to coincide with calcium deposits	None
Ishimura et al. (28)	167	M	Japan	Stable HD for >3 months	RIA	Total	CS	None	None
Dweck et al. (54)	30	M, F	UK	Valve replacement surgery or asymptomatic disease under surveillance	Immunostaining	Total	18F-NaF	Positive relationship between aortic valve 18F-NaF uptake and OC	None
Krzanowski et al. (51)	57	M, F	Poland	>18 years old, stable dialysis ≥2 months, negative history of malignant disease, and lack of active viral infection	ELISA	Total	PWV	Negative relationship between OC and PWV	Age, hypertension, MABP, AoPWV evaluation, hypertension, ln (dialysis therapy duration), dialysis fluid exchange method, and Ca Pi index
Ma et al. (24)	1,077	M	China	Males with and without NGT	ELISA	Total	C-IMT and plaques	Negative relationship between OC and carotid plaque in subgroup analysis with men with NGT (n = 638). No associations with C-IMT	Age, BMI, WHR, FBG, PPG, SBP, DBP, TG, HDL-c, LDL-c, smoking, logHOMA-IR, and logHOMA-%B
Prats-Puig et al. (32)	203	M, F	Spain	5–10 years old, MS families, and no pubertal development	ELISA	Total, ucOC	C-IMT	ucOC positively associated with C-IMT in MS+ family offspring. Total OC not associated	Age, gender, BMI, fat mass, HOMA-IR, serum lipids, and CRP
Choi et al. (41)	162	M, F	Korea	Healthy adults	ECL and ELISA	OC, ucOC	CACS	Positive relationship between OC and CACS in men only. No significant findings for ucOC	Age, BMI, smoking, hypertension, diabetes, SBP, HOMA2-IR (log-transformed), TG (log-transformed), HDL-c, and lumbar BMD

(*Continued*)

TABLE 1 | Continued

Reference	*n*	Sex	Location	Inclusion criteria	OC measurement	OC type	Calcification or atherosclerosis measurement	Study outcomes	Adjustments
Zhang et al. (42)	224	M, F	China	CA	Flow cytometry	OC + EPCs	CACS	No correlation between OC+ cells with calcification in stable angina pectoris patients. In unstable angina pectoris and acute myocardial infarction patients, the number of spotty calcium deposits was significantly positively correlated with the absolute numbers of OC+ cells	None
Maser et al. (60)	50	M	USA	>18 years old, T2DM	ELISA	Total, ucOC	CACS	None	Age, duration of diabetes, HOMA-IR, BMI, gender, SBP, HbA1c, leptin, and adiponectin
Collin et al. (52)	23	M, F	USA	18–85 years old, early atherosclerosis	Flow cytometry	OC + MNCs	CA, endothelium dependent coronary vasoreactivity	Positive relationship between OC+ cells and extent of necrotic core and calcification	None
Luo et al. (45)	476	M, F	China	BMI ≥18.5 and <25.0 kg/m^2, NGT, normotensive, and normal lipid status	ELISA	Total	C-IMT	None	Age, BMI, W, SBP, DBP, FPG, serum fasting insulin, CRP, smoking status, and CVD family history
Zhang et al. (22)	290	M, F	China	Non-dialysis CKD patients	ELISA	ucOC	C-IMT	Negative relationship between ucOC and carotid plaques[e] and IMT[†]	[e,f]
Janda et al. (21)	59	M, F	Poland	HD and PD patients	ELISA	Total	C-IMT	No significant correlations	FBG, PTX3, FRS, and dialysis status
Yang et al. (53)	421	M, F	China	CA and echocardiography	ELISA	Total	Echocardiography	Positive relationship between OC and aortic valve stenosis	None
Ramirez-Sandoval et al. (63)	76	M, F	Mexico	PD patients ≥6 months; stable clinical course ≥3 months	Luminometry	Total	CS	None	None
Golovkin et al. (64)	112	M	Russia	CAD patients	ELISA	Total	CS	Levels of OC higher in patients with mild CS than those with severe calcification when assessed by Agatston score, but not Syntax score	None
Barbarash et al. (65)	112	M	Russia	Age ≤75 years; diagnosis of stable angina according to the Canadian Cardiovascular Society guidelines	ELISA	Total	CS	None	None

(*Continued*)

TABLE 1 | Continued

Reference	*n*	Sex	Location	Inclusion criteria	OC measurement	OC type	Calcification or atherosclerosis measurement	Study outcomes	Adjustments
Yun et al. (43)	3,604	M, F	Korea	Healthy adults	ECL	Total	PWV	OC level independently related to arterial stiffness; inverse J shape relationship. At low OC levels, the relationship was negatively linear. However, after controls for age and metabolic factors, the relationship with arterial stiffness at high levels of OC was not significant	Age, BMI, SBP, glucose, TV, eGFR, smoking, drinking, exercise, menopause, history of hypertension and diabetes, and total hip BMD
Kim et al. (44)	122	M	Korea	CABG	ELISA	cOC, ucOC	CACS	No significant differences in ucOCN or cOCN levels between groups divided according to CAC score	Age, BMI, T2DM status, hypertension, SBP, DBP, HbA1c, TC, creatinine, and statin therapy
Yang et al. (62)	593	M, F	USA	Patients undergoing CA because of known or suspected CAD	Flow cytometry	Total	CAS	OC+ early EPCs associated with an increase in levels[g] and risk[h] of higher degree of severity of CAS	[g,h]

M, male; F, female; T2DM, type 2 diabetes mellitus; RIA, radioimmunoassay; ucOC, undercarboxylated osteocalcin; ACS, aortic calcification score; ELISA, enzyme-linked immunosorbent assay; C-IMT, carotid intima-media thickness; MS, metabolic syndrome; PD, peritoneal dialysis; CA, coronary angiography; PWV, pulse wave velocity; CS, calcification score; OC, osteocalcin; EPC, endothelial progenitor cells; FACS, fluorescence activated cell sorting; MNCs, mononuclear cells; cOC, carboxylated osteocalcin; CACS, coronary artery calcification score; ECL, electrogenerated chemiluminescence; CABG, coronary artery bypass grafting; BMI, body mass index; CKD, chronic kidney disease; HD, hemodialysis; ESRD, end-stage renal disease; OPCs, osteoprogenitor cells; LDL-c, low density lipoprotein cholesterol; BMD, bone mineral density; HOMA-IR, homeostasis model assessment index for insulin resistance; CVD, cardiovascular disease; PTH, parathyroid hormone; BSAP, bone-specific alkaline phosphatase; CRP, C-reactive protein; HDL, high-density lipoprotein; CHD, coronary heart disease; NGT, normal glucose tolerance; WHR, waist-to-hip ratio; FBG, fasting blood glucose; PPG, oral glucose challenge; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; HOMA-%B, beta cell function; eGFR, estimated glomerular filtration rate; W, weight; MABP, mean arterial blood pressure; TC, total cholesterol; PTX3, pentraxin 3; FRS, Framingham Risk Score; CAD, coronary artery disease; CAS, coronary artery stenosis.

[a]Duration of diabetes and Brinkman index.

[b]Age, duration of diabetes, gender, BMI, Brinkman index, SBP, serum creatinine, LDL-c, HDL-c, TG, and HbA1c.

[c]Age, gender, smoking, alcohol intake, duration of diabetes, BMI, waist circumference, SBP, DBP, HOMA-IR, CRP, FBG, serum creatinine, serum urea, serum cholesterol, TG, HDL-c, and LDL-c.

[d]Age, gender HbA1c, HOMA-IR, and serum CRP.

[e]Age, sex, BMI, smoking history, MABP, eGFR, therapeutic medication use, FBG, TC, TG, LDL-c, HDL-c, and hs-CRP levels.

[f]Age.

[g]None.

[h]Age, sex, hypertension, diabetes, hypercholesterolemia, smoking, obesity, and family history of premature CAD.

cytometry or fluorescence activated cell sorting methods, or examined OC by histological immunostaining (**Table 1**). OC was measured using luminometry in one study (63). ucOC was measured in five studies (22, 31, 32, 41, 44), cOC in one study (44), and total OC was measured in the remaining studies (**Table 1**). OC positive mononuclear cells, endothelial progenitor cells (EPCs), or osteoprogenitor cells (OPCs) were examined by seven studies (37, 38, 40, 42, 52, 61, 62).

Methods of calcification or atherosclerosis measurements used in OC analyses varied and ranged from calcification scoring methods ($n = 22$), intima-media thickness measurements ($n = 14$), pulse wave velocity (PWV) measurements ($n = 4$), plaque presence ($n = 2$), and coronary angiography or echocardiography ($n = 6$; **Table 1**). One study used 18F-Sodium Fluoride uptake as a marker of calcification (54).

Risk of Bias Assessment

Results of the risk of bias assessment for all 46 studies are presented in **Figure 2**. Due to the majority of studies being cross-sectional in design and limitations on sample sizes, only one study randomly selected participants. Since all the included studies were observational cohort studies, no risk of bias assessment for "Allocation concealment" could be performed. Forty-six percent of studies included a component of blinding. None of the studies were reported with high risk of attrition bias or other bias. Four studies were reported with high risk of reporting bias. Overall, most information was from studies at low risk of bias.

Relationship between OC and Markers of Atherosclerosis or Calcification

Results of the meta-analysis examining OC concentrations between groups with normal vascular parameters and those presenting with markers of calcification/atherosclerosis are detailed in **Figure 3**. There was no significant overall difference between OC concentration (total, ucOC, or cOC) in patients with "atherosclerosis" and control, though a trend toward lower OC concentrations was seen in the control group [overall mean difference 0.93 ng/mL (95% CI −0.28, 2.15), $p = 0.13$]. There was significant statistical heterogeneity, I^2 88%, $p < 0.00001$. Egger's test showed no publication bias present ($p = 0.279$, **Figure 4**).

Due to multiple end points and assessments within studies, results are reported in terms of total outcomes to avoid bias reporting of overall positive, negative or neutral findings per study in the semiquantitative analysis. Among the studies, the relationship between OC and markers of atherosclerosis or calcification was reported as positive for 26 outcomes and negative for 17 outcomes, while no relationship was established for 29 outcomes.

No significant relationship was established for cOC, which was measured by only one study (44). In seven studies that measured ucOC and markers of atherosclerosis or calcification, two positive outcomes were reported (31, 32), three negative outcomes (22, 29), and four non-significant outcomes (41, 44, 60). Fifty-four percent of studies did not adjust for any confounding variables (**Table 1**). Of these, 17 positive outcomes between OC and markers of atherosclerosis or calcification were reported, 5 negative outcomes, and 12 non-significant outcomes. Within the other 22 studies adjusting for age and/or other confounding variables including CVD risk factors, 8 positive outcomes were reported between OC and markers of atherosclerosis or calcification, 11 negative outcomes were reported, and 17 outcomes were non-significant.

All 13 studies measuring OC positive mononuclear cells, EPCs, or OPCs, or histological staining for OC, reported a positive relationship between OC and markers of atherosclerosis.

A number of different outcomes were reported within the same studies, depending on gender, type of OC measured, or type of calcification or atherosclerosis measurement. Ogawa-Furuya et al. (29) and Kanazawa et al. (35) found a negative association between OC and markers of atherosclerosis within men but no significant association in women (29, 35). Prats-Puig et al. (32) found a positive association with ucOC, but no significant association was reported for total OC (32). Reyes-Garcia et al. (34) found a positive association in women only. Ma et al. (24) found an association between OC and plaque scores within men with normal glucose tolerance, but no association was reported with C-IMT (24). Choi et al. (41) found a positive association in men with total OC but no association in women or with ucOC (41). Yun et al. (43) found a negative association at low levels of OC only (43), and Zhang et al. (42) found an association in unstable angina pectoris and acute myocardial infarction patients but not in pectoris patients with stable angina (42). The single longitudinal study by Kanazawa et al. (47) showed conflicting results as baseline measurements demonstrated that OC was significantly and positively correlated with plaque score; however, OC was then negatively correlated with changes in plaque score at the end of the study (47).

Overall, in studies that conducted gender sub-analyses, more positive relationship outcomes between OC and measurements of atherosclerosis or calcification were observed within males than negative outcomes, while the reverse was reported within females. A neutral outcome was the most common finding overall for males and females. For the remaining studies when the study population was analyzed as a total, a positive outcome was the most common finding.

Thirty-six percent of outcomes from studies conducted in Asia found a negative relationship between OC and markers of atherosclerosis or calcification, in contrast to 6% in European studies and 5% in American, Canadian, Mexican, or Australian studies. Outcomes examined by population characteristics, e.g., chronic kidney disease patients, healthy adults, vascular problems (vascular dysfunction/coronary heart disease/atherosclerosis) were mixed. When examining studies that only used blood samples to measure OC ($n = 33$), i.e., excluding the OC positive cell studies and histological studies, no trend became apparent.

No trend was observed for differing methods of measuring calcification or atherosclerosis, and results were similar when examining total studies and those that used blood samples to assess OC. Overall, the method of calcification scoring to assess calcification or atherosclerosis resulted in the most non-significant outcomes with OC measurements.

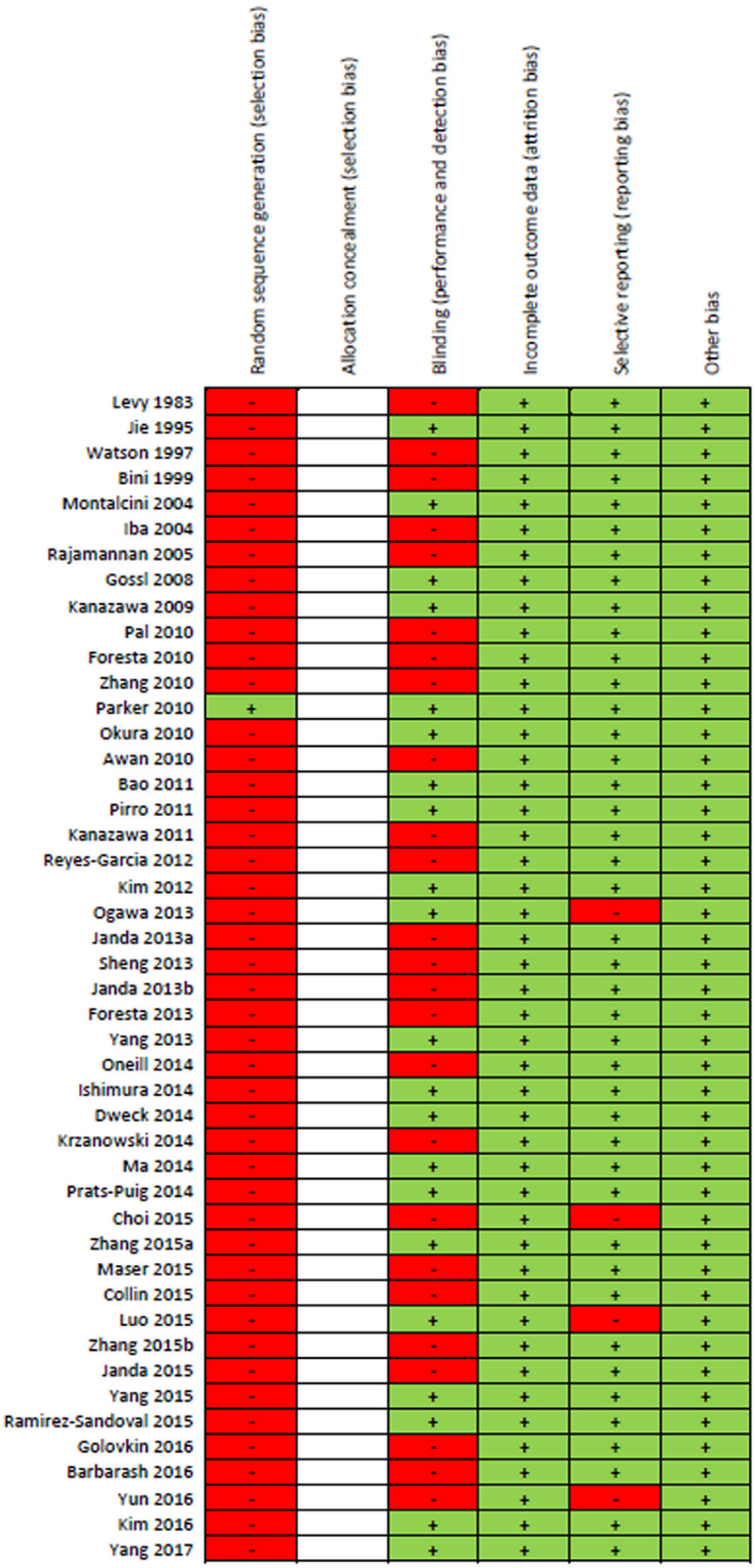

FIGURE 2 | "Risk of bias" summary: green (+) indicates low-bias risk and red (–) indicates high-bias risk. The studies included in this review were all observational in study design and thus the risk of bias for the item "allocation concealment" was not performed and spaces were left blank.

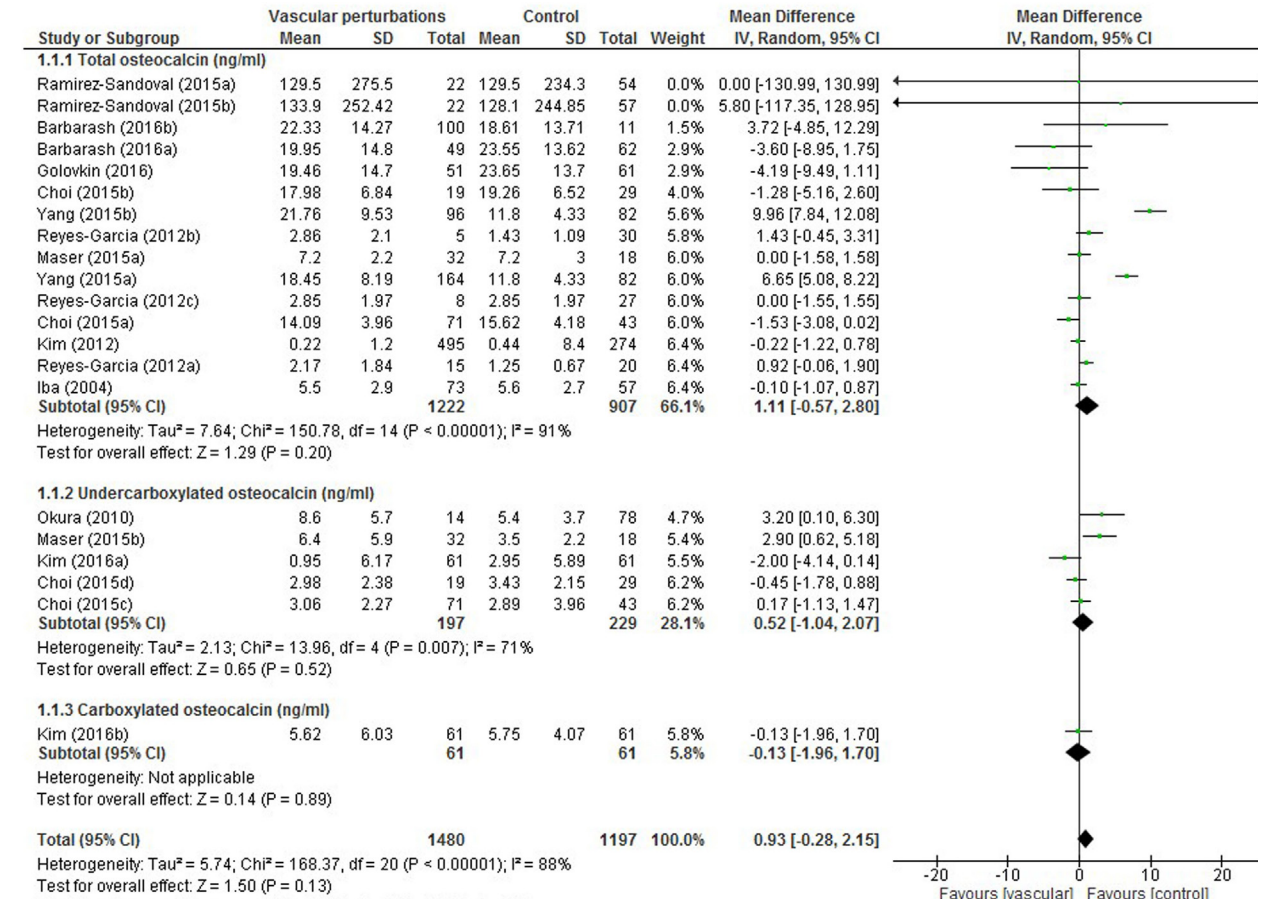

FIGURE 3 | Meta-analysis examining osteocalcin (OC) concentration (nanograms per millilitre) differences between groups with and without vascular perturbations (markers of calcification or atherosclerosis).

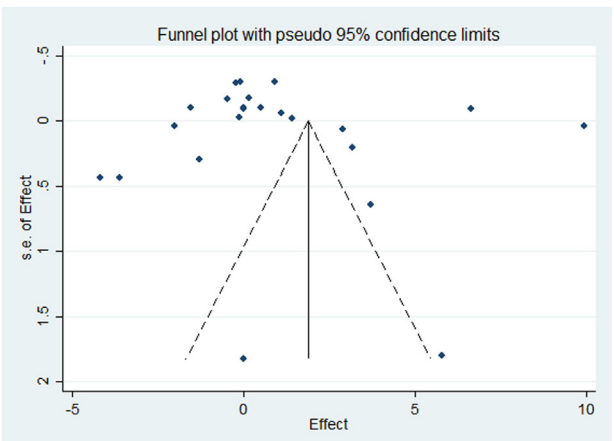

FIGURE 4 | Funnel plot evaluating publication bias on the effect of OC concentration on atherosclerosis or calcification. The SE of the mean difference in osteocalcin concentration for each study is plotted against its effect size (horizontal axis). Although the distribution of the studies within the funnel plot does not appear symmetrical, there was no statistical evidence of publication bias (Egger's statistic $p = 0.279$).

DISCUSSION

This review aimed to uncover whether there was a conclusive association between OC and vascular calcification or atherosclerosis in humans by performing a systematic review of the current literature. In total, 33 studies measuring blood OC concentrations and 13 studies measuring OC positive cells or histological staining of OC were found through the literature searches. Overall, no clear association could be made between OC and extent of calcification or atherosclerosis, which was confirmed by meta-analysis. However, all studies measuring OC positive cells or histological staining of OC showed a positive relationship with calcification or atherosclerosis. Some potential reasons for discrepancies in results were explored during the synthesis, including the method of OC measurement, variability in population characteristics and ethnicity, gender, and method of measuring calcification or atherosclerosis.

The majority of studies measured blood concentrations of total OC, ucOC, or both, while only one study measured cOC. No significant relationship was established for cOC, and the studies measuring total OC and/or ucOC resulted in a combination of positively and negatively correlated associations, as well as

non-significant outcomes. Within the current review, the role of the different forms of OC in the vasculature, i.e., ucOC or cOC, could not be determined as too few studies examined OC in its different presentations. Total OC may not be a valuable measurement for risk of vascular calcification as it is suggested that ucOC is the biologically active form. Future research may benefit from focusing on the various types of OC to ascertain whether there is a relationship present. However, there has been difficulty in measuring ucOC and cOC as few assays exist and it is unclear which assay system provides the most accurate measurements due to problems with comparability and heterogeneity of OC (14, 66–68). OC also displays a circadian rhythmicity with a nocturnal peak and thus timing of blood sampling may also contribute to variations in results (69, 70).

Circulating mononuclear cells, EPCs, and OPCs expressing OC were used by studies included in this review as a form of OC measurement. These cells are found in the bloodstream and released by bone marrow. EPCs can differentiate into endothelial cells and play a role in angiogenesis (71). It has been hypothesized that OC positive EPCs are involved in the mechanism of calcification by mediating abnormal vascular repair. This is thought to be as a result of the activation of osteogenic genes within the EPCs. EPCs are considered to be part of the initial response to vessel damage; however, instead of promoting normal repair they express an "osteogenic transcriptosome" which promotes calcification. This is further supported by gene expression analyses of CD34+ cells showing expression of bone mineralization related proteins such as Runx2 and BMP-2. It has been proven that OC is expressed by atherosclerotic plaques and VSMCs, which have differentiated as part of the process of calcification already (72) and so studying the association between cells expressing OC and calcification could provide further direction for future studies. All the articles reviewed in this study found a significant, positive correlation between OC positive cells and increased calcification or atherosclerosis.

Histological staining for OC resulted in similarly positive findings. The positive correlation found in all these studies between OC and calcification supports the hypothesis that OC expressed by these cells may contribute to the initial calcification of the vessels. This is comparable to Idelevich et al. (16) whose observations suggest OC is an active contributor to the mineralization process and stimulates differentiation of chondrocytes and VSMCs (16). These observations indicate the potential clinical implications of OC in detecting subclinical atherosclerosis and spotty calcifications. Conflicting results arise only when OC is measured in blood samples, suggesting a need for ucOC and cOC to be measured separately with reliable reproducible assays to disentangle their functions.

All the studies examined in this systematic review, except two, were cross-sectional observational studies. These, although useful, are also limited in their interpretation as a cause–effect relationship cannot be concluded from the results. The mechanism behind calcification remains very much unresolved and so the role, if any, of OC in the process is difficult to identify. The longitudinal study by Kanazawa et al. showed conflicting results between baseline measurements and final measurements. These results demonstrated that initially, total OC was significantly and positively correlated with carotid plaque score (47). However, OC was negatively correlated with changes in plaque score even after adjustment with atherosclerosis-related risk factors at the end of the study. This suggested that OC was relevant to calcification at both extremes, forming a U-shaped association. It was therefore hypothesized that atherosclerotic plaques may initially promote OC secretion but eventually the increased level of OC may suppress the progression of atherosclerosis or calcification. Furthermore, the longitudinal study by Yang et al. (62) reported that very high numbers of early circulating OC positive EPCs tended to be associated with to the risk of all-cause mortality (62). Further studies in a similar prospective longitudinal style should be carried out to confirm the hypotheses suggested. This may provide a reason for the numerous conflicting cross-sectional studies that have studied populations at different phases of disease.

No clear trends could be seen as a result of gender, although it can be noted more negative than positive outcomes between OC and calcification or atherosclerosis were observed in men, while the opposite was observed for women. Gender differences in OC actions have been reported elsewhere, for example in diabetes and fertility (5, 73). Due to the variety of population characteristics included the studies reviewed, associations could not be concluded between particular populations and study results. Most participants were over 50 years of age (data not shown), and this may seem reasonable as the risk of vascular calcification and atherosclerosis increases with age; however, with an increase in age also comes an increase in comorbidities, which could have influenced the results found in some studies. In addition, OC concentrations are influenced by medication including glucocorticoid therapy, antiresorptive agents and vitamin D treatment (74). Not all the studies accounted for these being potential confounding factors.

An interesting study by Namba et al. (75) examined the effect on bone metabolism markers and atherosclerosis measures in patients with atrial fibrillation when switching from warfarin (a vitamin K antagonist) to rivaroxaban (75). This study found ucOC concentrations to decrease after 6 months of rivaroxaban treatment as vitamin K was no longer prohibited. Concomitantly, osteopontin (an atherosclerosis-related marker) was decreased, bone alkaline phosphatase (a bone formation marker) was increased and PWV and augmentation index were significantly decreased. The availability of vitamin K allows for γ-carboxylation of ucOC to cOCN, and the reported improvements in atherosclerosis markers suggest and allude to the importance and potential clinical relevance of the differing presentations of OC, and their usefulness to detect at risk populations.

Ethnicity may play a role in the conflicting results of the studies in this review. Thirty-seven percent of studies conducted in Asia reported negative relationships between OC and calcification or atherosclerosis, compared to 6% of European studies. Studies in both populations used a combination of endpoints measuring calcification or atherosclerosis, showing that this variation did not affect this comparison. Within 10 studies that had a sample size >300, 1 reported a positive outcome, 7 reported negative outcomes, with the remaining two finding no significant outcomes.

Eight of these larger studies were conducted in Asia, thus the negative outcomes may be reflective of higher statistical power or ethnicity or a combination of both.

The meta-analysis performed providing adequate data on OC concentration confirmed findings from the qualitative component of this systematic review. The large heterogeneity reported again questions the reliability of serum or plasma measurements of OC concentration, the accuracy of methods of measurement of total OC and its undercarboxylated and carboxylated forms, and the need for well-defined studies with a primary aim of assessing OC's role in vascular calcification and atherosclerosis. The heterogeneity present in the meta-analysis can be further explained by the variety of study populations (kidney disease, diabetes or glucose intolerance, postmenopausal women, and hypertension) and the different methods employed to distinguish between those with and without vascular calcification or atherosclerosis and varying severities therein.

There are a few limitations in this review, which should be considered. Despite a thorough search of the two databases chosen, the addition of more databases may have widened the search to increase the number of results and hence improve the reliability and validity of the findings. However, the review was carried out by two independent reviewers, and searches generated were analyzed separately and then compared. Only one study analyzed cOC, which limits the results reported here. Furthermore, due to the observational nature of the studies included, only associations can be drawn and no causal relationships can be concluded.

In conclusion, no clear association can be made between OC and vascular calcification or atherosclerosis from the currently available published research. This review has highlighted themes, which may influence OC within differing populations leading to inconclusive results. In addition, the various forms of circulating OC should be separately measured and considered in future studies. Longitudinal studies may provide more insightful results as to the potential pathological effects of OC.

AUTHOR CONTRIBUTIONS

SM and SO: substantial contributions to the conception or design of the work. All the authors: the analysis and interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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6. Human vascular cell responses to the circulating bone hormone osteocalcin

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ORIGINAL RESEARCH ARTICLE

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Human vascular cell responses to the circulating bone hormone osteocalcin

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Abstract

The purpose of this study was to characterize the direct effects of uncarboxylated osteocalcin (ucOCN) on vascular cell biology in vitro, to assess its potential function in pathophysiological conditions such as atherosclerosis. Human aortic endothelial cells (HAECs) and smooth muscle cells (HASMCs) were treated with ucOCN (0.1–50 ng/ml) and changes in phosphorylation of intracellular signaling proteins, angiogenesis, proliferation, migration, monolayer permeability, and protein secretion were measured. In HAECs, phosphorylated JNK and CREB were decreased with ucOCN ($p < 0.05$). In HASMCs, phosphorylated p70S6K and NF-KB were increased by ucOCN ($p < 0.05$). Cell proliferation increased in both cell types dose dependently which was blocked by AKT and ERK pathway inhibitors. ucOCN did not affect cell permeability, angiogenesis, or migration. The direct activity of ucOCN on vascular cells is recognized, particularly its proliferative effects. However, at least in physiological settings, it does not appear that osteocalcin may directly promote atherogenesis based on the outcomes measured.

KEYWORDS
AKT, aortic endothelial cells, ERK, osteocalcin, p70s6k

1 | INTRODUCTION

In recent years, the skeleton's role as an endocrine organ has been increasingly acknowledged and investigated, in particular its cross-talk with glucose and lipid metabolism (Ferron & Lacombe, 2014; Lee et al., 2007). These links have been attributed in part to a vitamin K dependent protein called osteocalcin (OCN), also known as bone Gla protein or BGLAP. OCN is the most abundant noncollagenous protein found in the bone matrix, predominantly produced by osteoblasts (Hauschka, Lian, Cole, & Gundberg, 1989). Posttranslational γ-carboxylation of glutamic acid residues results in a carboxylated form of OCN (cOCN) which induces a high affinity for calcium ions. This promotes binding of cOCN to hydroxyapatite crystal lattices present in the bone extracellular matrix. Uncarboxylated osteocalcin (ucOCN) is released into the circulation, as well as some cOCN, following a number of conditions such as decarboxylation, vitamin K deficiency, or by the acidic environment during bone resorption (Cairns & Price, 1994; Motyl, Mccabe, & Schwartz, 2010; Plantalech, Guillaumont, Vergnaud, Leclercq, & Delmas, 1991).

OCN has an established extra-skeletal function through involvement with whole body metabolism, fertility, and cognition (Ferron et al., 2010; Hauschka et al., 1989; Lee et al., 2007; Oury et al., 2011, 2013), reviewed elsewhere (Karsenty, 2017). A new line of enquiry into the wide-ranging function of OCN is its role in the vasculature, and it has been proposed as a biomarker for cardiometabolic risk, and a potential therapeutic target (Tacey et al., 2018). It has been reported that in older men, a lower ratio of ucOCN/total OCN predicted myocardial infarction independently of

Abbreviations: OCN, osteocalcin; ucOCN, uncarboxylated osteocalcin; HAEC, human aortic endothelial cell; HASMC, human aortic smooth muscle cell.

conventional cardiovascular risk factors (Yeap et al., 2015). However, other studies have found no association between OCN and cardiovascular disease incidence (Holvik et al., 2014; Hwang et al., 2015). Elsewhere, Yeap et al. (2012) found that total osteocalcin predicted all-cause mortality and cardiovascular disease-related mortality in community-dwelling older men, however the relationship was U-shaped with men at both ends of the distribution at increased risk. Similarly, Kanazawa, Yamaguchi, and Sugimoto (2011) performed a longitudinal study in which a U-shaped association between OCN and plaque score was determined. It appears OCN may protect vascular function, which is often mediated through an improved metabolic state involving insulin signaling and glucose regulation (Dou et al., 2014; Jung et al., 2013; Tacey et al., 2018). *In vivo* studies in rats and mice found that ucOCN may be protective against atherosclerosis and promotes normal vascular function (Tacey et al., 2018). Direct effects of OCN on vascular cells remains to be confirmed through *in vitro* investigation. Other bone hormones such as FGF-23, and more recently sclerostin, have already been demonstrated to influence vascular cells directly (Cianciolo et al., 2018; Oranger et al., 2017).

Another particular area of interest is the relationship between OCN and vascular calcification. This is based on the underlying resemblance of vascular calcification to bone mineralization and limited direct investigations with osteocalcin and the vasculature (Evrard, Delanaye, Kamel, Cristol, & Cavalier, 2015; Idelevich, Rais, & Monsonego-Ornan, 2011; Millar, Patel, Anderson, England, & O'Sullivan, 2017). Cross-sectional epidemiological data surrounding osteocalcin has reported conflicting associations and a meta-analysis of data in humans correlating OCN and markers of calcification and atherosclerosis was inconclusive (Millar et al., 2017).

In vitro examination of OCN in human cells and exploration of its potential role and mechanisms of actions is needed as our knowledge is remarkably limited. To date, it has been reported that ucOCN is the active form of OCN involved in its endocrine functions (Lacombe & Ferron, 2015). Therefore, we undertook comprehensive *in vitro* experiments in human aortic endothelial cells (HAECs) and human aortic smooth muscle cells (HASMCs), assessing their response to ucOCN, with the hypothesis that it can initiate direct intracellular signaling, and promote angiogenesis. We aimed to report ucOCN related intracellular pathways and cellular functions to progress understanding of its importance under normal physiological conditions, and any indications that ucOCN may be a promoter or suppressor of normal vascular function. It is important to explore the effects of ucOCN on both HAECs and HASMCs as each cell type has their distinct characteristics and role in maintaining vascular function and homeostasis, and equally have their own responses and involvement in vascular pathologies.

2 | MATERIALS AND METHODS

2.1 | Materials

Human uncarboxylated osteocalcin (ucOCN; amino acids 1–49, [Glu17,21,24]) was purchased from US Biological (O8060-09C-USB; Ely, UK) and AnaSpec Inc. (AS-65307; Ely, UK). The amino acid sequence of purchased osteocalcin was as follows: Tyr-Leu-Tyr-Gln-Trp-Leu-Gly-Ala-Pro-Val-Pro-Tyr-Pro-Asp-Pro-Leu-Glu-Pro-Arg-Arg-Glu-Val-Cys-Glu-Leu-Asn-Pro-Asp-Cys-Asp-Glu-Leu-Ala-Asp-His-Ile-Gly-Phe-Gln-Glu-Ala-Tyr-Arg-Arg-Phe-Tyr-Gly-Pro-Val.

2.2 | Cell culture

HAECs and HASMCs were purchased from PromoCell (UK) and maintained at 37°C in a humidified incubator supplemented with 5% CO_2 in commercially available endothelial cell growth media and smooth muscle cell growth media (PromoCell), containing 1% Penicillin–Streptomycin (Sigma-Aldrich, UK). Cells were used between passages 3 and 5. Human ovarian cancer cell line, SKOV-3 (American Type Culture Collection [ATCC] HTB-77) obtained from ATCC were cultured in Roswell Park Memorial Institute-1640 media (Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% Penicillin–Streptomycin. SKOV-3 cells (passage 21) were used as a negative control when identifying the GPRC6A receptor during western blotting. Human osteoblasts (HOBs) were originally isolated from human femoral head trabecular bone and have been characterized previously (Anderson, Downes, Perry, & Caballero, 1998; Henstock, Ruktanonchai, Canham, & Anderson, 2014; Huang, Silvio, Wang, Tanner, & Bonfield, 1997). HOBs were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 1% Penicillin–Streptomycin, 200 nM L-glutamine, and 15 µg/ml ascorbic acid (all Sigma-Aldrich) and were used as a positive control for measuring osteocalcin secretion from cells by enzyme-linked immunosorbent assay (ELISA) and for GPRC6A receptor identification during western blotting. After experimental treatments, cell media was collected and cells were washed with phosphate buffered saline (PBS; pH 7.4, Gibco™, Loughborough, UK). Radioimmunoprecipitation assay buffer (Sigma-Aldrich) with protease and phosphatase inhibitors (A32959; Thermo Fisher Scientific, Loughborough, UK) was added to lyse the cells which were then collected and centrifuged at 14,000*g* for 5 min at 4°C. Cell supernatants were frozen at − 80°C or analyzed immediately, unless otherwise stated.

2.3 | Vascular permeability

HAECs were grown until confluent in 12-well plates on 12 mm diameter, 0.4 µM pore polycarbonate membrane inserts (Corning® Costar®; Sigma-Aldrich). Transepithelial electrical resistance (TEER) was measured using EVOM™ voltohmmeter (World Precision Instruments, Sarasota, FL) to evaluate paracellular permeability of cells treated with vehicle or ucOCN (10 ng/ml).

2.4 | Enzyme-linked immunosorbent assays

Human osteocalcin DuoSet ELISA (R&D Systems; DY1419) was used to measure total secreted osteocalcin in cell culture media. Endothelin Pan Specific, ICAM-1/CD54, VCAM-1/CD106, and total MMP-3 DuoSet ELISAs were performed on cell culture media according to the manufacturer's instructions (Catalog numbers DY1160, DY720, DY809, and DY513; R&D Systems, Abingdon, UK).

2.5 | Proliferation assay

Cell Titre 96 AQueous One Solution Cell Proliferation Assay (Catalog No. G3581; Promega, Southampton, UK) was performed according to manufacturer's instructions in HAECs and HASMCs. Cells were seeded at approximately 5,000 cells per well in 96-well plates and left to adhere for at least 4 hr. Cells were then treated with control media, or media supplemented with 0.1 ng/ml, 0.5 ng/ml, 1 ng/ml, 10 ng/ml or 50 ng/ml of ucOCN for 48 or 72 hr. The proliferation assay was repeated with 10 ng/ml of ucOCN on confluent cells to establish if the effects of OCN were due to an increase in cell proliferation as opposed to an increase in cell metabolism.

The proliferation assay was repeated using 10 ng/ml of ucOCN alone or in the presence of 3 μM PI3K inhibitor (LY294002 hydrochloride; Catalog No. 1130; Tocris Bioscience, Bristol, UK) or MAPK (MKK/MEK) inhibitor (PD98059; Catalog No. 1213; Tocris Bioscience).

2.6 | Scratch wound/migration assay

An *in vitro* scratch assay with HAECs and HASMCs was performed as described previously (Liang, Park, & Guan, 2007). Cells were seeded in six-well cell culture plates and left to adhere until confluent in usual cell growth media. For the HAEC experiments, cells were then sustained with endothelial cell basal medium (PromoCell) without growth or supplement factors but containing 10% FBS (F9665, Sigma-Aldrich) to allow cell survival but limiting proliferation. For the HASMC experiments, once confluent, cells were switched to smooth muscle cell basal medium (PromoCell) without growth or supplement factors but supplemented with 0.1% FBS. After 24 hr, a scratch was marked on each well with a P200 pipette tip, washed with PBS, and replaced with media alone, or media containing ucOCN (10 ng/ml). Images of the scratch were captured at baseline and at various time points to monitor cell migration. The area of the scratch wound at baseline and at each time point was measured using the ImageJ software.

2.7 | Angiogenesis assay

Confluent (80–100%) or sub-confluent (50–60%) HAECs were treated with ucOCN (10 ng/ml) or with media alone for 72 hr. The MILLIPLEX MAP Human Angiogenesis Assay (Catalog No. HAGP1-MAG-12K; Merck Millipore, Hertfordshire, UK) was performed according to the manufacturers' instructions to detect changes in angiopoietin-2 (ANG-2), vascular endothelial growth factor d (VEGFd), hepatocyte growth factor (HGF), VEGFc, interleukin-8 (IL-8) and fibroblast growth factor-2 (FGF-2) in cell lysates.

2.8 | GPRC6A expression

HAECs, HASMCs, SKOV-3s, and HOBs were grown until confluent and cultured for an additional 24 hr. Cell lysates were collected and protein samples (5 μg/lane) were resolved by electrophoresis on 10% Mini-protean TGX precast gels (Bio-Rad Laboratories, Inc., Hertfordshire, UK). The proteins were wet transferred to a nitrocellulose membrane and incubated in blocking buffer (2% bovine serum albumin in tris buffered saline with tween 20 [TBST]) overnight at 4° C. The membrane was then incubated with rabbit anti-human GPRC6A primary antibody (Abbexa, Cambridge, UK; 1:1,000 in 3% blocking buffer) for 2 hr at room temperature. The membrane was then washed and incubated for 1.5 hr with alkaline phosphatase conjugated antirabbit secondary antibody (Catalog No. A3937, 1:25,000; Sigma-Aldrich). Immunoreactive bands were visualized by chemiluminescence (Bio-Rad Immun-Star™ AP Substrate Pack #1705012). Protein bands were visualized using the ChemiDoc™ MP Imaging system with Image Lab™ software (Bio-Rad Laboratories, Inc.). The membrane was then stripped for reblotting using ReBlot Plus Mild Antibody Stripping Solution (Sigma-Aldrich) and then blocked with 5% marvel in TBST for 2 hr at room temperature. The membrane was then incubated with rabbit anti-human beta-actin (Abcam, Cambridge, UK; Catalog No. 8227, 1:5,000 in 3% marvel in TBST) overnight at 4°C. Finally, the membrane was washed and incubated with secondary antibody in 3% marvel in TBST and visualized and quantified as above.

2.9 | Cell signaling assay

HAECs and HASMCs were grown until confluent and treated with ucOCN (10 ng/ml) or with control media for 2, 5, 10, and 30 min. The MILLIPLEX MAP 9-plex Multi-Pathway Magnetic Bead Signaling Kit (48-680MAG; Merck Millipore) was performed according to the manufacturers' instructions to detect changes in phosphorylated ERK/MAP kinase 1/2 (Thr185/Tyr187), AKT (Ser473), STAT3 (Ser727), JNK (Thr183/Tyr185), p70s6 kinase (Thr412), NF-kB (Ser536), STAT5A/B (Tyr694/699), CREB (Ser133), and p38 (Thr180/Tyr182) in cell lysates using Luminex® xMAP® technology. Phosphorylated- and total-mTOR (Ser2448) were also measured in the lysate samples using the Luminex system (48-625MAG, Merck Millipore).

2.10 | Protein content assay

A bicinchoninic acid protein (BCA) assay was performed to quantify the total protein content in cell lysates (Smith et al., 1985). The BCA working reagent was prepared by mixing BCA solution with copper (II) sulfate pentahydrate 4% solution (Sigma-Aldrich) at a 50:1 ratio. Protein concentrations of samples were interpolated against a bovine serum albumin standard curve. Secreted protein concentrations, intracellular signaling proteins, and intracellular angiogenesis markers were corrected for total protein content unless otherwise indicated.

2.11 | Statistical analysis

For cell signaling analyses, two-way analysis of variances (ANOVAs) were performed using treatment (ucOCN or control) and time point (2, 5, 10, and 30 min) as factors. One-way ANOVAs were performed to detect differences in protein secretions and angiogenesis markers

between treatment groups. A one-way ANOVA was used to detect differences in cell viability between groups. Results are presented as mean % change to control and standard error of the mean. Multiple comparisons were adjusted for by Dunnett's or Sidak's statistical hypothesis test. All statistical analyses were performed using Prism 7 for Windows (Version 7.01, GraphPad Software Inc.). Adjusted p values were considered significant at $p < 0.05$.

3 | RESULTS

3.1 | Osteocalcin directly activates intracellular signaling responses in HAECs and HASMCs

It was found that secreted osteocalcin was not detectable in untreated HAECs or HASMCs following 72 hr of confluence (Figure S1A).

In HAECs, treatment with ucOCN reduced phosphorylation levels of CREB at 30 min ($p < 0.05$; Figure 1a). Phosphorylated JNK was decreased at 10 min with ucOCN ($p < 0.05$; Figure 1b). ucOCN appeared to decrease p38 phosphorylation and increase ERK phosphorylation but this did not reach significance (Figure 1c,d). There were no significant differences in phosphorylation of NF-kB, AKT, p70s6k, STAT3, STAT5, or mTOR with ucOCN treatment compared with control (Figure 1e–j).

In HASMCs, 10-min treatment with ucOCN increased NF-kB phosphorylation levels compared to control ($p < 0.05$; Figure 2e). In contrast, after 30 min, ucOCN decreased NF-kB phosphorylation compared to control ($p < 0.05$; Figure 2e). Phosphorylated p70s6k concentrations were increased at 10 min following ucOCN treatment ($p < 0.05$; Figure 2g). Phosphorylated STAT5 was decreased after 30 min ($p < 0.05$; Figure 2i). Phosphorylated AKT levels appeared increased with ucOCN after 10 min but this did not reach significance (Figure 2f). No significant differences were observed for levels of phosphorylated CREB, JNK, ERK, p38, STAT3, or mTOR with ucOCN treatment compared to control (Figure 2a–d,h,j).

3.2 | Osteocalcin increases cell proliferation but not migration or angiogenesis markers in HAECs

As our results indicated a direct effect of ucOCN in vascular cells, we went on to examine whether OCN influenced stages of angiogenesis of endothelial cells (matrix degradation, proliferation, and migration). There were no significant differences in cell migration/wound closure rates between treatments (control or ucOCN; Figure 3a,b). The potential influence on matrix degradation was assessed by matrix metalloproteinase-3 (MMP-3) production after 24–72 hr. MMP-3 secretion was not affected by ucOCN compared to control (Figure 3e). However, after 72 hr of treatment ucOCN increased proliferation (0.5, 1.0, and 10.0 ng/ml; $p < 0.05$; Figure 3c). The effect of ucOCN on HAEC proliferation was no longer observed when cells were cotreated with AKT or ERK inhibitors (Figure 3d). When a similar experiment was conducted in already confluent cells treated with ucOCN, there were no significant differences between groups, suggesting OCN is not affecting cellular metabolism (Figure S1b,c). In addition, a panel of angiogenesis markers were measured after treatment with ucOCN in confluent and subconfluent cells. After 72 hr of incubation, there were no differences in angiogenic regulators (ANG-2, HGF, FGF-2, VEGFc, IL-8, and VEGFd) between treatment groups in confluent experimental conditions nor in still growing, subconfluence cells (Figure 4a–f).

3.3 | Osteocalcin increases cell proliferation in HASMCs but not migration

Our results showed that ucOCN did not affect the rate of wound closure (Figure 5a,b). Supporting this, we found ucOCN did not affect production

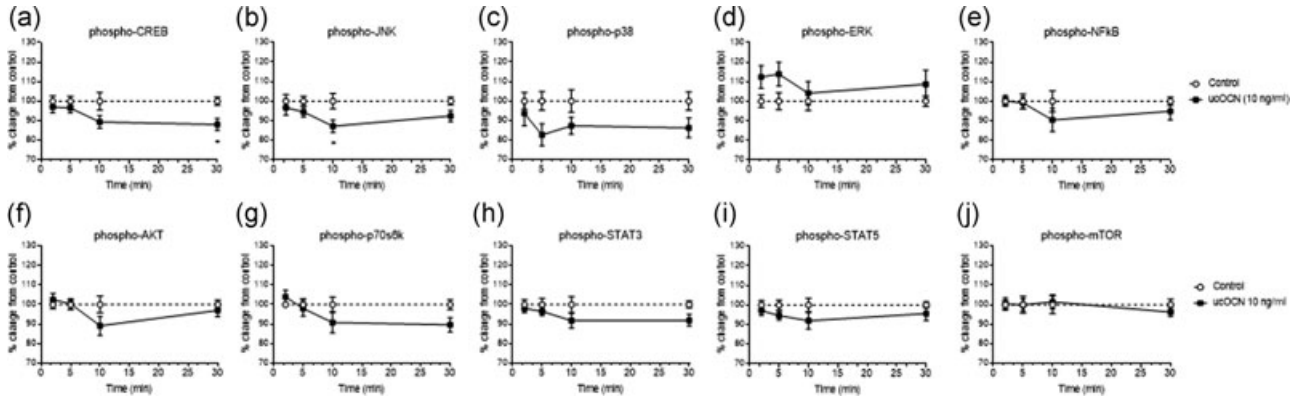

FIGURE 1 The effects of osteocalcin on human aortic endothelial cell signaling. (a–i) Luminex® xMAP® technology was used to detect changes in phosphorylated CREB (pS133), JNK (pT183/pY185), NFkB (pS536), p38 (pT180/pY182), ERK (pT185/pY187), Akt (pS473), p70 S6K (pT412), STAT3 (pS727), and STAT5A/B (pY694/699) (Milliplex™, 48-680MAG, Merck Millipore) in cell lysates when treated with ucOCN (10 ng/ml) for 2, 5, 10, and 30 min ($n = 10$ for control and $n = 20$ for ucOCN from three experimental repeats, normalized to total protein). (j) Changes in phosphorylated mTOR in cell lysates were also detected using the Luminex system ($n = 8$ for control and $n = 16$ for ucOCN from three experimental repeats, normalized to total mTOR; Milliplex™, 48-625MAG, Merck Millipore). Data were analyzed by two-way ANOVA. Multiple comparisons were adjusted for by Sidak's statistical hypothesis test. Data are given as means with error bars representing SEM. *Denotes a significant difference compared to control ($p < 0.05$). ANOVA: analysis of variance; SEM: standard error of mean; ucOCN: uncarboxylated osteocalcin

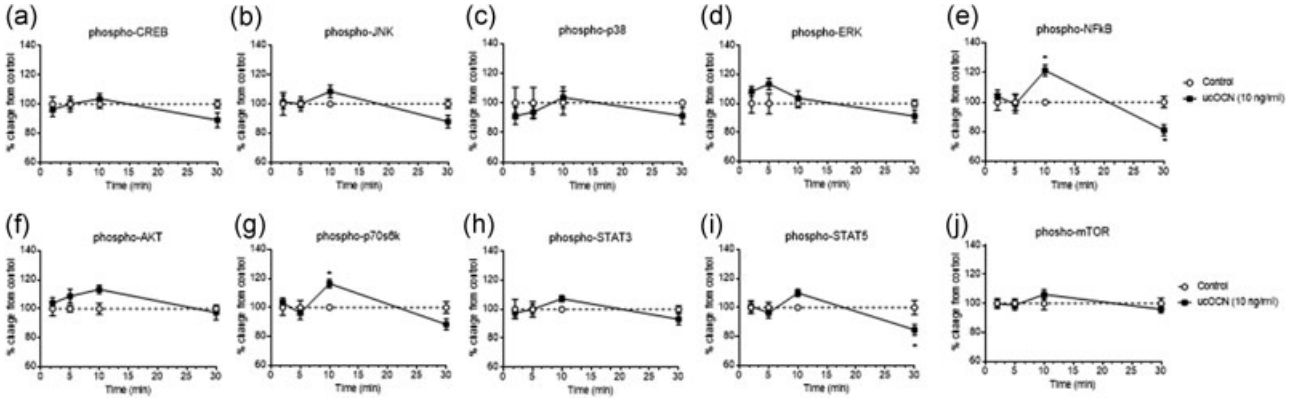

FIGURE 2 The effects of osteocalcin on human aortic smooth muscle cell signaling. (a–i) Luminex® xMAP® technology was used to detect changes in phosphorylated CREB (pS133), JNK (pT183/pY185), NFkB (pS536), p38 (pT180/pY182), ERK (pT185/pY187), Akt (pS473), p70 S6K (pT412), STAT3 (pS727), and STAT5A/B (pY694/699) (Milliplex™, 48-680MAG, Merck Millipore) in cell lysates when treated with ucOCN (10 ng/ml) for 2, 5, 10, and 30 min ($n = 10$ for control and $n = 20$ for ucOCN, from three experimental repeats, normalized to total protein). (j) Changes in phosphorylated mTOR in cell lysates were also detected using the Luminex system ($n = 6$ for control and $n = 12$ for ucOCN from three experimental repeats, normalized to total mTOR; Milliplex™, 48-625MAG, Merck Millipore). Data were analyzed by two-way ANOVA. Multiple comparisons were adjusted for by Sidak's statistical hypothesis test. Data are given as means with error bars representing SEM. *Denotes a significant difference compared to control ($p < 0.05$). ANOVA: analysis of variance; SEM: standard error of mean; ucOCN: uncarboxylated osteocalcin

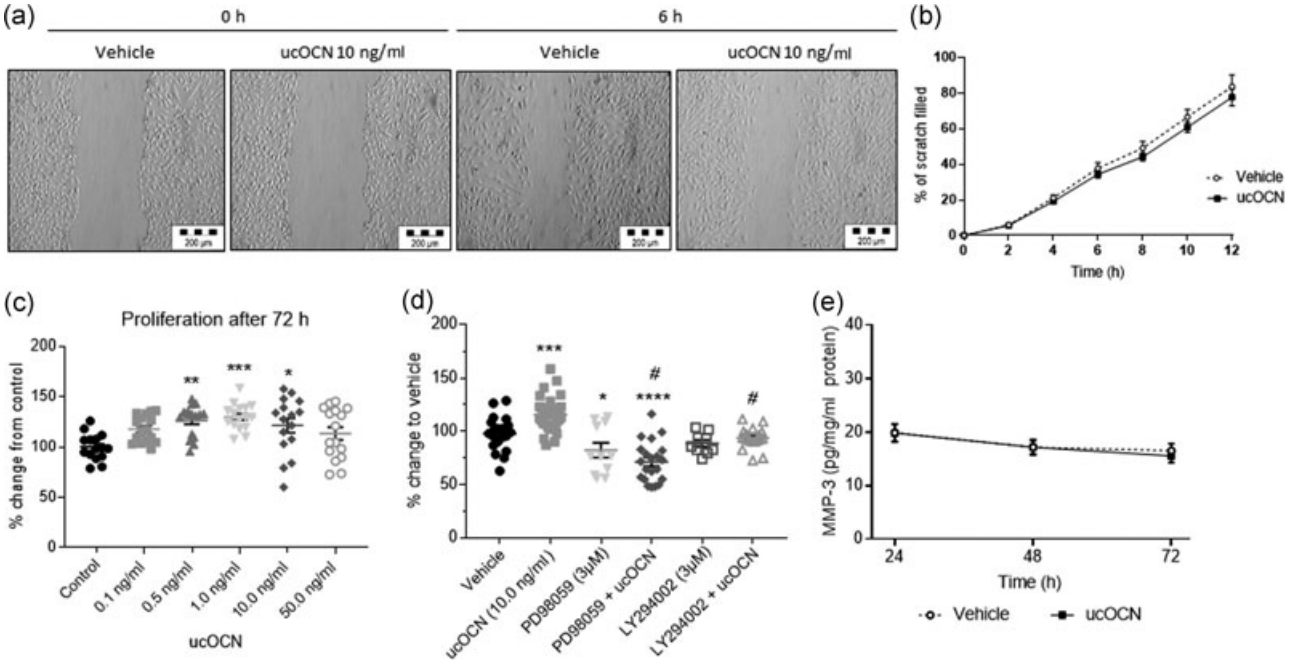

FIGURE 3 The effects of osteocalcin on human aortic endothelial cell (HAEC) migration and proliferation. (a) Representative images from a scratch wound assay performed in HAECs in which the scratch area was monitored for up to 12 hr. (b) Rate of migration/closure of scratch of HAECs treated with either vehicle or ucOCN (10 ng/ml; $n = 12$ for control and $n = 24$ for ucOCN, from three experimental repeats). (c) ucOCN concentration-effect (0.1–50.0 ng/ml) on cell proliferation as measured by MTS assay after 72 hr ($n = 16$ from three experimental repeats). Control media and osteocalcin prepared media was replaced after 48 hr. (d) Cell proliferation was further assessed using PI3K inhibitor (LY294002 hydrochloride 3 μM) and MAPK (MKK/MEK) inhibitor (PD98059 3 μM) (three repeats, total at least $n = 9$). (e) MMP-3 secretion over 24–72 hr by HAECs treated with vehicle or ucOCN (10 ng/ml; $n = 9$ for control and $n = 18$ for ucOCN, from three experimental repeats). Data are given as means with error bars representing SEM. Data were analyzed by one-way ANOVA and multiple testing corrected for by Dunnet's statistical test. *Denotes a significant difference compared to control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). #Denotes significant difference compared to ucOCN. ucOCN, uncarboxylated osteocalcin; MMP-3, matrix metalloproteinase-3; MTS: Cell Titre 96 AQueous One Solution Cell Proliferation Assay

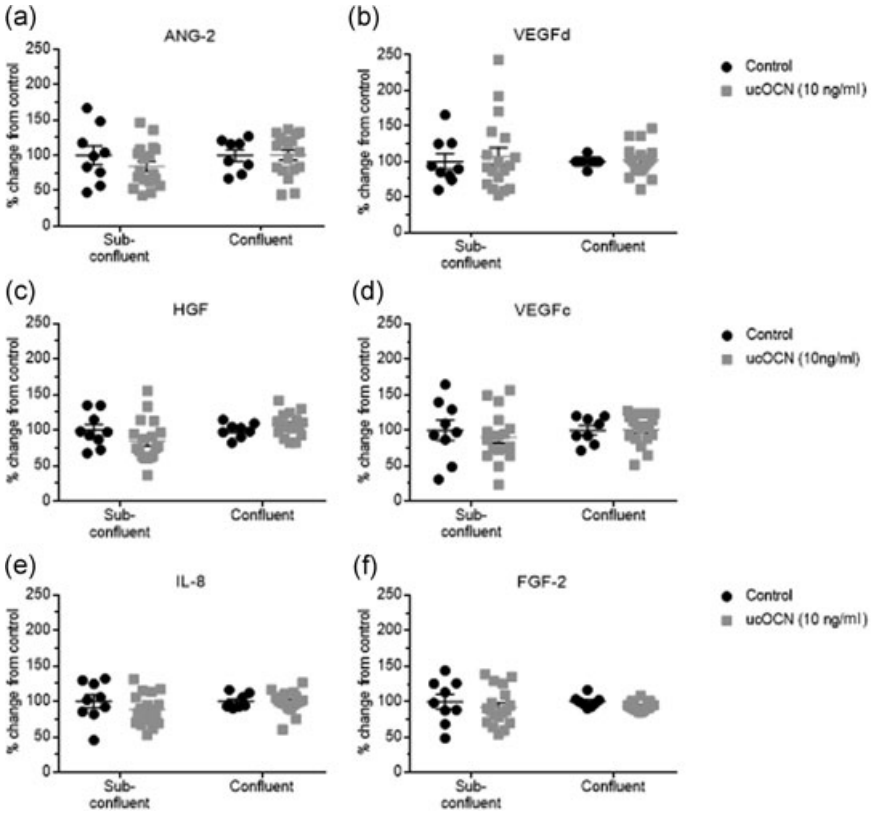

FIGURE 4 The effects of osteocalcin on markers of angiogenesis in human aortic endothelial cells. (a–f) Luminex® xMAP® technology (Milliplex™, 48-680MAG, Merck Millipore) was used to detect changes in markers of angiogenesis (angiopoietin-2, VEGFd, HGF, VEGFc, IL-8, and FGF-2) in cell lysates following treatment with ucOCN (10 ng/ml, 72 hr) on subconfluent and confluent cells ($n = 9$ for control and $n = 18$ for ucOCN, from three experimental repeats). In the confluent cells, control media and osteocalcin containing media was replaced every 24 hr. In subconfluent cells, media was not replaced during the 72 hr. ucOCN: uncarboxylated osteocalcin; ANG-2: angiopoietin-2; VEGFd: vascular endothelial growth factor d; HGF: hepatocyte growth factor; VEGFc: vascular endothelial growth factor c; IL-8: interleukin-8; FGF-2: fibroblast growth factor-2

of MMP-3 after 24–72 hr incubation (Figure 5e). ucOCN (0.5, 1.0, and 10.0 ng/ml) significantly increased cell proliferation after 48 hr compared to control (Figure 5c). This effect was blocked when cells were cotreated with either AKT or ERK pathway inhibitors (Figure 5d).

3.4 | Osteocalcin does not affect endothelial cell permeability or adhesion makers

Acute treatment with ucOCN did not affect endothelial cell permeability, as measured using TEER over 7.5 hr (Figure 6a). Adhesion molecules VCAM-1 and ICAM-1 were measured in HAECs and HASMCs respectively. ucOCN did not affect their secretion over time (Figure 6b,d). In addition, ucOCN did not affect production of endothelin, a vasoconstrictor, in HAECs (Figure 6c).

3.5 | Posited osteocalcin receptor, GPRC6A, is present in HAECs and HASMCs

The proposed osteocalcin receptor, GPRC6A, was detected in HAECs, HASMCs, and HOBs (positive control), and not in SKOVS (negative control) via western blotting (Figure 7).

4 | DISCUSSION

The aim of this study was to examine the direct vascular effects of physiologically relevant concentrations of osteocalcin (Binkley, Krueger, Engelke, Foley, & Suttie, 2000; Hannemann et al., 2013; Luukinen et al., 2000). ucOCN activated acute cell signaling cascades in HAECs and HASMCs, suggesting there is a molecular target for OCN on these cells. In both cell types, ucOCN increased proliferation through ERK and AKT signaling pathways. OCN does not appear to play a role in migration, endothelial cell angiogenesis or permeability.

In humans, the BGLAP (bone gamma-carboxyglutamate protein) gene for encoding OCN is ubiquitously expressed in brain, colon, appendix, and many other tissues (NCBI,). However, data on protein expression is less widely available. OCN has been implicated in vascular calcification plaques (Bini, Mann, Kudryk, & Schoen, 1999; Dweck et al., 2014; Foresta et al., 2013; O'Neill & Adams, 2014; Rajamannan et al., 2005), and differentiated osteoblast-like HASMCs are known to produce OCN during vascular calcification and mineralization (Dhore et al., 2001). Our results found that OCN expression was not detectable in HASMC or HAEC supernatants or intracellularly in normal physiological conditions, suggesting that OCN production from HASMCs is restricted to a pathophysiological setting exclusive to transdifferentiated HASMCs.

ucOCN is found within the circulation although there are no standardized reference values. Total OCN concentrations have been reported by Hannemann et al. (2013) by age and gender. However, reported serum ucOCN concentrations largely varied between <1 and >30 ng/ml (Binkley et al., 2000; Iki et al., 2012; Plantalech et al., 1991; Sowers et al., 1999). We therefore used 10 ng/ml which has also been used in previous *in vitro* studies (Dou et al., 2014; Jung et al., 2013).

We aimed to examine whether exogenously added fully uncarboxylated OCN can directly affect intracellular signaling in HAECs and HASMCs. We found that ucOCN significantly altered

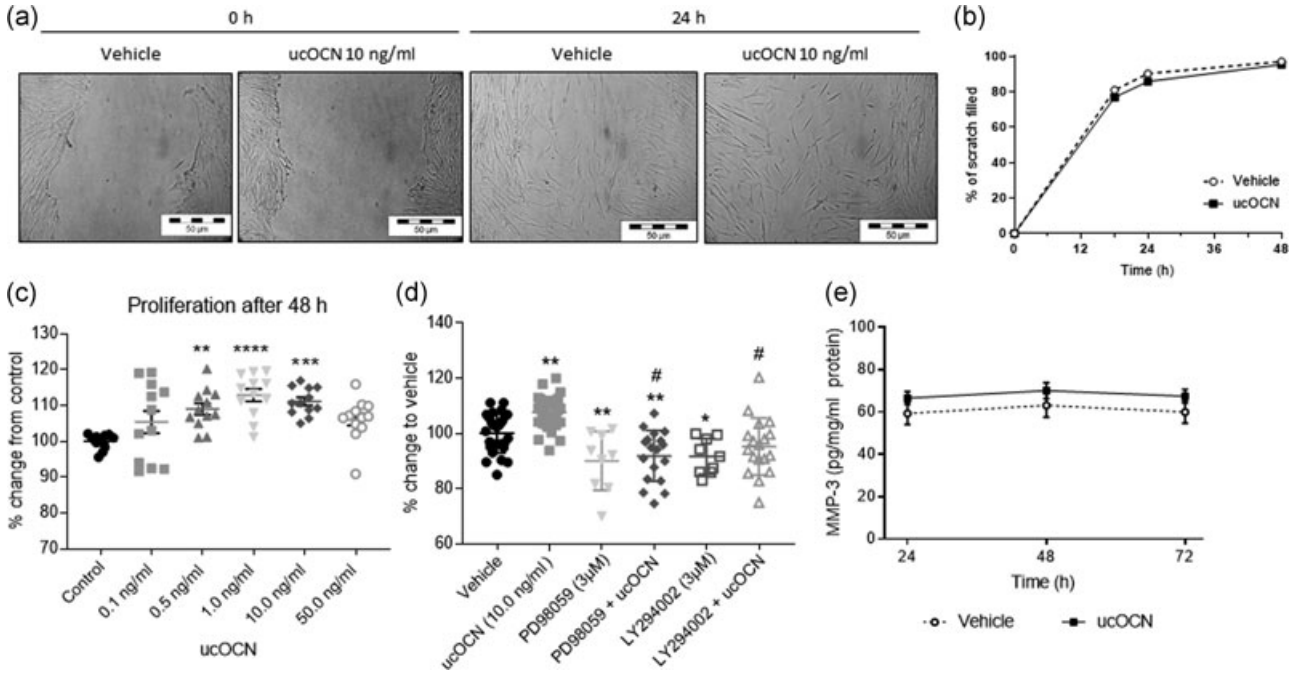

FIGURE 5 The effects of osteocalcin on human aortic smooth muscle cell (HASMC) migration and proliferation. (a) Representative images from a scratch wound assay performed in HASMCs in which the scratch area was monitored for up to 48 hr. (b) Rate of migration/closure of scratch of HASMCs treated with either vehicle or ucOCN (10 ng/ml; $n = 23$ from four experimental repeats). (c) ucOCN concentration-dependent effect (0.1–50.0 ng/ml) on cell proliferation as measured by MTS assay after 48 hr ($n = 12$ from three experimental repeats). (d) Cell proliferation was further assessed using PI3K inhibitor (LY294002 hydrochloride) and MAPK (MKK/MEK) inhibitor (PD98059) (three experimental repeats, total at least $n = 9$). (e) MMP-3 secretion over 24–72 hr by HAECs treated with vehicle or ucOCN (10 ng/ml; $n = 12$ for control and $n = 24$ for ucOCN, from three experimental repeats). One-way ANOVAs were used to analyze proliferation data and multiple testing corrected for by Dunnett's statistical test. Data are given as means with error bars representing SEM. *Denotes a significant difference compared to control ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$). #Denotes significant difference compared to ucOCN. ANOVA: analysis of variance; SEM: standard error of mean; MMP-3, matrix metalloproteinase-3; MTS: Cell Titre 96 AQueous One Solution Cell Proliferation Assay; ucOCN, uncarboxylated osteocalcin

phosphorylation levels of numerous signaling proteins including CREB and JNK in HAECs, and NF-kB and p70s6K in HASMCs. Jung et al. (2013) has previously reported an increase in AKT phosphorylation and eNOS and nitric oxide (NO) production in a PI3-kinase dependent manner in HAECs when treated with ucOCN for 1 hr (0.3–30 ng/ml; Jung et al., 2013). Overall, these results evidence that there is a direct and rapid intracellular response to osteocalcin in HAECs and HASMCs, likely to be receptor-mediated.

The G protein-coupled receptor, class C, group 6, subtype A (GPRC6A) is a recently "de-orphaned" receptor co-activated by cations, for example, calcium, and basic L-α-amino acids (Clemmensen, Smajilovic, Wellendorph, & Brauner-Osborne, 2014; Wellendorph & Brauner-Osborne, 2004). Osteocalcin and testosterone have been proposed to also be agonists to this receptor, which is present in rat arteries (Harno et al., 2008; Oury et al., 2011; Pi, Parrill, & Quarles, 2010; Pi, Wu, & Quarles, 2011). However, others have failed to reproduce and confirm these propositions with osteocalcin (Jacobsen et al., 2013; Rueda et al., 2016). In this paper, we present evidence for the existence of the GPRC6A receptor in both HAECs and HASMCs. Delineation of the specificity and functional response of GPRC6A to OCN in the human vasculature remains to be shown, however there is a lack of a selective receptor antagonist. Attempts at siRNA knock-down of the receptor may prove fruitful but we did not achieve successful knockdown in our attempts (data not shown). Another receptor, GPR158 has also been recently shown to mediate OCN's effect of cognitive function in mice (Khrimian et al., 2017) which also merits investigation in vascular cells.

Furthermore, we examined whether ucOCN affected stages of angiogenesis such as matrix degradation, migration, and proliferation. ucOCN did not affect MMP-3 production or wound healing *in vitro*. However, ucOCN was shown to increase proliferation in both HAECs and HASMCs, in a dose-dependent, bell-shaped manner, which adds to previously reported antiapoptotic effects of osteocalcin in HAECs (Jung et al., 2013). ucOCN has been previously reported as protective against free fatty acid induced apoptosis and low concentrations of ucOCN (0.3 ng/ml) has been shown to increase markers of pancreatic β-cell proliferation (Ferron, Hinoi, Karsenty, & Ducy, 2008). We revealed that osteocalcin is working through both AKT and ERK converging pathways to translate the increase in proliferation (Mendoza, Er, & Blenis, 2011). We chose these pathways to examine as it has been previously demonstrated by our group (unpublished data) that OCN increases AKT and ERK phosphorylation at longer time points (e.g. 24 hr) in HASMCs, and another study has also demonstrated increases in AKT phosphorylation in HAECs with OCN (Jung et al., 2013).

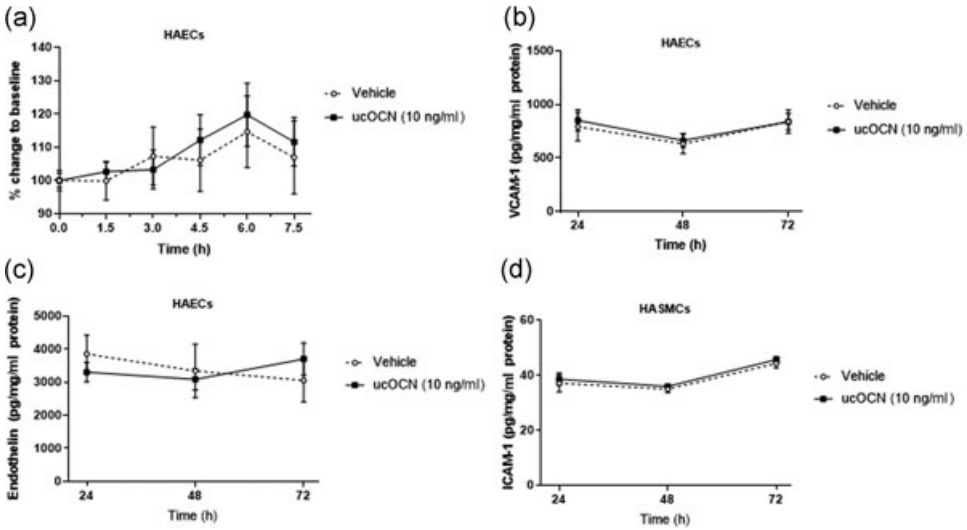

FIGURE 6 The effects of osteocalcin on vascular cell permeability, endothelin and adhesion markers. (a) Transepithelial electrical resistance measurement was used to evaluate the paracellular permeability of confluent human aortic endothelial cell monolayers treated with or without ucOCN and cOCN ($n = 10$ for control and $n = 20$ for ucOCN, from five experimental repeats). ucOCN (10 ng/ml) did not affect secretion of VCAM-1 (b) or endothelin-1 (c) after 24, 48 and 72 hr ($n = 14$ for control and $n = 28$ for ucOCN, from three experimental repeats) from confluent human aortic endothelial cells. (d) Secretion of ICAM-1 did not differ over 24–72 hr between vehicle or ucOCN (both 10 ng/ml) in confluent human aortic smooth muscle cells ($n = 12$ for control and $n = 24$ for ucOCN, from three experimental repeats). Data are given as means with error bars representing SEM. ICAM-1: intracellular adhesion molecule-1; SEM: standard error of mean; ucOCN: uncarboxylated osteocalcin; VCAM-1: vascular cell adhesion molecule-2

It has been previously shown in an *in vivo* chick embryo angiogenesis model that osteocalcin promotes angiogenesis (Cantatore, Crivellato, Nico, & Ribatti, 2005). Interestingly, ucOCN did not affect any of the measured angiogenesis regulators. The discrepancy may be due to the use of human cells in our study, within which the effects of OCN may be less pronounced or nonexistent. Our results may indicate that osteocalcin is working to promote proliferation, at least in HAECs, via mechanisms independent of key angiogenic markers. ucOCN also does not appear to acutely increase mTOR phosphorylation, which is a central regulator of cell growth required for angiogenesis and proliferation. These novel findings suggest that OCN may affect vascular cells in a limited range of function, pertaining to proliferation in normal physiological conditions.

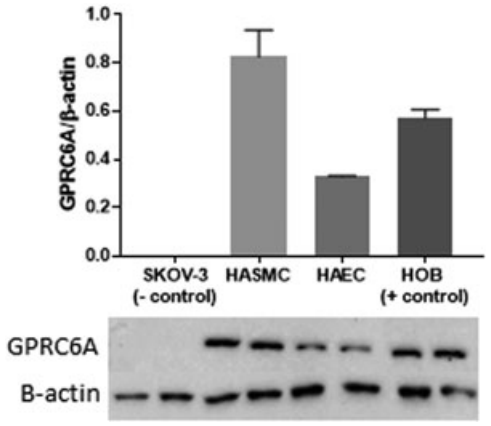

FIGURE 7 Posited osteocalcin receptor, GPRC6A, is present in human aortic endothelial (HAEC) and smooth muscle cells (HASMC). Western blot data showing lack of presence of GPRC6A in ovarian carcinoma cells (SKOV-3; negative control), and presence in HASMCs, HAECs, and human osteoblasts (HOBS; positive control). Data are given as means with error bars representing SEM ($n = 2$). SEM: standard error of mean

OCN did not affect cell permeability. This is a pivotal finding as cell layer integrity is essential to vascular function and degradation is a key, early event in atherogenesis, plaque formation and cell barrier infiltration. We also demonstrated that ucOCN does not influence two key endothelial cell proteins; endothelin, a potent vasoconstrictor, and VCAM-1. VCAM-1 expression is increased during inflammation and by cytokines, and as such we have found no evidence to suggest that osteocalcin is involved in the adhesion of monocytes and other cells to the endothelium, nor acts as a promotor of vasoconstriction. In the aortic smooth muscle cells, ICAM-1, which functions as a leukocyte recruiter and may be thought of as pro-inflammatory, was not affected by osteocalcin.

There are several circulating forms of OCN that can be found in the circulation including carboxylated OCN to varying degrees (0–2 carboxylated glutamic acid residues), and N-terminal fragments (Garnero, Grimaux, Seguin, & Delmas, 1994). It is unknown the extent to which structural differences may play in their biological functions (Li, Zhang, Yang, Li, & Dai, 2016). It is a limitation of the current work that not all present circulating forms could be examined. We recommend *ex vivo* and *in vivo* studies be performed to fully understand the effects of OCN on the vasculature.

The *in vitro* investigations conducted here lay a foundation for further research into the vascular effects of osteocalcin. ucOCN initiates direct responses in human aortic endothelial and smooth muscle cells and is proproliferative. However, the lack of effect of OCN on a range of endpoints such as VCAM-1, MMP-3, endothelin-1,

and cell permeability, do not suggest a direct involvement of OCN in atherogenesis or blood vessel disease at least in physiological settings. Further investigations within pathological settings and examination of the biological activity of other various fragments of osteocalcin should be undertaken.

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CONFLICT OF INTERESTS

The authors declare that there are no concflict of interersts.

AUTHOR CONTRIBUTIONS

S. O. S and S. M. designed the experiments which were carried out by S. M. S. M. wrote the manuscript with input from S. O. S and S. A. S. A. and S. O. S supervised the project.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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7. Osteocalcin does not influence acute or chronic inflammation in human vascular cells

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Osteocalcin does not influence acute or chronic inflammation in human vascular cells

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Abstract

Some human observational studies have suggested an anti-inflammatory role of osteocalcin (OCN). An inflammatory protocol using interferon-γ and tumor necrosis factor-α (10 ng/ml) was employed to examine the acute (24 hr) and chronic (144 hr) effects of uncarboxylated OCN (ucOCN) in commercial, primary, subcultured human aortic endothelial cells (HAEC), and human smooth muscle cells (HASMCs). The inflammatory protocol increased phosphorylation of intracellular signaling proteins (CREB, JNK, p38, ERK, AKT, STAT3, STAT5) and increased secretion of adhesion markers (vascular cell adhesion molecule-1, intracellular adhesion molecule-1, monocyte chemoattractant protein-1) and proinflammatory cytokines (interleukin-6 [IL-6], IL-8). After acute inflammation, there were no additive or reductive effects of ucOCN in either cell type. Following chronic inflammation, ucOCN did not affect cell responses, nor did it appear to have any pro- or anti-inflammatory effects when administered acutely or chronically on its own in either cell type. Additionally, ucOCN did not affect lipopolysaccharide (LPS)-induced acute inflammation in HAECs or HASMCs. The findings of this study do not support a causal role for OCN within the models of vascular inflammation chosen. Further confirmatory studies are warranted.

KEYWORDS
bone hormone, endothelial, inflammation, osteocalcin, smooth muscle

1 | INTRODUCTION

Atherosclerosis is a progressive inflammatory disease, involving revolving phases of inflammation leading to macrophage infiltration and microcalcifications (Hutcheson & Aikawa, 2014; Ross, 1999). Inflammatory cytokines, chemoattractant proteins, and adhesion molecules are present throughout the atherosclerotic process, of which the endothelium and smooth muscle cell layers are both pivotal. The process of atherosclerotic calcification is also crucially triggered by inflammatory-related pathways (Bessueille & Magne, 2015; Mazzini & Schulze, 2006). A paradox vascular-bone axis exists as while bone formation decreases with age, biomineralization of the vasculature is increased, which is suggested to be due to a shared etiology of inflammation (Bessueille & Magne, 2015; Sage, Tintut, & Demer, 2010). The endocrine function of bone, particularly osteocalcin (OCN), is increasingly under investigation due to its link with whole body metabolism and far-reaching extra-skeletal effects (Lee et al., 2007; Oldknow, Macrae, & Farquharson, 2015; Oury et al., 2011; Oury et al., 2013).

OCN is produced predominantly by osteoblasts and is the most abundant, noncollagenous protein found in the mineralized matrix of bone (Hauschka, Lian, & Gallop, 1975). Posttranslational γ-carboxylation of three glutamic acid residues within OCN results in carboxylated OCN (cOCN). However, OCN can be

carboxylated to varying degrees, allowing for undercarboxylated forms to be present in the circulation (one or two carboxylated residues), and uncarboxylated OCN (ucOCN; no carboxylated residues). ucOCN has previously been regarded as the "active form," and it has less affinity to bind to hydroxyapatite crystals in bone than cOCN.

Cross-sectional observational studies in humans have demonstrated inverse associations between OCN and systemic inflammatory markers, such as C-reactive protein (CRP) and interleukin-6 (IL-6; Bao et al., 2013; Chen et al., 2013; Liao et al., 2015; Lucey et al., 2013; Pittas, Harris, Eliades, Stark, & Dawson-Hughes, 2009; Sarkar & Choudhury, 2013). A study including 108 newly-diagnosed Type 2 diabetic patients reported that total serum OCN levels were inversely associated with IL-6 and CRP (Sarkar & Choudhury, 2013), these results were also evidenced in an older healthy population (n = 380; Pittas et al., 2009). Two large studies in Chinese males (n = 2,043 and n = 1,768) with metabolic syndrome or normal metabolic state also found an inverse association between total serum OCN and CRP (Bao et al., 2013; Liao et al., 2015). In young obese and overweight women, as well as in postmenopausal women, these findings have also been replicated (Lucey et al., 2013; Chen et al., 2013). It is also known that subclinical inflammation is associated with altered bone metabolism, which further potentiates a role for OCN within the vasculature during inflammation (Ding, Parameswaran, Udayan, Burgess, & Jones, 2008; Kim, Kim, & Sohn, 2010; Koh et al., 2005).

In white-adipose tissue from ucOCN-treated obese mice, several inflammatory genes and transcription factors were down-regulated including tumor necrosis factor (TNF); IL-1β; IL-6; chemokine (C-C motif) ligand, Ccl2; caspase 1; and NLR family, pyrin domain containing 3, Nlrp3 (Guedes, Esteves, Morais, Zorn, & Furuya, 2017). Within the same study, ucOCN-treated (20 ng/ml) mouse adipocytes similarly displayed a reduction in expression of inflammatory genes following stimulation with TNF-α (Guedes et al., 2017).

OCN has previously been shown to increase nitric oxide production and prevent free fatty acid-induced apoptosis in human aortic endothelial cells (HAECs); increase angiogenesis in a chick embryo in vivo model; increase proliferation in HAECs and HASMCs; and increase glucose metabolism and promote vascular calcification in OCN-over expressing mouse chondrocytes and vascular smooth muscle cells (Cantatore, Crivellato, Nico, & Ribatti, 2005; Idelevich, Rais, & Monsonego-Ornan, 2011; Jung et al., 2013; Millar, Anderson, & O'sullivan, 2019). Interestingly, Hill et al. (2014) demonstrated an anti-inflammatory role of both cOCN and ucOCN (20 ng/ml) in isolated rat adipocytes by decreasing TNF-α secretion, while cOCN also decreased IL-6 secretion (Hill et al., 2014). Furthermore, in whole tissue extracts, IL-10 (an anti-inflammatory cytokine) was increased (Hill et al., 2014).

The potential role for OCN in the development of atherosclerosis has been recently extensively reviewed (Tacey et al., 2018). The question remains however whether OCN is a pathological bystander or in fact mediator. To our knowledge, no in vitro studies to date have investigated the inflammatory role of OCN in human vascular cells. The aim of the current experiments was to investigate the distinct role of ucOCN in HAECs and human aortic smooth muscle cells (HASMCs) after acute and chronic administration, with and without stimulated inflammation. We hypothesized that ucOCN may reduce proinflammatory markers when administered on its own and may reduce the stimulated inflammatory responses of HAECs and HASMCs.

2 | MATERIALS AND METHODS

2.1 | Osteocalcin

Human ucOCN (amino acids 1–49, [Glu17,21,24]) was purchased from AnaSpec Inc. CA (AS-65307). The amino acid sequence of purchased OCN was as follows: Tyr-Leu-Tyr-Gln-Trp-Leu-Gly-Ala-Pro-Val-Pro-Tyr-Pro-Asp-Pro-Leu-Glu-Pro-Arg-Arg-Glu-Val-Cys-Glu-Leu-Asn-Pro-Asp-Cys-Asp-Glu-Leu-Ala-Asp-His-Ile-Gly-Phe-Gln-Glu-Ala-Tyr-Arg-Arg-Phe-Tyr-Gly-Pro-Val. The same batch of ucOCN has been previously shown to be biologically active in vascular cells in our previous work (Millar et al., 2019).

2.2 | Cell culture

HAECs and HASMCs were purchased from PromoCell (UK) and maintained at 37°C in a humidified incubator supplemented with 5% CO_2. Cells were cultured in endothelial cell growth media and smooth muscle cell growth media, respectively, containing 1% penicillin–streptomycin (Sigma-Aldrich, UK) and supplemental mix (PromoCell, UK). In all experiments, cells were used between passages 3 and 5. After experimental treatments, cell media was collected and cells were washed once with phosphate-buffered saline (PBS; pH 7.4; Gibco™). Radioimmunoprecipitation buffer (Sigma-Aldrich) with protease and phosphatase inhibitors (A32959; Thermo Fisher Scientific) was added to lyse the cells and the plates were shaken at 4°C for an hour. The cells were then collected and centrifuged at 14,000g for 5 min at 4°C and cell supernatants were frozen at −80°C.

2.3 | Acute and chronic inflammation

Acute inflammation was induced by treating cells with 10 ng/ml interferon-γ (IFN-γ) or vehicle (ethanol) for 8 hr, followed by addition of 10 ng/ml TNF-α or vehicle (1% ethanol) for 16 hr, as described previously (Alhamoruni, Wright, Larvin, & O'sullivan, 2012). Cells were cotreated with or without ucOCN (10 ng/ml; Hannemann et al., 2013; Hu et al., 2013; Withold, Friedrich, & Degenhardt, 1997) for 24 hr in the acute inflammation protocol. The chronic inflammatory protocol included 8 hr of IFN-γ (5 ng/ml) followed by addition of TNF-α (5 ng/ml). After 48 hr, the media was replaced and treatment repeated up until 144 hr. Cells were treated with or without ucOCN (10 ng/mL). Media was collected at each time point and stored at −80°C until analyzed.

To test whether ucOCN could effect the production of endogenously induced inflammation, in a subset of experiments, lipopolysaccharide (LPS; 10 ng/ml) was added to HAECs and HASMCs for 24 hr with and without ucOCN (10 ng/ml). Media was collected after this time and stored at −80°C until analyzed.

2.4 | Total protein content

A bicinchoninic acid protein assay was performed to quantify the total protein content in the cell lysates collected at the end of the experiments (Smith et al., 1985).

2.5 | Cell signaling assay

The MILLIPLEX MAP 9-plex Multi-Pathway Magnetic Bead Signaling Kit (catalog no. 48-680MAG; Merck Millipore) was performed per the manufacturers' instructions to detect changes in phosphorylated ERK/MAP kinase 1/2 (Thr185/Tyr187), AKT (Ser473), STAT3 (Ser727), JNK (Thr183/Tyr185), p70s6 kinase (Thr412), NFkB (Ser536), STAT5A/B (Tyr694/699), CREB (Ser133), and p38 (Thr180/Tyr182) in cell lysates using the Luminex® xMAP® technology (48-680MAG; Milliplex™; Merck Millipore).

2.6 | Enzyme-linked immunosorbent assays

Total OCN, endothelin pan specific, intracellular adhesion molecule-1 (ICAM-1)/CD54, vascular cell adhesion molecule-1 (VCAM-1)/CD106, total MMP-3, CCL2/monocyte chemoattractant protein-1 (MCP-1), IL-8/CXCL8, and IL-17 DuoSet enzyme-linked immunosorbent assays (ELISA) were performed on cell culture media as per the manufacturer's instructions (catalog no. DY1419; R&D systems, DY1160, DY720, DY809, DY513, DY279, DY208, and DY317). IL-6 ELISA Ready-SET-Go! was performed on cell culture media as per the manufacturer's instructions (catalog no. 88-7066-22; Affymetri; eBioscience).

2.7 | Lactate dehydrogenase activity assay

Lactate dehydrogenase activity (LDH) Colorimetric Assay kit (category no. ab102526; Abcam) was performed on cell media from the chronic inflammation experiment as per manufacturer's instructions.

2.8 | Haematoxylin and eosin staining

After 48 hr of the chronic inflammation protocol, a representative selection of HAECs and HASMCs was washed with PBS and fixed with ice cold methanol/acetone (50:50) for 10 min at room temperature. Fixed cells were then washed with PBS and stained with 0.1% Mayer's haematoxylin and counterstained with 1% eosin Y solution to allow visualization of the nuclei and cytoplasm.

2.9 | Statistical analysis

One-way analysis of variances (ANOVAs) were performed to assess differences in protein secretions and protein phosphorylation after 24 hr. Data were mostly normally distributed and nonparametric *t* tests (Mann–Whitney) were performed in a few cases were the data were not normalized. Results were normalized to protein content. Two-way ANOVAs were performed to detect differences in IL-6 secretion and LDH activity using time and treatment as factors for the chronic inflammation experiment. Multiple comparisons were adjusted for by Dunnett's statistical hypothesis test. All statistical analyses were performed using Prism 7 for Windows (Version 7.01; GraphPad Software Inc.). *p* Values were considered significant at $p < .05$.

3 | RESULTS

3.1 | HAEC responses to acute OCN

No changes in protein secretions (VCAM-1, IL-8, ICAM-1, IL-10, IL-6, or MCP-1) were observed in response to ucOCN in HAECs after 24 hr (Figure 1a–f). Furthermore, total protein content levels did not differ between ucOCN and vehicle (Figure S1a). No intracellular signaling protein phosphorylation (CREB, NFkB, p38, JNK, AKT, ERK, STAT3, STAT5, or p70s6k) was affected by ucOCN (Figure 2a–i).

3.2 | HAEC responses to acute inflammation

Acute inflammation significantly increased the secretion of ICAM-1, VCAM-1, IL-6, IL-8, and MCP-1 ($p < .001$; Figure 1a,b,d–f). IL-10 secretion was not affected (Figure 1c). Phosphorylation of CREB, JNK, p38, ERK, AKT, STAT3, and STAT5 was increased with inflammation (Figure 2a,b,d–f,h,i). Phosphorylation of NFkB and p70s6k was not affected (Figure 2c,g). There were no significant differences in cell signaling (CREB, p38, JNK, ERK, AKT, STAT3, STAT5, NFkB, p70s6k), protein secretion, or total protein content between cells treated with or without ucOCN alongside inflammation after 24 hr.

3.3 | Human aortic endothelial cell responses to chronic OCN

In the chronic experiments, IL-6 secretion increased over time but was not affected by OCN (10 ng/ml) after 48, 96, or 144 hr incubation (Figure 3a). Similarly, no effects of ucOCN were observed on LDH activity, IL-10 secretion, or total protein content (Figure 3c–f).

3.4 | Human aortic endothelial cell responses to chronic inflammation

Because we found no anti-inflammatory effects of ucOCN in the acute inflammatory protocol, we next established whether any effects become apparent after prolonged inflammation and ucOCN treatment. IL-6 secretion increased over time with the inflammatory protocol, with

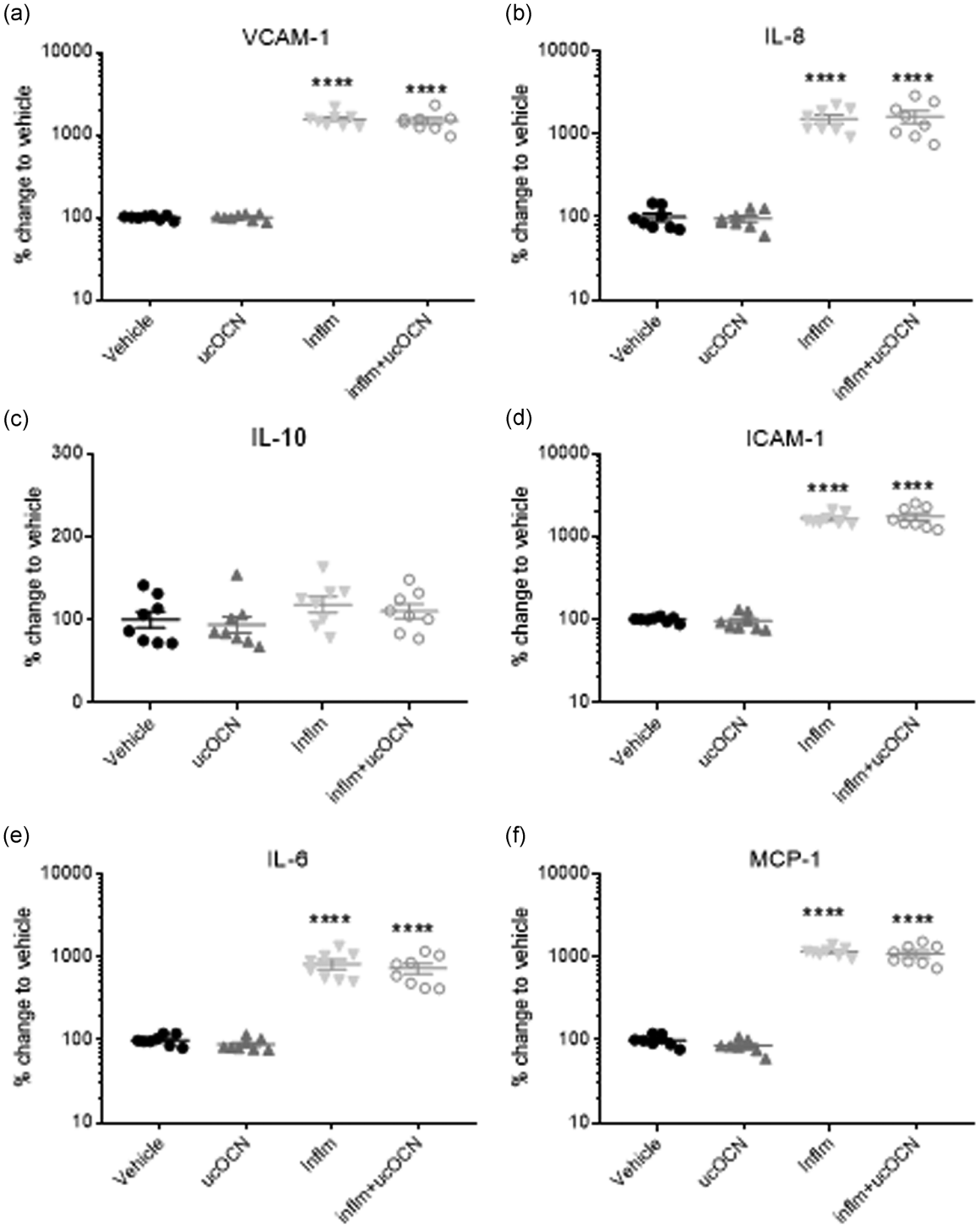

FIGURE 1 Protein secretion responses in HAECs after acute inflammation. Mean % change relative to vehicle and *SEM* of secreted cell proteins VCAM-1, ICAM-1, IL-6, endothelin, IL-10, IL-8, and MCP-1 when treated with vehicle, ucOCN (10 ng/mL), an inflammatory protocol (IFN-γ and TNF-α, both 10 ng/ml, 8 hr followed by 16 hr, respectively), or inflammatory protocol and ucOCN. Data were normalized to total protein content. Data were analyzed by one-way ANOVA. **** indicates $p < .001$ compared with vehicle. HAEC, human aortic endothelial cell; ICAM-1, intracellular adhesion molecule-1; IL, interleukin; inflm, inflammatory protocol; MCP-1, monocyte chemoattractant protein-1; ucOCN, uncarboxylated osteocalcin; *SEM*, standard error of mean; VCAM-1, vascular cell adhesion molecule-1

a peak observed at 48 hr, but ucOCN demonstrated no additional affects (Figure 3b). Nicotinamide adenine dinucleotide H concentration was measured as an indicator of LDH activity. LDH activity increased sharply following 48 hr of inflammation in HAECs, following a similar trajectory as with IL-6 production (Figure 4d). There were no differences detected between cells treated with ucOCN alongside inflammation and those treated with inflammation alone (Figure 3d). IL-10 secretion at 144 hr did not differ between treatment groups

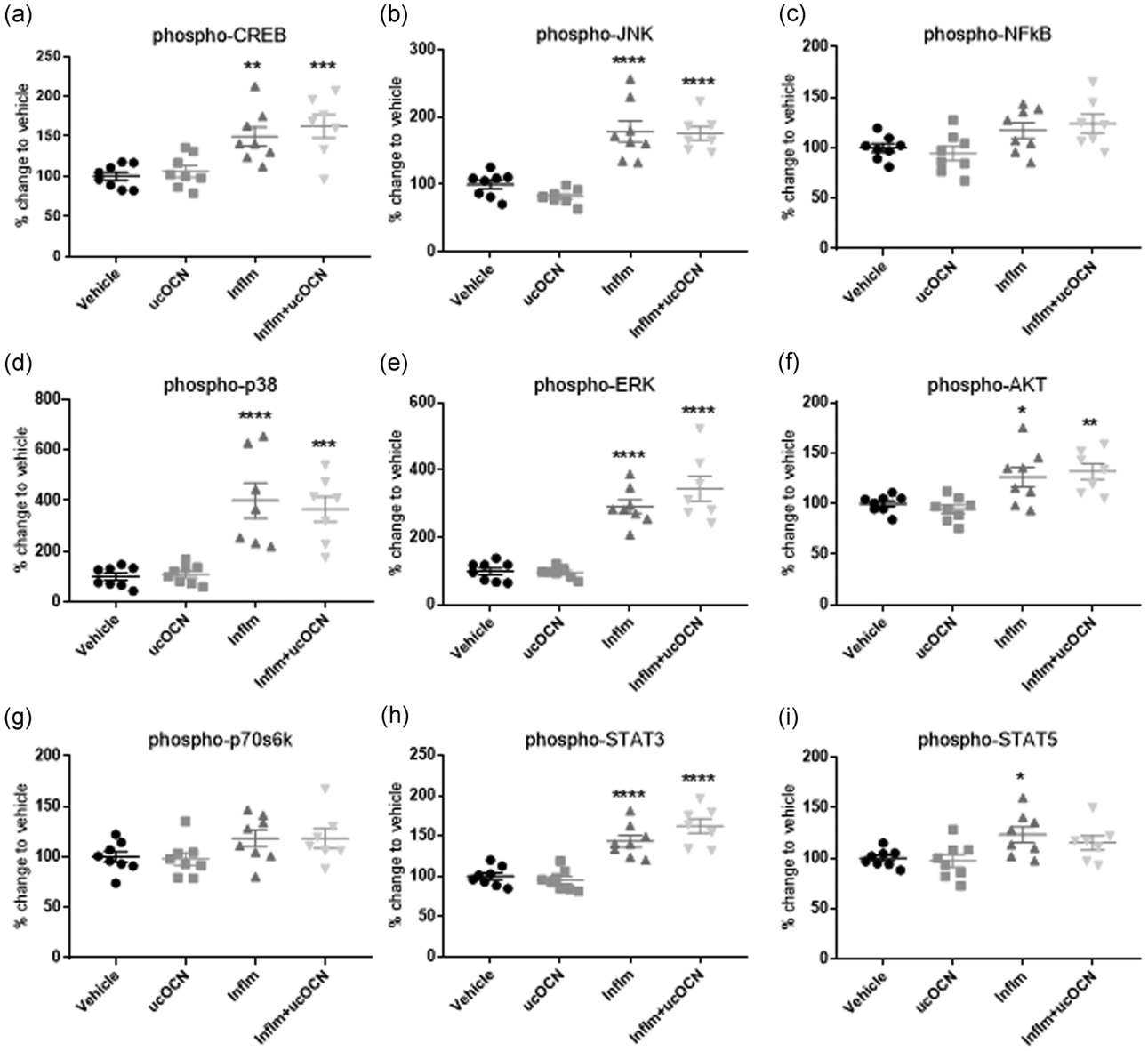

FIGURE 2 Intracellular signaling responses in HAECs. Luminex® xMAP® technology was used to detect changes in phosphorylated CREB (pS133), JNK (pT183/pY185), NFkB (pS536), p38 (pT180/pY182), ERK (pT185/pY187), Akt (pS473), p70 S6K (pT412), STAT3 (pS727), and STAT5A/B (pY694/699; 48-680MAG; Milliplex™; Merck Millipore) in cell lysates when treated with vehicle or ucOCN (10 ng/ml) with and without inflammatory stimulus (IFN-γ and TNF-α) for 24 hr. Data were analyzed by one-way ANOVA with multiple comparisons to vehicle corrected for by Dunnett's test. Data are given as means with error bars representing *SEM*. *denotes a significant difference compared with vehicle (*$p < .05$. **$p < .01$, ***$P < .005$, ****$p < .001$). HAEC, human aortic endothelial cell; IFN-γ, interferon-γ; inflm, inflammatory protocol (8 hr of IFN-γ 10 ng/ml followed by addition of TNF-α 10 ng/mL for 16 hr); *SEM*, standard error of mean; TNF-α, tumor necrosis factor-α; ucOCN, uncarboxylated osteocalcin

(Figure 3e). Total protein content (as an indicator of cell death) was decreased with the inflammatory protocol ($p < .05$) but no additional effect of ucOCN was observed (Figure 3f).

After 48 hr of inflammatory protocol, HAECs adopted an activated, spindle-shaped morphology as opposed to the characteristic cobblestone appearance of noninflamed cells (Figure S2).

3.5 | HASMC responses to acute OCN

No changes were observed in the secretion of ICAM-1, IL-8, MCP-1, IL-17, MMP-3, or endothelin after 24 hr treatment with ucOCN compared with vehicle (Figure 4a–f). Phosphorylation of p38, ERK, AKT, CREB, JNK, NFkB, p70s6k, STAT3, and STAT5 was not affected by ucOCN exposure (Figure 5a–i). Total protein content did not differ with ucOCN treatment (Figure S1B).

3.6 | HASMC responses to acute inflammation

ICAM-1, MCP-1, IL-8, and MMP-3 secretion was significantly increased by inflammation in HASMCs compared to vehicle ($p < .001$), however no effect of ucOCN was observed (Figure 4a–c,e). There was no significant effect of inflammation

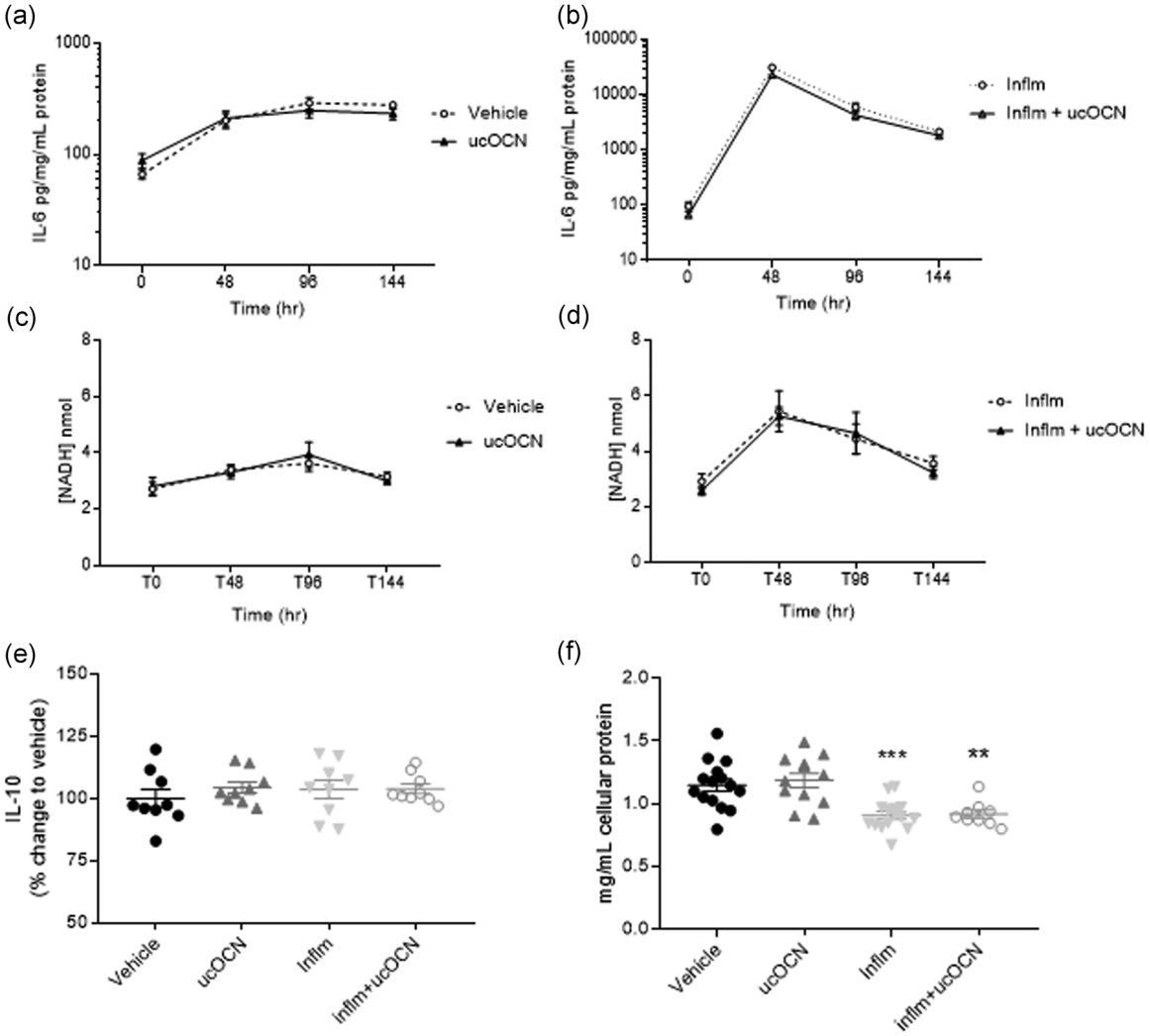

FIGURE 3 IL-6 secretion, LDH activity, IL-10 secretion, and total protein content in HAECs after chronic inflammation with or without OCN. The effects of ucOCN on secretion of IL-6 without inflammation (a) and with inflammation (b) after 0, 48, 96, and 144 hr. The effect of ucOCN on LDH activity was measured by NADH concentration without inflammation (c) and with inflammation (d) after 0, 48, 96, and 144 hr. IL-10 secretion was measured after 144 hr (e). Total protein content was measured by BCA assay at the end of the experiment. ucOCN (10 ng/ml) was added every 48 hr. Total $n = 9$ from three experimental repeats. Data are given as means with error bars representing *SEM*. *denotes a significant difference compared with vehicle, analyzed by one-way ANOVA (**$p < .01$, ***$p < .005$). ANOVA, analysis of variance; HAEC, human aortic endothelial cell; IL, interleukin; IFN-γ, interferon-γ; inflm, inflammatory protocol (8 hr of IFN-γ followed by addition of TNF-α; both 5 ng/ml); LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide H; *SEM*, standard error of mean; TNF-α, tumor necrosis factor-α; ucOCN, uncarboxylated osteocalcin

nor OCN on endothelin production or IL-17 secretion (Figure 4d,f). Phosphorylation of CREB, p38, ERK, and STAT3 was increased with inflammation but no additional effect of ucOCN was observed (Figure 5a–i).

3.7 | HASMC cell responses to chronic OCN

In the chronic experiments, IL-6 secretion increased over time but was not affected by OCN after 48, 96, or 144 hr incubation (Figure 6a). LDH activity increased over time, with no additional effect of ucOCN observed (Figure 6c). Similarly, IL-10 secretion and total protein content was not affected by OCN after 144 hr (Figure 6e,f).

3.8 | HASMC responses to chronic inflammation

Following chronic exposure to inflammation, IL-6 secretion increased in HASMCs until 96 hr and remained elevated (Figure 6b). Addition of ucOCN did not affect IL-6 levels. Similarly, LDH activity was increased with inflammation but was not affected by the coadministration of ucOCN (Figure 6d). IL-10 and total protein content were not altered by the inflammation protocol nor ucOCN (Figure 6e,f).

3.9 | OCN secretion is not detected following chronic inflammation

Finally, total OCN was measured in cell media after chronic inflammation (144 hr) to test whether inflammation induced OCN

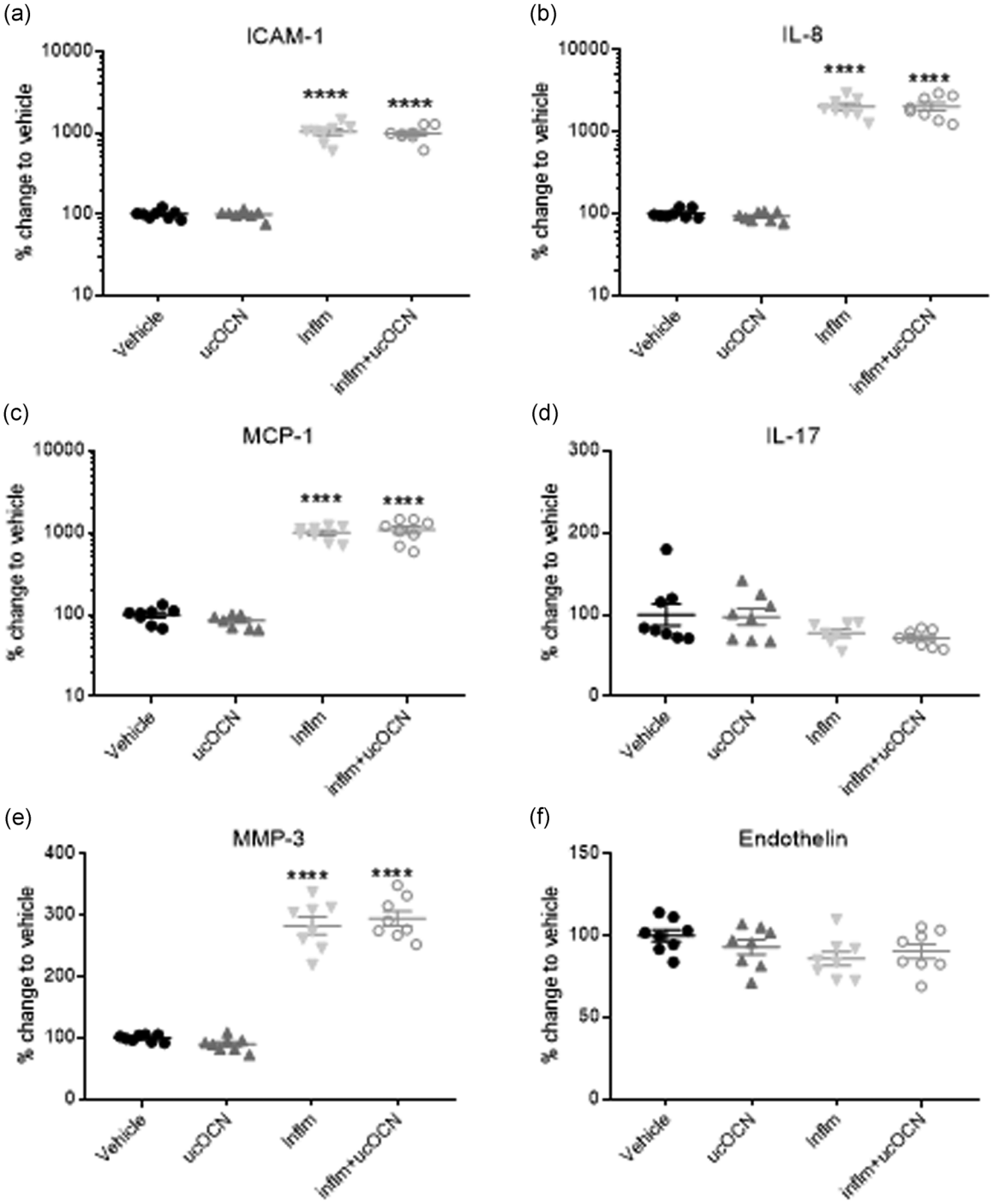

FIGURE 4 Protein secretions in HASMCs after acute inflammation. Mean % change relative to vehicle and *SEM* of secreted cell proteins ICAM-1, MCP-1, endothelin, MMP-3, IL-17, and IL-8 when treated with vehicle, ucOCN (10 ng/ml), an inflammatory protocol (IFN-γ and TNF-α, both 10 ng/ml, 8 hr followed by 16 hr, respectively), or inflammatory protocol and ucOCN. Data were normalized to total protein content. Data were analyzed by one-way ANOVA. ****denotes a significant difference compared with vehicle ($p < .005$). ANOVA, analysis of variance; HASMC, human aortic smooth muscle cell; ICAM-1, intracellular adhesion molecule-1; IL, interleukin; IFN-γ, interferon-γ; MCP-1, monocyte chemoattractant protein-1; MMP-3, matrix metalloproteinase; *SEM*, standard error of mean; TNF-α, tumor necrosis factor-α; ucOCN, uncarboxylated osteocalcin; inflm, inflammatory protocol

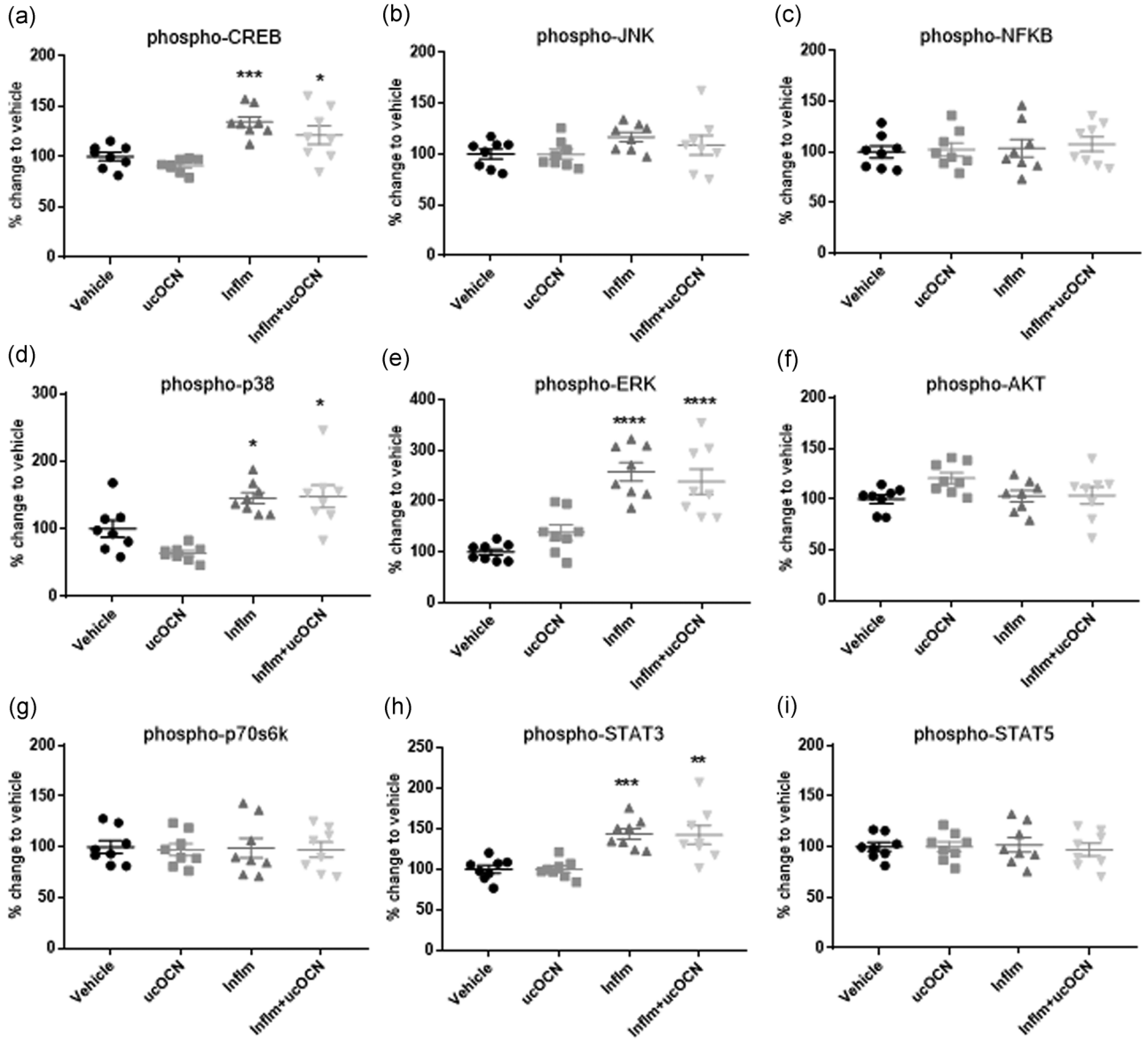

FIGURE 5 Intracellular signaling responses in HASMCs. Luminex® xMAP® technology was used to detect changes in phosphorylated CREB (pS133), JNK (pT183/pY185), NFkB (pS536), p38 (pT180/pY182), ERK (pT185/pY187), Akt (pS473), p70 S6K (pT412), STAT3 (pS727), and STAT5A/B (pY694/699; 48-680MAG; Milliplex™; Merck Millipore) in cell lysates when treated with vehicle or ucOCN (10 ng/ml) with and without inflammatory stimulus (IFN-γ and TNF-α) for 24 hr. Data were analyzed by one-way ANOVA with multiple comparison to vehicle corrected for by Dunnett's test. Data are given as means with error bars representing *SEM*. *denotes a significant difference compared with vehicle (*$p < .05$. **$p < .01$, ***$p < .005$, ****$p < .001$). ANOVA, analysis of variance; HASMC, human aortic smooth muscle cell; IFN-γ, interferon-γ; inflm, inflammatory protocol (8 hr of IFN-γ 10 ng/ml followed by addition of TNF-α 10 ng/ml for 16 hr); *SEM*, standard error of mean; TNF-α, tumor necrosis factor-α; ucOCN, uncarboxylated osteocalcin

secretion. OCN was not detectable in media from HAECs nor HASMCs at the end of the experiments (data not shown).

3.10 | OCN does not prevent LPS-induced inflammation

As ucOCN did not affect inflammation induced by exogenously added cytokines, we then tested whether LPS-induced inflammation elicited a different response. Acute LPS treatment (24 hr) significantly increased secretion of VCAM-1 and ICAM-1 in HAECs and HASMCs, respectively, compared with control (Figure 1c,d). In both HAECs and HASMCs, there were no differences in VCAM-1 or ICAM-1 secretion respectively between LPS treated and LPS + ucOCN-treated cells (Figure 1c,d).

4 | DISCUSSION

OCN is synthesized predominantly by osteoblast cells, which acts as an extra-skeletal hormone known to effect insulin sensitivity, insulin

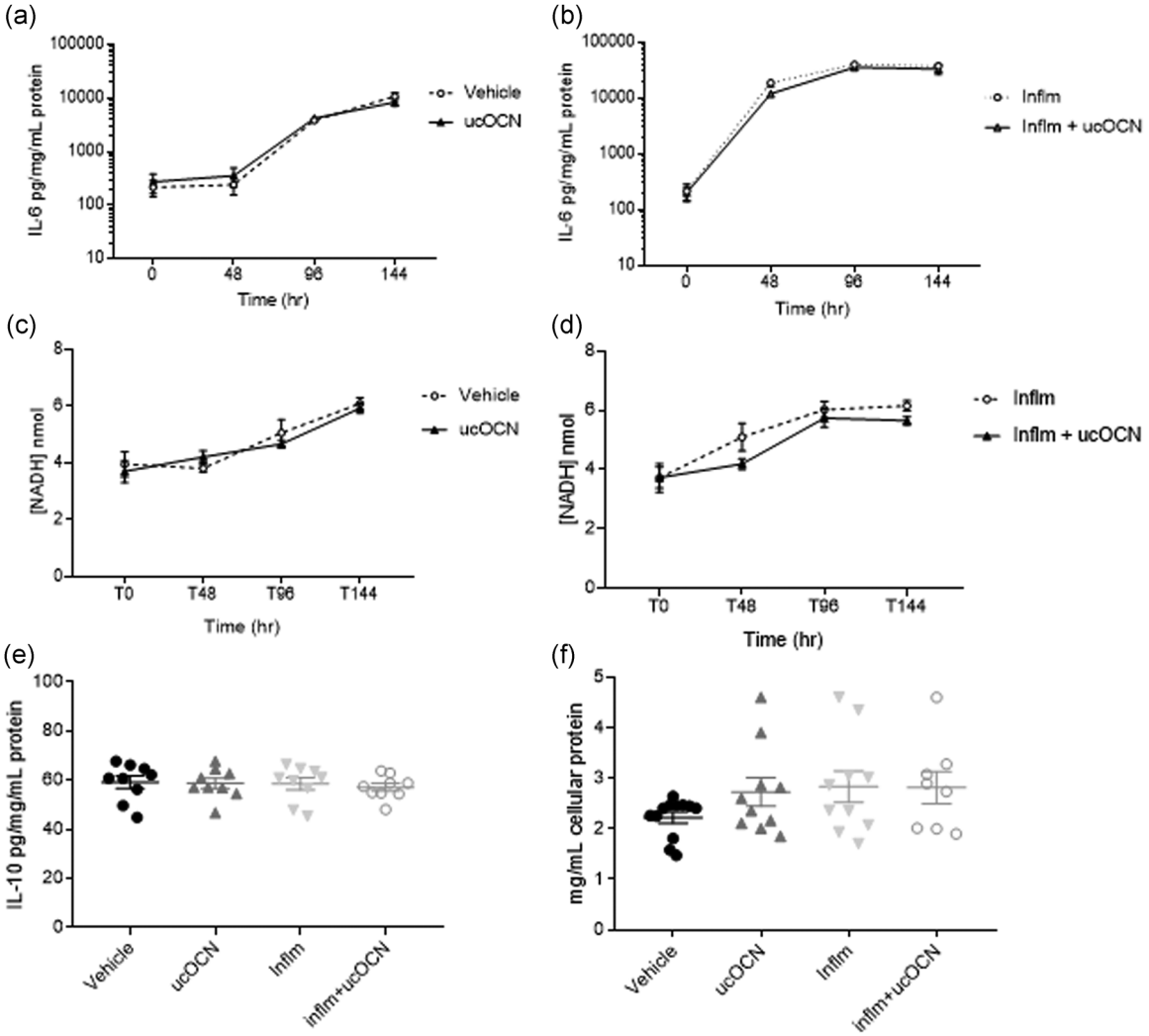

FIGURE 6 IL-6 secretion, LDH activity, IL-10 secretion, and total protein content in HASMCs after chronic inflammation with or without OCN. The effects of ucOCN on secretion of IL-6 without inflammation (a) and with inflammation (b) after 0, 48, 96, and 144 hr. The effect of ucOCN on LDH activity was measured by NADH concentration without inflammation (c) and with inflammation (d) after 0, 48, 96, and 144 hr. IL-10 secretion was measured after 144 hr (e). Total protein content was measured by BCA assay at the end of the experiment. ucOCN (10 ng/ml) was added every 48 hr. Total $n = 9$ from three experimental repeats. BCA, bicinchoninic acid; HASMC, human aortic smooth muscle cell; LDH, lactate dehydrogenase; IFN-γ, interferon-γ; IL, interleukin; inflm, inflammatory protocol (8 hr of IFN-γ followed by addition of TNF-α; both 5 ng/ml); NADH, nicotinamide adenine dinucleotide H; TNF-α, tumor necrosis factor-α; ucOCN, uncarboxylated osteocalcin

secretion, energy metabolism, cognition, and fertility (Lee et al., 2007; Oury et al., 2011; Oury et al., 2013). Population-based cross-sectional studies have reported a negative or inverse association between OCN and markers of systemic inflammation, such as IL-6 and CRP, suggesting a potential anti-inflammatory role (Chen et al., 2013; Kim et al., 2010; Liao et al., 2015; Pittas et al., 2009; Sarkar & Choudhury, 2013; Schett et al., 2006). However, the in vitro effects of OCN on inflammation within the vasculature have not yet been described. Therefore, we decided to try and reveal mechanisms of action of ucOCN during inflammation, as well as investigate the effects of acute and chronic administration of ucOCN on its own.

Here, we demonstrate for the first time using two human vascular cell types that ucOCN does not regulate or alter the inflammatory responses of HAECs or HASMCs during acute inflammation. Our observational results presented here do not reflect an anti-inflammatory role of OCN suggested by some cross-sectional, human studies, at least within the experimental models used. ucOCN did not affect inflammatory cytokine production nor inflammatory signaling pathways in HASMCs and HAECs. This may be representative of the nonbinary nature of OCN which cannot be limited to having a positive or negative influence overall in vascular pathology and physiology in humans. It may transpire that OCN plays a protective role in the later stages of atherosclerosis and have more of an impact on the process of calcification.

As no overall anti-inflammatory effects of OCN were reported in the acute inflammatory protocol, we next established whether any effects of OCN become apparent after prolonged inflammation and OCN treatment. Pro- and anti-inflammatory cytokine production as well as LDH activity were not affected by chronic ucOCN treatment, alone or in combination with the inflammatory protocol. Lastly, we further showed that in another model of inflammation using LPS,

ucOCN did not affect VCAM-1 or ICAM-1 secretion in HAECs and HASMCs respectively. An interesting observation from this study was the transition of HAECs after 48 hr of chronic inflammation to an activated morphology that is characteristic of endothelial cells under sheer stress and inflammation, representative of the early stages of atherosclerosis (Hunt & Jurd, 1998). This corresponds to the upregulation of activated endothelium markers VCAM-1 and ICAM-1 reported at 24 hr. Additionally we demonstrated that inflammation does not induce secretion of OCN itself.

The role of OCN in cardiovascular disease has had conflicting results in humans. Some longitudinal studies have reported U-shaped associations between OCN and cardiovascular-related or all-cause mortality (Kanazawa, Yamaguchi, & Sugimoto, 2011; Yang et al., 2017; Yeap et al., 2012). However, other studies have found no association between OCN and cardiovascular disease risk (Holvik et al., 2014; Hwang et al., 2015). A systematic review and meta-analysis on the association between OCN and markers of atherosclerosis or calcification in humans found no conclusive relationship (Millar, Patel, Anderson, England, & O'sullivan, 2017). Although a causal role of ucOCN in vascular inflammation was not identified in this study, further work should explore whether OCN may influence systemic inflammation via directly acting on immune cells. Additionally, the exploration of the possible biological activity of other forms of circulating OCN should not be disregarded. We recognize that phenotypic drift occurs with the isolation and subculturing of cells and we acknowledge this as a limitation of the current study. Further work should explore both the effect of OCN on intact vessels and on cells from different vascular beds including resistance and conduit vessels.

In conclusion, in our models of vascular inflammation, OCN did not display a role in the direct inflammatory responses of primary subcultured HAECs or smooth muscle cells in either an acute or chronic setting.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

S. O. S. and S. M. designed the experiments which were carried out by S. M. and I. Z. (I. Z. carried out the IL-6 and L. D. H. measurements for the chronic experiments). S. A. and S. O. S supervised the project. S. M. wrote the manuscript with input from S. O. S. and S. A.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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8. An investigation into the role of osteocalcin in high phosphate induced human arterial smooth muscle cell calcification

This chapter is presented in manuscript format and is under review in the Journal: Journal of Bone and Mineral Research. Figures and table are at the end of the document.

Statement of personal contribution: conception/design of the work, along with contributions from SOS and SA; performed the *in vitro* experiments; analysis of all data; preparation of figures and table; drafting of manuscript with input from all authors.

An investigation into the role of osteocalcin in high phosphate induced human arterial smooth muscle cell calcification

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Disclosure Page

Nothing to disclose.

Abstract

Osteocalcin (OCN) is a bone-derived protein that is detected within human calcified vascular sections. Calcification is particularly prevalent in chronic kidney disease (CKD) patients but the role of OCN in calcification, whether active or passive, has not been elucidated. The relationship between OCN, CKD and vascular calcification was assessed in CKD patients (n=28) and age-matched controls (n=19). *In vitro*, we analysed whether addition of uncarboxylated osteocalcin (ucOCN) influenced the rate or extent of vascular smooth muscle cell (VSMC) calcification. Human aortic VSMCs were cultured in control media or mineralisation inducing media (MM) containing increased phosphate with or without ucOCN (10 ng/mL or 30 ng/mL) for up to 21 days. Markers of osteochondrogenic differentiation and calcification were determined (alkaline phosphatase [ALP] activity, total intracellular OCN, Runx2 expression, α-SMA expression, alizarin red calcium staining, and calcium quantification). Calcification was present in our sample population (mean age 76 years), but no differences were detected between CKD patients and controls. Plasma OCN was increased in CKD patients compared to controls (14 ng/mL vs 9 ng/mL; $p<0.05$) and correlated to estimated glomerular filtration rate ($p<0.05$), however no relationship was detected between OCN and calcification. *In vitro*, ALP activity, α-SMA expression and calcium concentrations were significantly increased in MM treated VSMCs at day 21, but no effect of ucOCN was observed. Cells treated with control media+ucOCN for 21 days did not show increases in ALP activity nor calcification. In summary, although plasma OCN was increased in CKD patients, this study did not find a relationship between OCN and calcification in CKD and non-CKD patients, and found no *in vitro* evidence of an active role of ucOCN in vascular calcification as assessed over 21 days. OCN may rather be a by-product than mediator of vascular calcification, but further investigation is warranted.

Key words: osteocalcin, calcification, CKD, vascular, mineralisation

Introduction

Vascular calcification is a known major risk factor for mortality and morbidity, particularly within chronic kidney disease patients, and is an independent risk factor for cardiovascular disease (1-3). CKD-mineral bone disorder (CKD-MBD) entails derangements in mineral metabolism, bone remodelling abnormalities, and accelerated medial and intimal calcification, which worsens under haemodialysis (4). In stage 5 CKD patients, calcification in particular is driven by vascular apoptosis and osteochondrogenic differentiation triggered by increased phosphate levels (4). Calcification involves the progressive deposition of calcium within vessels, reducing elasticity and impairing cardiovascular function by promoting mechanical failure (5). As the human population continues to age and increase longevity, the consequences of such diseases are further pronounced. Long believed to be a passive part of ageing 'wear and tear', vascular calcification is now considered an active, cell-mediated complex process that is a regulated form of extracellular matrix bio-mineralisation but is not yet fully understood (5, 6).

In bone, bio-mineralisation occurs via endochondral ossification or membranous ossification programmed by chondrocytes and osteoblasts, initiated by matrix vesicles whose function is nucleation and growth of calcium crystals. Vascular smooth muscle cells (VSMCs) can trans-differentiate into osteoblast or chondrocyte-like cells displaying osteogenic fingerprints generally characterised by a decrease in smooth muscle cell markers (e.g. α-SMA, SM-MHC) and an increase in osteochondrogenic markers such as alkaline phosphatase (ALP), Runx2, SOX9, and osteocalcin (6, 7). In a remarkably similar way to bone, differentiated VSMCs demonstrate hydroxyapatite production and mineralisation (6). Hydroxyapatite crystals form within matrix vesicles secreted from the membranes of osteoblasts, odontoblasts, and chondrocytes (8). These buds provide a nidus for calcium, phosphate and mineral nucleation which is then deposited in the extracellular matrix between collagen fibrils (8). This active osteogenic process can be triggered by oxidative stress, oxylipids, phosphate, inflammatory oxylipids and oxLDL (6).

Osteocalcin (OCN) is the most prominent non-collagenous protein found in the bone extracellular matrix, predominantly produced by osteoblasts and can be found in the circulation following bone resorption (9, 10). Osteocalcin has two main forms, carboxylated osteocalcin (cOCN) and uncarboxylated osteocalcin (ucOCN). The carboxylation process is vitamin K dependent and induces a high affinity of osteocalcin for the calcium ions present in

hydroxyapatite. OCN is additionally expressed by differentiated osteoblast-like VSMCs (7, 11). Interestingly, it has been shown that OCN is not required for bone mineralisation in mice (12, 13). It has been reported that OCN may delay nucleation and growth of hydroxyapatite in pig bone, and if this is also the case in differentiated VSMCs, OCN may be viewed as a vascular calcification inhibitor (14). Embedded OCN in calcified vascular regions has been positively correlated to the extent of vascular calcification in humans, but circulating concentrations have had conflicting reports (15).There has been very little experimental evidence documented on the role of OCN in calcification, and none to date in human cells nor using ucOCN. In mice, it has been demonstrated that OCN stimulates glucose utilisation and promotes VSMC mineralisation and osteochondrogenic differentiation, in particular through HIF-1α activation (16).

Investigation of OCN in human VSMCs is required to clarify its physiological importance. We measured circulating concentrations of OCN in chronic kidney disease patients and in controls, and analysed this alongside vascular calcification data. *In vitro*, we hypothesised that the addition of ucOCN at physiological and pathophysiological concentrations to human aortic VSMCs may increase the speed or extent of osteochondrogenic calcification.

Materials and Methods

Patients

Investigations were performed on baseline data from a single-centre cohort of blood pressure-controlled hypertensive CKD patients (n=28) and age-matched controls (n=19) (see Table 1 for patient demographics) (17, 18). The study was originally approved by the Local Regional Ethics Committee and all patients gave informed consent. Plasma levels of osteocalcin (OCN) were measured using a commercially available assay (Milliplex MAP Human Bone Magnetic Bead Panel Cat no HBNMAG-51K, MerckMillipore). Multislice computed tomography (MSCT) was used to quantify calcification in a standardized section of the superficial femoral artery. Each slice was scored individually and a calcification score was generated. Calcification was considered to be present if an area ≥1 mm displayed a density >130 Hounsfield units (19).Validation studies confirmed that the scoring technique is highly reproducible. Inter-observer reproducibility between the investigator and a consultant radiologist was assessed in a 1-in-20 sample. The intraclass correlation was 1 (confidence interval [CI] 1 to 1) and the CoV was 3.9%. Repeatedly scored scans showed an intra-observer intraclass correlation of 1 (CI 1 to 1) and a CoV of 2.4%. Carotid-femoral pulse wave velocity (PWVcf) was assessed by ECG-gated applanation tonometry using a SphygmoCor® (AtCor Medical Pty Ltd., Australia). Non-invasive continuous pulse wave analysis was used to determine hemodynamic variables, described previously (17).

Cell culture

Primary human aortic smooth muscle cells (HASMCs) were obtained from PromoCell (UK) and maintained at 37°C in a humidified incubator supplemented with 5% CO2 in commercially available smooth muscle cell growth media (PromoCell, UK). Cells were not used beyond passage 5. Human osteoblasts (HOBs) were originally isolated from human femoral head trabecular bone and have been characterised previously (20-22). HOBs were cultured in osteoblast growth media (PromoCell, UK) and maintained as above. After experimental treatments, cell media and cell lysates were collected and frozen at -80°C prior to analysis.

Osteocalcin

Human fully uncarboxylated osteocalcin (ucOCN; amino acids 1-49, [Glu17,21,24]) was purchased from AnaSpec Inc. CA (AS-65307). The same batch of ucOCN has been

previously shown to be biologically active in vascular cells in our previous work (Millar et al., 2019). Additionally, samples of ucOCN were routinely measured by duoset ELISA (see osteocalcin quantification section below) to monitor stability and consistent concentration throughout the experimental period. We used ucOCN as it has previously been deemed the 'active' form of osteocalcin within the circulation and is present in higher concentrations than cOCN. Based on our patient data (Figure 1B), 10 ng/mL and 30 ng/mL concentrations of ucOCN were chosen.

Calcification experiments

For inducing calcification, cells were grown in commercially available mineralisation media (PromoCell, UK; C-27020) containing elevated phosphate concentrations similar to those used in the published literature to induce calcification (personal communication with PromoCell) for up to 21 days. Cells were treated with or without ucOCN (10 ng/mL or 30 ng/mL). Media and ucOCN were replaced every 3rd day. All experiments were performed independently at least three times, with a minimum n=2 for each condition at each time point, with the exception of the HOBS experiments which were performed twice.

Osteocalcin, MMP-3 and IL-1β and quantification

Total human intracellular and extracellular osteocalcin was measured using an enzyme linked immunosorbent (ELISA) duoset assay (R&D systems, DY1419). Whole cell lysates and spent cell culture media were collected on days 0, 6, 12 18 and 21. Secreted human total matrix metalloproteinase-3 (MMP-3) and interleukin-1β (IL-1β) were measured using ELISA kits (R&D systems, DY513 and DY201). Spent cell culture media were collected on days 0, 6, 12, 18 and 21. Assays were performed according to manufacturer's instructions.

Total protein quantification

A bicinchoninic acid protein (BCA) assay was performed to quantify the total protein content in cell lysates at days 0, 6, 12, 18 and 21 (23). The BCA working reagent was prepared by mixing BCA solution with copper (II) sulphate pentahydrate 4% solution (Sigma-Aldrich, UK) at a 50:1 ratio. Protein concentrations of samples were determined by interpolation against a bovine serum albumin standard curve.

Alizarin red staining and calcium quantification

Alizarin Red, or 1,2-dihydroxyanthraquinone was used to stain hydroxyapatite mineralized matrixes in cell monolayers producing a red-orange colour. Alizarin Red powder (Sigma Aldrich) was dissolved in dH_2O to make a 40mM solution, and pH adjusted to 4.1-4.3 with 0.5% ammonium hydroxide. Cells were fixed with 10% (v/v) formaldehyde (Sigma Aldrich) at room temperature for 15 minutes. The monolayers were then washed twice with excess dH_2O. Alizarin Red solution was then added to each well and incubated at room temperature for 20 minutes. The unincorporated dye was then removed and the plates were washed 4 times with excess dH_2O. To extract and quantify the incorporated dye, 10% (v/v) acetic acid was added to each well. The cell layer mixture in acetic acid was then collected into eppendorfs, vortexed, and overlaid with mineral oil. The eppendorfs were heated to 85◦C for 10 minutes and transferred to ice to cool. The samples were centrifuged at 20,000 x g for 15 minutes and the supernatants removed and neutralised with ammonium hydroxide (10 % v/v). Colorimetric detection was then carried out at 405 nm and data expressed as absorbance.

Calcium content was measured using a calcium detection assay kit (Abcam, ab102505) according to manufacturer's instructions. Briefly, cells were decalcified overnight with 0.6M hydrochloric acid (HCL). The calcium contents of the supernatants were then quantified using the 0-cresolphthalein method in which a chromogenic complex is formed between calcium ions and 0-cresolphthalein and then measured at 575 nm using a spectrophotometric plate reader (24). Calcium quantification was performed on day 0 and day 21.

Alkaline phosphatase (ALP) activity

ALP activity was measured using an ALP detection assay kit (Abcam, ab83369) according to manufacturer's instruction. Briefly, p-nitrophenyl phosphate (pNPP) was used as a phosphatase substrate which turns yellow when dephosphorylated by ALP and absorbance was measured at 405 nm using a spectrophotometric plate reader. ALP activity was measured on days 0, 6, 12, 18 and 21.

α-SMA, Runx2 and Sox9 protein expression

Cell lysate supernatants were collected and protein samples (10 µg/lane) were resolved by electrophoresis on 10% Mini-protean TGX precast gels (Bio-Rad Laboratories, Inc., UK). The proteins were wet transferred to a nitrocellulose membrane. Protein bands were visualised by staining with Ponceau S stain and imaged to quantify total lane protein as

previously described (25). Membranes were then incubated in blocking buffer followed by incubation with either rabbit anti-human smooth muscle alpha actin (Abcam, ab32575, 1:2500 dilution), goat anti-human Runx2 (R&D systems, AF2006, 1:2000 dilution), or goat anti-human Sox9 (R&D systems, AF3075, 1:400 dilution) overnight at 4◦C. The membrane was then washed and incubated for 1.5 hours at room temperature with alkaline phosphatase conjugated anti-rabbit secondary antibody (Sigma, Catalogue No. A3937, 1:25000 dilution in 3% marvel in TBST) or anti-goat secondary antibody (Abcam, ab97097, 1:5000 dilution in 3% marvel in TBST). Immunoreactive bands were visualised by chemiluminescence (Bio-Rad Immun-Star™ AP Substrate Pack #1705012). Protein bands were visualised using the ChemiDoc™ MP Imaging system with Image Lab™ software (Bio-Rad). Proteins were normalised to total lane protein as determined by Ponceau S staining.

Statistical analysis

For the population data, univariate comparisons of continuous variables between CKD patients and non-CKD controls were performed using parametric or non-parametric (Mann-Whitney) t-tests with or without Welch's correction depending on distribution and variance as appropriate. A one-way ANOVA was used to assess differences in OCN concentrations between controls, CKD stage 3, and CKD stage 4 patients correcting for multiple comparisons with Dunnett's multiple comparison test. Spearman's correlation tests were performed to assess the relationships between OCN and other biological measurements including estimated glomerular filtration rate (eGFR) and cardiovascular parameters. Data are presented as means and standard deviation (SD) for parametric data, and median and interquartile range for non-parametric data are presented. For the *in vitro* data, two-way ANOVAs were used to assess differences between groups using day and treatment as factors for ALP activity, OCN quantification, MMP-3 quantification, IL-1β quantification and total protein quantification. One-way ANOVAs were used to assess differences between groups for Runx2, α-SMA, and Sox9 quantification, calcium quantification, and alizarin red staining quantification. Data are presented as means and standard error of the mean (SEM). Multiple comparisons were adjusted for by Dunnett's statistical hypothesis test. All statistical analyses were performed using Prism 8 for Windows (Version 8.01, GraphPad Software Inc.). P-values were considered significant at $p<0.05$.

Results

Patient characteristics

The clinical characteristics of CKD patients and age-matched non-CKD controls are summarised in Table 1. CKD patients (stage 3 and 4, n=29) had significantly higher serum creatinine and urinary protein to creatinine ratio and significantly lower eGFR and haemoglobin compared to controls (n=19), as expected (Table 1). Other demographics and clinical parameters were similar between the two groups.

Mean plasma OCN concentration in controls was 9 ng/mL (± 4 ng/mL SD; n=19), mean OCN in stage 3 CKD patients was 11 ng/mL (± 5 ng/mL SD; n=20), and stage 4 CKD patients had a mean OCN concentration of 22 ng/mL (± 7 ng/mL SD; n=6). OCN was significantly increased in CKD stage 4 patients compared to controls ($p<0.001$; Figure 1A), and in CKD patients as a whole (mean 14 ng/mL ± 9 ng/mL) compared to controls ($p<0.05$). OCN was significantly correlated to eGFR in the total population, in that there was a significant increase in OCN concentrations when eGFR decreased (n=43; $p<0.05$; Figure 1B). Vascular calcification was detected in our sample population (mean age 76 years, Table 1) but calcium scores were not significantly different between CKD patients as a whole and controls, or CKD patients divided into stages compared to controls (Figure 1C). OCN was not correlated with calcium score, calcium density or pulse wave velocity within the total population (Supplemental Figure 1).

Cell morphology and protein content

VSMCs maintained in usual smooth muscle cell growth media maintained classical spindle-shaped morphology of contractile smooth muscle cells throughout the experimental time points irrespective of treatment with or without ucOCN (Figure 2). In contrast, cells maintained in osteoblast MM acquired a more cobble-stone synthetic phenotype appearing by day 4 which progressed until the end of the experiment (Figure 2). There were no visual differences between cells treated with MM with or without ucOCN.

A BCA assay showed an increase in protein content between day 0 and day 21 (Figure 3A). No significant differences were detected between any of the treatment groups and MM or ucOCN did not affect total protein content.

ALP activity

ALP regulates pyrophosphate levels and promotes calcification by reducing pyrophosphate levels, as pyrophosphate is a potent inhibitor of calcification through inhibition of hydroxyapatite formation (26). Increased ALP activity is therefore used as a classical marker of transdifferentiated smooth muscle cells and of mineralisation and calcification. ALP activity was increased in MM treated cells with and without ucOCN, significantly apparent from day 6 with continued gradual increase until day 21 (Figure 3B; $p<0.001$; days 6, 12, 18 and 21 compared to control). Cells treated with MM with and without ucOCN followed an identical trend, while cells maintained in normal smooth muscle cell media did not increase ALP activity throughout the experiment.

In a subset of experiments, cells were treated with normal smooth muscle cell media and ucOCN alone (10 ng/mL) to assess if ucOCN alone could stimulate calcification. There was no significant increase in ALP activity levels in cells treated with ucOCN which was undiscernible compared to control cells without ucOCN (Figure 3C) over 21 days.

Intracellular and secreted osteocalcin

Intracellular total osteocalcin appeared raised and fluctuated slightly over time in MM treated cells particularly at days 6 and 12 but this did not reach significance and there was no effect of ucOCN (Figure 3D). Extracellular secreted total osteocalcin, which is a marker of vascular smooth muscle cell osteoblastic differentiation, was not detected in any media samples after removing background levels already present in culture media over 21 days (data not shown).

Secreted MMP-3 and IL-1β

MMP-3 and IL-1β are associated with vascular calcification (27, 28). MMP-3 secretion increased with time in control media treated cells, and was increased compared to MM treated cells at day 21 ($p<0.0001$; Figure 3E). MMP-3 secretion did not increase over time in MM treated cells, and no differences were detected between those treated with or without ucOCN (10 ng/mL). IL-1β secretion was also higher in control cells than those treated with MM ($p<0.05$, day 21; Figure 3F). There were no differences between those treated with and without ucOCN (10 ng/ml).

Alizarin red staining and calcium quantification

Alizarin red staining was used to detect calcification. In half of the experiments performed, only mild calcification could be detected by alizarin red staining in MM treated cells after 21 days (Figure 4A), while in the other half of experiments strong calcification was detected (Figure 4B). A calcium quantification assay was performed which detected an increase in calcium in MM treated cells after 21 days, corresponding to the alizarin red staining ($p<0.01$, Figure 4C). In the experiments which displayed strong calcification by alizarin red staining this was confirmed by large significant increases in calcium detected using the calcium assay ($p<0.01$, Figure 4D). No calcium was detected in day 21 cells maintained in normal smooth muscle cell media. There was no differences between MM treated cells with or without ucOCN (10 or 30 ng/mL) in either the mild or strongly calcified cell experiments (Figure 4). In a subset of experiments, cells were maintained in normal smooth muscle cell media and treated with ucOCN alone (10 ng/mL). There was no detection of calcification visually or by quantification of alizarin red staining in these cells, and no differences were observed between those treated with or without ucOCN (data not shown). As a positive control, human osteoblasts (HOBs) were maintained in normal growth media or MM media. Both mild and moderate calcification was observed by visual alizarin red staining, quantification of alizarin red staining, and also by calcium quantification (Supplemental Figure 2).

Runx2 and α-SMA expression

Runx2/Cbfa1 is a master transcriptional regulator essential for ossification and is classically used as an osteochondrogenic marker. α-SMA is a classical smooth muscle cell marker and is usually decreased in osteochondrogenic differentiated VSMCs. Runx2 expression was not increased in our MM treated cells and there were no significant differences between groups (Figure 5B). However, in our positive control experiment in HOBs, runx2 was significantly increased in MM treated cells as expected (Figure 5A, $p<0.05$ compared to control). Additionally, smooth muscle cells treated with MM displayed an unexpected increased expression of α-SMA (Figure 5C). MM treated cells with ucOCN (10 ng/mL and 30 ng/mL) showed a more variable but significantly increased expression compared to control ($p<0.05$). Sox9 expression was not detectable in our samples.

Discussion

Due to conflicting epidemiological data and limited *in vitro* data in mice on the relationship between OCN and vascular calcification (15, 16), we aimed to investigate for the first time in human aortic smooth muscle cells whether OCN affects the speed or extent of vascular calcification, and to further assess any relationship between plasma OCN and CKD patients. This study found a significant relationship between circulating OCN concentrations and renal function (eGFR) of CKD patients, but found no *in vitro* evidence of an active role of ucOCN at physiological and pathological concentrations during vascular calcification as assessed over 21 days.

In our data, circulating total OCN concentrations were significantly higher in CKD patients compared to age-matched control patients, and were inversely correlated with eGFR. This is consistent with previous studies in pre-dialysis CKD patients which also found a negative relationship between OCN and GFR (29, 30). Our circulating concentrations were similar to those reported elsewhere for CKD haemodialysis patients (31). Levels of OCN-positive circulating endothelial progenitor cells have been found to be increased in haemodialysis patients compared to controls (32). OCN was not however correlated with calcification scoring or carotid-femoral pulse wave velocity, a measure of arterial stiffness, in our group as a whole. There were no significant differences between our control group and CKD group in cardiovascular measurements but perhaps if more prominent calcification was present in our sample a correlation may have been identified. There also may be a distinct difference in the role of ucOCN versus cOCN, however these different forms are not routinely measured and could not be investigated. The patients assessed were stage 3 and stage 4 CKD patients, and it is possible that stage 5 CKD patients would have higher concentrations of circulating OCN, and thus a relationship with calcification may become apparent. In our meta-analysis of the relationship between OCN and vascular calcification, no conclusion could be drawn on the relationship due to heterogeneous data and conflicting results (15). In the present study, the results also do not promote the viewpoint of OCN having a causal effect on vascular calcification and is not recommended for use as a sensitive marker of calcification.

Our *in vitro* findings further reject a hypothesis for the direct involvement of OCN in vascular calcification. After 21 days, cells cultured in mineralisation inducing media had calcium depositions and increased ALP activity, alongside distinctly altered morphology. Control cells did not have any calcium detected, nor increases in ALP, and retained classical

vascular smooth muscle cell morphology. The addition of ucOCN at two concentrations (based on levels measured in our CKD patients) did not affect the endpoints examined, suggesting that OCN does not have a direct role in vascular calcification but is rather a by-product of osteochondrogenic transdifferentiation. Furthermore, addition of ucOCN alone to control cells did not increase ALP activity nor induce calcification.

Despite components of the osteogenic fingerprint of transdifferentiated vascular smooth muscle cells being observed (such as increased ALP, calcium, and OCN), a couple of unexpected results were also obtained. Differentiated VSMCs displayed an increased expression of α-SMA, which is usually decreased in osteochondrogenic differentiated cells, and no changes in Runx2 expression, which is usually increased (6). Although these findings were unexpected it is widely appreciated that VSMCs possess remarkable phenotypic flexibility and it may simply transpire that the cells in these experiments represent an earlier phenotype before complete differentiation and widespread calcification. The mechanisms of vascular calcification and endochondral transitioning is a complex research area that is not fully understood. The increase in α-SMA however is interesting and it has been reported elsewhere that a higher expression has been observed in mineralised nodules in aortic VSMCs (33). Of note, in calcific aortic stenosis, the smooth muscle cell phenotype remains, and in myofibroblast differentiation and calcification, Runx2 and α-SMA are dually increased in the osteogenic/osteoblastic phenotype (34, 35). As the primary endpoint for our study was to examine the effect of ucOCN on vascular calcification, we did not further investigate the effects on other osteogenic markers. As multiple sub-populations of VSMCs exist, including synthetic, contractile, and particularly calcifying prone cell phenotypes, we recommend other sources of VSMCs are investigated with ucOCN to confirm our findings (36, 37). Lastly, it may transpire that carboxylated osteocalcin (cOCN), which has a higher affinity for calcium ions, may be more relevant in vascular calcification and further studies should also address this specifically.

In mildly-calcified cells ucOCN did not increase calcification, and in strongly-calcified cells ucOCN did not decrease calcification. Previously, OCN has been proposed to be involved in the regulation of arterial calcification as it is present in calcified regions in humans (15). In a rabbit *in vivo* model, OCN was detected in 8- and 14-day calcified structures but not earlier, suggesting OCN may not be involved in the initiation of calcification but rather later regulation (Gadeau et al., 2001). OCN levels increased with osteochondrogenic differentiation in two different mice cell lines, chondrocytes and VSMCs, and when

overexpressed, OCN functions as a stimulator of differentiation and mineralization, upregulating Sox9, Runx2, collagen type X, ALP, proteoglycans, and mineral content in both of these cell types (Idelevich et al., 2011). In matrix GLA protein null mice ($MGP^{-/-}$) OCN did not display any anti-mineralisation function in arteries nor did over-expression of OCN in osteoblasts inhibit normal mineralisation in bone (13). A correlation between aortal calcification and elevation of OCN in 1,25(OH)2D3-treated rats, which was hampered by OCN siRNA silencing, has also been shown (Idelevich et al., 2011). Importantly however, these studies have not been performed in humans or human cells, thus this study is the first to examine the effects of ucOCN in human VSMCs.

Our examination of IL-1β and MMP-3 secretion revealed some interesting insights. IL-1β has emerged in recent years as a potential stimulator of vascular calcification for example by increasing ALP activity, and has been proposed as a marker of inflammatory calcification (27, 38). In contrast, we found significantly increased secretion of IL-1β over time in control cells only, compared to mineralisation media treated cells. It may transpire that immune cell secreted IL-1β contributes to calcification of VSMCs, but IL-1β secreted from VSMCs themselves does not induce calcification. At least in our experiments, VSMC secreted IL-1β may even be protective, as calcified cells did not have increased levels. Differential cell specific actions of IL-1β have previously been demonstrated, for example addition to chondrocytes *in vitro* was shown to inhibit ALP activity (38). Similarly, MMP-3 secretion was increased over time in control cells only, particularly apparent at day 21. MMP-3 is required for the degradation of the extracellular matrix and has been associated with vascular calcification, particularly within atherosclerotic plaques (28). However, our results would suggest that MMP-3 secretion from VSMCs may be protective against calcification as control cells did not calcify. This may be due to differences in models of calcification used, and more pro-inflammatory and atherosclerotic models may show different effects of IL-1β and MMP-3. Importantly however, there was no inhibitory or stimulatory effect of ucOCN on either IL-1β or MMP-3 secretion.

Conclusions

OCN has been consistently detected in vascular calcification plaques. However, circulating OCN levels were not correlated with calcification or pulse wave velocity in our study population. ucOCN over 21 days did not, in either mild or strong calcification instances, increase the speed or extent of osteochondrogenic calcification of VSMCs, nor showed any

inhibitory effects. The results presented in this study suggest that OCN is likely a by-product of calcification and transdifferentiated osteochondrogenic vascular cells, and is unlikely to contribute directly to the process itself. Further investigations of other circulating forms of OCN and in other vascular cell types and conditions are recommended to confirm these findings.

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Authors' roles

Study design: SM and SOS. Study conduct: SM, SOS, CWM, SGJ. Data analysis: SM. Data interpretation: All authors. Drafting manuscript: SM. Revising manuscript content: All authors. Approving final version of manuscript: All authors.

Figure legends

Figure 1. (A) Mean (± SD) osteocalcin (OCN) concentrations of stage 3 CKD patients, stage 4 CKD patients and age-matched controls. Differences were assessed by one-way ANOVA. **** indicates $p<0.001$. (B) The osteocalcin-eGFR (estimated glomerular filtration rate) relationship, assessed by Spearman's correlation ($r = -0.32$; $p<0.05$). (C) Mean (± SD) calcification scores of CKD patients and age-matched controls.

Figure 2. Human aortic smooth muscle cells (SMCs) were cultured in usual growth media (control) with or without ucOCN (10 ng/mL), or mineralisation inducing media (MM) with or without ucOCN (10 ng/mL). Photos (10X magnification) taken at days 0, 6 and 18 visualised by light microscopcopy. Control media treated cells maintained classical a SMC phenotype while MM treated cells displayed a differentiated synthetic phenotype distinctly different to control cells.

Figure 3. Human aortic smooth muscle cells were grown in usual growth media (control), or mineralisation inducing media (MM) with or without ucOCN (10 ng/mL). Total protein content (A), ALP activity (B), intracellular total osteocalcin (D), secreted MMP-3 (E), and secreted IL-1β (F) were measured at days 0, 6, 12, 18 and 21. ALP activity was also measured in control and control+ucOCN treated cells (C). Data are represented by means with error bars representing SEM. Data were analysed by two-way ANOVA using mixed effects analysis and Dunnett's test for multiple comparisons with * indicating $P<0.05$ and *** indicating $p<0.0001$ compared to control.

Figure 4. Human aortic smooth muscle cells (SMCs) were maintained in control media or mineralisation inducing media (MM). After 21 days, alizarin red staining was used to visualise calcification and calcium quantification was determined using a calcium assay. In some experiments SMCs mildly or moderately calcified (A, C) while in others they were strongly calcified (B, D). Data are presented as means with error bars representing SEM. Data were analysed by one-way ANOVA with ** indicating $p<0.01$ and *** $p<0.001$ compared to control.

Figure 5. Human aortic smooth muscle cells (SMCs) were cultured in usual growth media (control), or mineralisation media (MM) with or without ucOCN (10 ng/mL or 30 ng/mL). (A) Human osteoblasts (HOBs) were also cultured in control or MM and Runx2 expression was measured by western blotting. Expression of Runx2 (B) and α-SMA (C) in SMCs were measured by western blotting on day 21. Data are presented as means with error bars representing SEM. Data were analysed by one-way ANOVAs or t-tests with * indicating $p<0.05$.

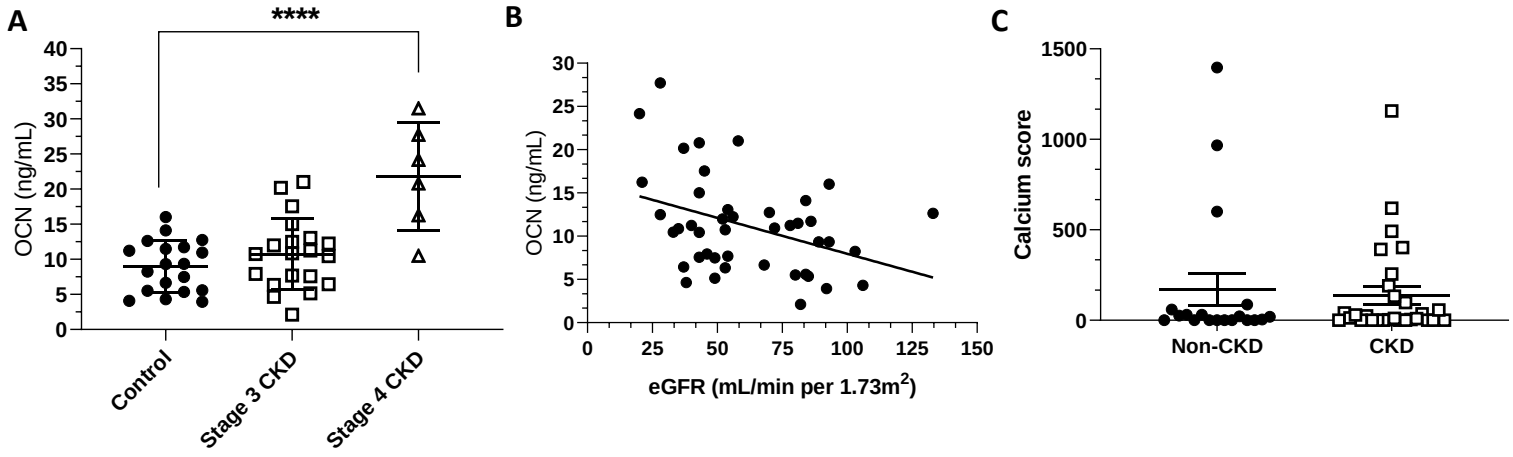
Supplemental Figure 1. Circulating total osteocalcin (OCN) concentrations were not correlated with calcification score (A), calcium density (B), or carotid-femoral pulse wave velocity (PWFcf) (C) in chronic kidney disease patients and age-matched controls (n=44).

Supplemental Figure 2. Human osteoblasts (HOBs) were maintained in control media or mineralisation inducing media (MM). After 21 days, alizarin red staining was used to visualise calcification. In some experiments HOBs mildly or moderately calcified (A) while in others they were strongly calcified (B). (C) Alizarin red staining quantification of HOBS after 21 days from 2 experiments reflecting mild and strong calcification. (D) Calcium quantification of HOBs reflecting mild and strong calcification. Data are presented as means with error bars representing SEM. Data were analysed by t-tests between control and MM within respective experiments. **** $p<0.0001$ compared to respective control.

Table 1. Population characteristics

	Non-CKD controls (mean ± SD)	**CKD patients (mean ± SD)**	**T-test (p-value)**
	N=19	**N=29**	
Gender (F/M)	7/12	13/16	
Age (years)	76 ± 4.8	76 ± 4.4	NS
BMI (kg/m²)	25.90 ± 4.00	25.40 ± 3.50	NS
Mean blood pressure (mmHg)	103.50 ± 9.80	104.00 ± 12.30	NS
Serum creatinine (µmol/L)	73.80 ± 21.00	143.70 ± 56.60	<0.0001
eGFR (mL/min per 1.73m^2)	93.11 ± 35.85	42.97 ± 13.73	<0.0001
Urine protein/creatinine ratio	0.11 ± 0.04	0.31 ± 0.42	<0.05
Haemoglobin (g/dL)	13.98 ± 1.69	12.76 ± 1.74	<0.01
Urea (mmol/L)	5.81 ± 1.79	9.67 ± 2.95	<0.0001
Corrected calcium (mmol/L)	2.37 ± 0.09	2.33 ± 0.08	NS
Phosphate (mmol/L)	1.07 ± 0.16	1.09 ± 0.14	NS
PWVcf (m/s)	13.63 ± 2.73	12.93 ± 2.40	NS
Calcium score[1]	19.00 (0.00 – 59.00)	13.99 (0.00 – 161.50)	NS
Calcification density[1]	2 (0 – 3)	2 (0 – 4)	NS

Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; PWVcf, carotid-femoral pulse wave velocity; NS, not significant. [1]*Median and interquartile range.*



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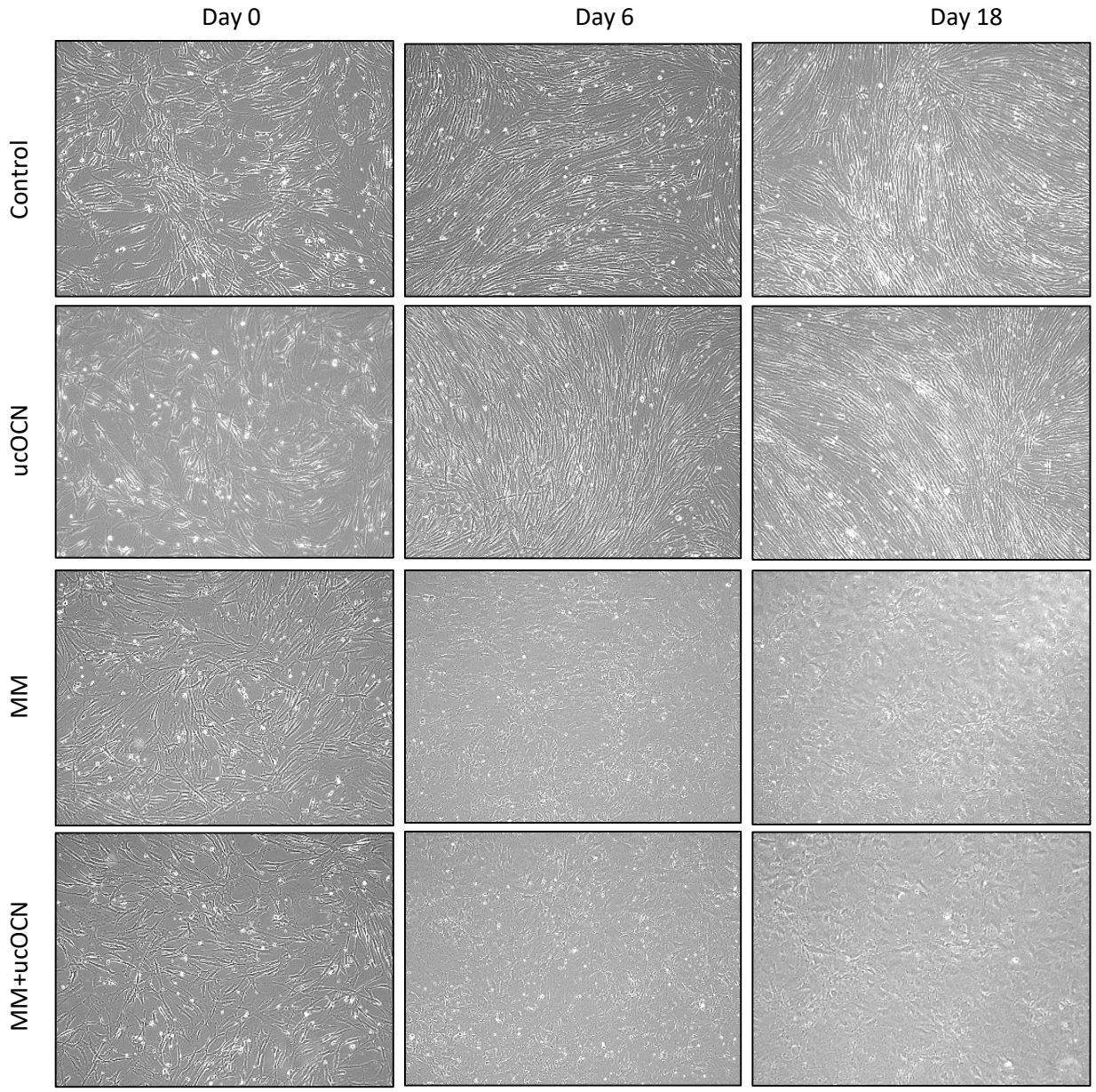

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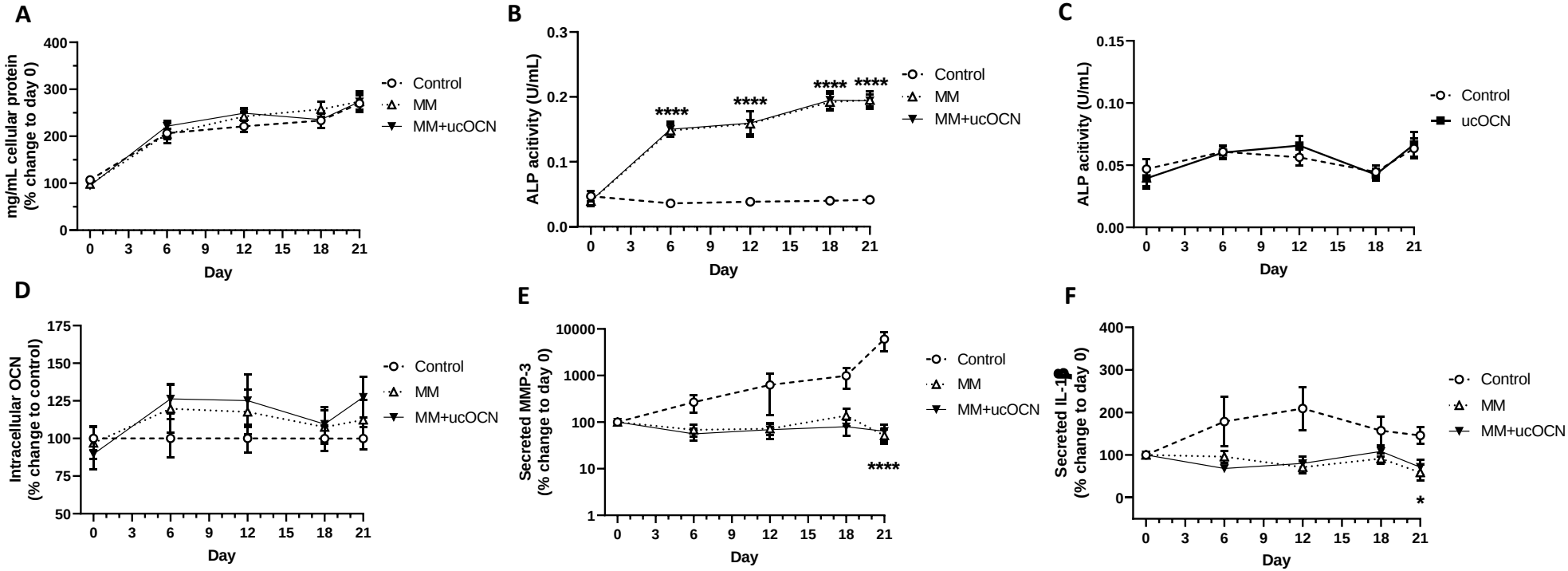

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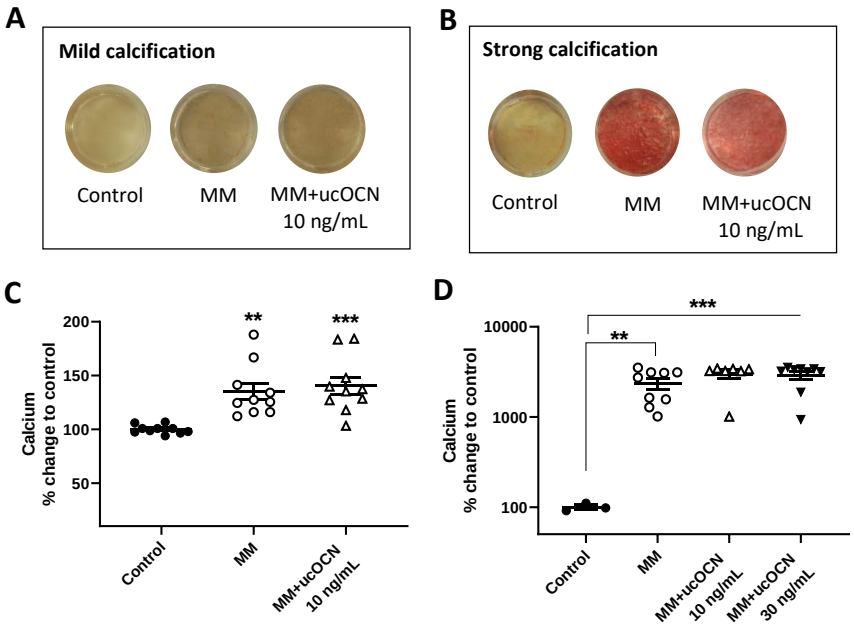

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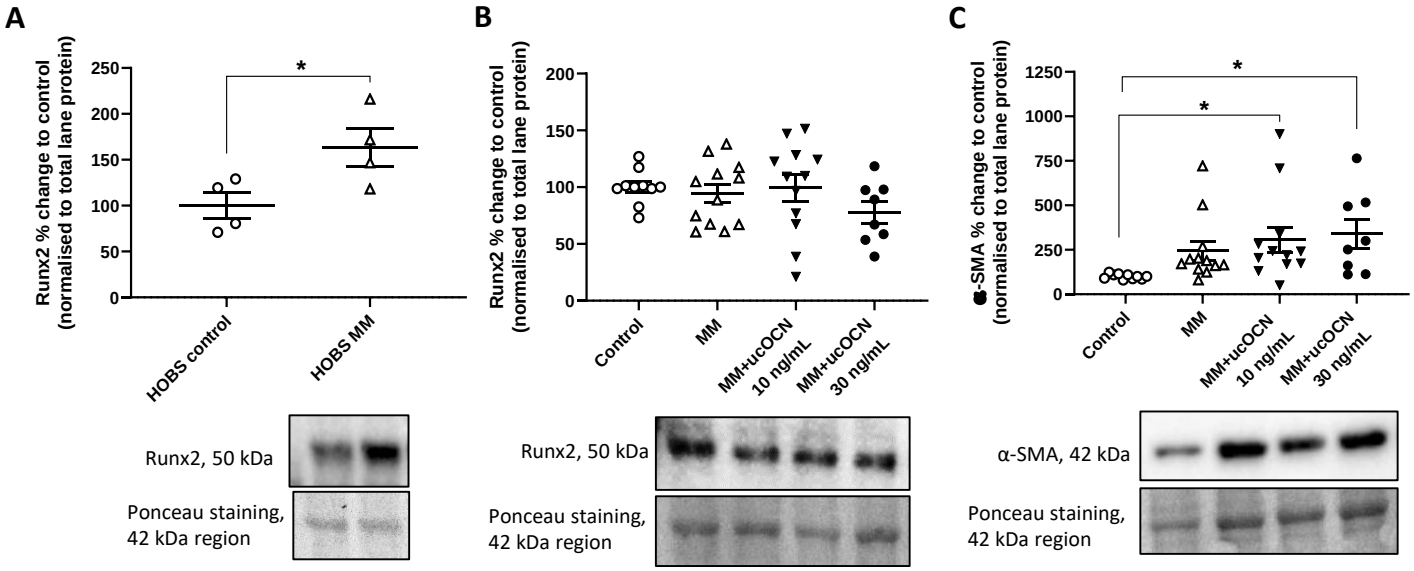

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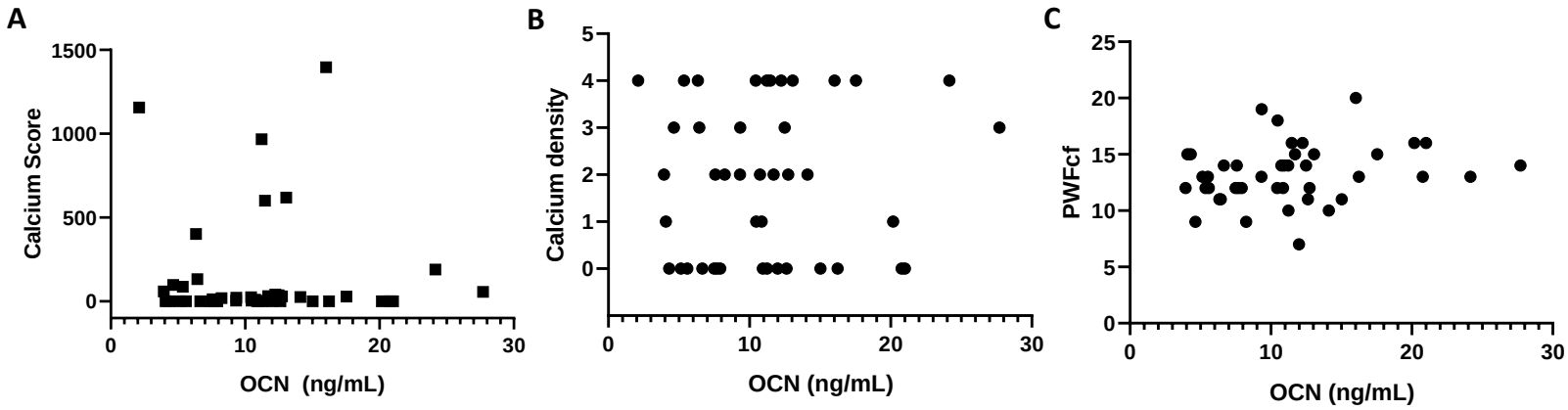
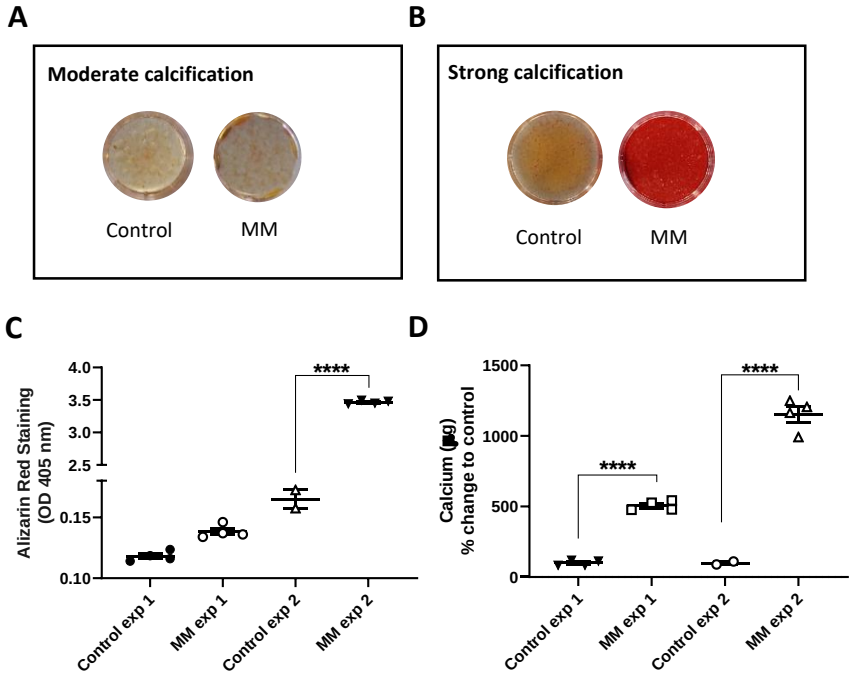


Supplemental Figure 1. Circulating total osteocalcin (OCN) concentrations were not correlated with calcification score (A), calcium density (B), or carotid-femoral pulse wave velocity (PWFcf) (C) in chronic kidney disease patients and age-matched controls (n=44), as analysed by Spearman's correlations.



Supplemental Figure 2. Human osteoblasts (HOBs) were maintained in control media or mineralisation inducing media (MM). After 21 days, alizarin red staining was used to visualise calcification. In some experiments HOBs mildly or moderately calcified (A) while in others they were strongly calcified (B). (C) Alizarin red staining quantification of HOBS after 21 days from 2 experiments reflecting mild and strong calcification. (D) Calcium quantification of HOBs reflecting mild and strong calcification. Data are presented as means with error bars representing SEM. Data were analysed by t-tests between control and MM within respective experiments. **** $p<0.0001$ compared to respective control.

9. General discussion

The revelation of the skeleton as an endocrine organ created the foundation on which the extra-osseous exploration of the osteoblast-derived peptide, osteocalcin, was established. Key studies revealed the energy regulating properties of this circulating hormone, and interest in its wider application in various disciplines including fertility, cognition, and diabetes has continued to advance (Lee et al., 2007, Karsenty, 2017).

Human observational data has reported conflicting evidence between osteocalcin concentrations and atherosclerosis and calcification, and osteocalcin has been consistently detected in atherosclerotic and calcified plaques (Millar et al., 2017). Thus, the role, if any, of osteocalcin in cardiovascular health and disease has been debated. Following extensive review of the literature (chapters 4 and 5) it was hypothesised that uncarboxylated osteocalcin (ucOCN) may be protective against atherosclerosis and inflammation, and may promote calcification (Millar et al., 2017, Millar et al., 2019c). Experiments were performed (chapters 6, 7 and 8) in order to reveal effects of osteocalcin in vascular endothelial and smooth muscle cells which may be underlying the conflicting human data, and to build upon existing animal studies. The data from the experimental work undertaken in this thesis indicated that despite initiating changes in intracellular signalling, ucOCN does not affect processes of vascular angiogenesis, protect against inflammation, or promote calcification (Millar et al., 2019b, Millar et al., 2019a).

Previous investigations into the effects of osteocalcin in vascular cells have been largely restricted to murine cell types or HUVECs (human umbilical vein endothelial cells), which have uncertain relevance to the human adult endothelium (Mestas and Hughes, 2004). As such, it was valuable to perform the experiments in this thesis using human aortic endothelial cells (HAECs) and smooth muscle cells (HASMCs). Indeed it has recently been suggested that osteocalcin bioavailability is controlled differently between humans and mice (Ferron et al., 2010, Dirckx et al., 2019). It may also be relevant to note that it is most common for rodent studies to be conducted in males only. Based on limited information, osteocalcin may have a more prominent role in males as has been noted in diabetes and fertility (Dhore et al., 2001, Oury et al., 2011). In our systematic review (chapter 5) we also noted that more inverse associations were

reported between osteocalcin and vascular calcification or atherosclerosis in men, whereas more positive associations were noted in women (Millar et al., 2017).

Two previous studies in HAECs demonstrated a protective anti-apoptotic effect of ucOCN and increases in eNOS and NO following treatment (Jung et al., 2013, Kondo et al., 2016). In agreement with this, we demonstrated in chapter 6 a proliferative effect of ucOCN in a concentration dependent and AKT- and ERK-pathway dependent manner in HAECs and HASMCs (Millar et al., 2019b). The remaining previous *in vitro* studies in murine cells and HUVECs demonstrated positive effects of ucOCN through improved metabolic status and improving insulin signalling (Idelevich et al., 2011, Zhou et al., 2013, Dou et al., 2014, Guo et al., 2017). However, the potential protective effects of ucOCN against hyperglycaemia and high glucose induced impairment in insulin signalling in HAECs have not yet been investigated.

ucOCN had been inversely associated in the human population with circulating inflammatory cytokines, and has been prominently demonstrated to be anti-inflammatory in both mice and rat adipose tissue and adipocytes (Guedes et al., 2017, Sarkar and Choudhury, 2013, Pittas et al., 2009). Thus we hypothesised that ucOCN may exhibit similar properties in vascular cells. However, we found no evidence of this following acute and chronic inflammatory stimulus in HAECs and HASMCs (chapter 7) (Millar et al., 2019a). These results potentiate osteocalcin as a hormone that lacks a direct effect in vascular inflammation. It may be worth investigating whether osteocalcin affects atherosclerosis via other related mechanisms such as immune cell infiltration. Additionally, osteocalcin protein and mRNA has been identified in human adipocytes and it would be interesting to examine whether the anti-inflammatory effects seen in rodent adipose tissue is also apparent in human adipose (Foresta et al., 2010). Adipose tissue can influence the cardiovascular system in a number of ways including impacting glucose and lipid metabolism, inflammation, and adipokine secretion (Timar et al., 2014, Freitas Lima et al., 2015). Adipokines, such as adiponectin and leptin, are similar to osteokines in that they are considered endocrinal biomolecules originating from one origin and affecting another. Osteocalcin increases adiponectin production in mice, which in turn supresses the inflammatory responses elicited by TNF-α (Ferron et al., 2008). In humans, osteocalcin also predicts adiponectin levels, which has been shown to stimulate angiogenesis in endothelial cells (Ouchi et al., 2004, Kuzniewski et al., 2016). These observations, along with the well demonstrated

influence of osteocalcin on energy metabolism, raises the possibility that osteocalcin in humans may be affecting the cardiovascular system and vascular endothelium indirectly, without significant interaction with the vascular cells themselves.

In chapter 8 we next turned our attention to the possibility that ucOCN may have importance in human vascular smooth muscle cell calcification, specifically cellular differentiation and biomineralisation. In our systematic review (chapter 5), we had found conflicting associations between circulating osteocalcin and vascular calcification in humans. However, calcified plaques were consistently positively associated with osteocalcin (Millar et al., 2017). Before conducting our cell culture experiments with ucOCN, I had trialled both commercial calcification media and lab recipe made calcification media. After 21 days the two media treatments in HASMCs were compared, and the commercial product was deemed more suitable as it gave better results. These outcomes were presented as a poster presentation entitled 'An evaluation of methodologies to induce calcification in vitro in human primary cells' at the British Cardiovascular Society Annual conference in 2019.

Over a period of 21 days, we examined the direct effects of ucOCN on human smooth muscle cell calcification. Despite total OCN being demonstrated to increase mineralisation and osteochondrogenic differentiation in mice vascular smooth muscle cells, this was not replicated in our human cells (Idelevich et al., 2011). We concluded that ucOCN may simply be a by-product of osteochondrogenic transdifferentiated cells as no active role was observed among multiple classical calcification endpoints and differentiation markers. This conclusion eludes to the fact that osteocalcin produced from differentiated osteogenic-like vascular cells is an *in situ* marker of calcification but circulating levels originating from bone are independent from this association and may not hold relevance to calcification.

Also in chapter 8, we included some analyses between osteocalcin circulating levels and calcification and arterial stiffness in chronic kidney disease patients. Within these data, no correlation was observed with osteocalcin. It must be noted that within our patients, only a small extent of calcification and arterial stiffness was detected. This may suggest that vascular disease was not severe or extensive enough to allow for inferences with circulating osteocalcin as has been reported previously (Nagata et al., 2015).

The heterogeneity of associations between circulating levels and calcification found in our systematic review (chapter 5) may be partly explained by osteocalcin gene polymorphisms. For example, in a cohort of older women, two polymorphisms were significantly associated with circulating total osteocalcin concentrations and fracture (McGuigan et al., 2010). Similarly, polymorphisms of the proposed osteocalcin receptor gene, GPRC6A, may further potentiate inter-individual differences. In a cohort of obese and control subjects, the polymorphism rs2274911 was associated with features of insulin resistance, even after adjustment of osteocalcin levels and features of the metabolic phenotype (Di Nisio et al., 2017). Our *in vitro* calcification study (chapter8) was the first of its kind, and further studies are warranted, as well as longitudinal observational studies in humans.

Limitations and future work

An inherent limitation of the cell culture work performed is that it does not fully represent human vascular physiology *in vivo*. Future work may address this by performing *ex vivo* myography studies which allows investigation of the relaxation and contraction responses of vessels to osteocalcin. It is also important to recognise that although the cells used came from a primary source, the passaging of the cells may incur some phenotypic drift. Furthermore, the cells were obtained from donors either during heart surgery or forensic medicine, thus intrinsically the cells may not be fully representative of a healthy physiological phenotype.

As osteocalcin is a circulating hormone, ucOCN and its other related forms are present in the foetal bovine serum used to supplement the cell culture growth media. Thus, osteocalcin may already be conferring effects at basal levels, and further addition of e.g. 10 ng/mL may not extend or alter these effects. To bypass this limitation, future studies could examine the response of cells to osteocalcin without serum for short term exposure experiments. Alternatively, a blocking agent may be manufactured in the future that could be added in the media to bind with osteocalcin and block its interaction with the cells. There is also less homology between goat osteocalcin and human osteocalcin (35%) compared to bovine (85%), therefore goat serum could be used to supplement media in future experiments.

This thesis found that ucOCN does not appear to have a fundamental role in vascular physiology within a number of conditions. In order to confirm these findings, future work could include use of microvascular cells and cells from other vascular origins and

multiple donors. Around 25% of circulating osteocalcin consists of intact osteocalcin, the remaining comprising N-terminal, mid-region, mid-region-C-terminal and C-terminal fragments. Thus, the examination of other osteocalcin fragments, including varying ratios of carboxylated and uncarboxylated OCN, still remains to be investigated.

Confirmatory studies are also needed to demonstrate if the vascular receptor of osteocalcin is indeed GPRC6A, and investigation into the effects of polymorphisms of the GPRC6A gene, and the osteocalcin gene, may be insightful. During the PhD, the proposed antagonist for GPRC6A, Z90240217, was used to investigate ucOCN cell signalling mechanisms. However, unfortunately through personal correspondence with the antagonist manufacturers, it transpired that it was not selective or specific enough for GPRC6A. Thus, our results were invalid, additionally as adding the antagonist on its own without ucOCN initiated cell signalling cascades and so was disregarded. siRNA knock down of the receptor may be a better alternative approach.

Another remaining area of investigation is examining whether osteocalcin is protective against hyperglycaemia induced endothelial dysfunction including insulin signalling impairment. These additional experiments alongside the work conducted for this thesis would conclusively answer whether there is a direct role for OCN in vascular biology.

Conclusions

This thesis focused on examining the direct effects of osteocalcin on vascular cells. Despite stimulating rapid intracellular signalling pathways and promoting proliferation, no direct role of ucOCN was observed within atherosclerosis, inflammation, or calcification *in vitro*. Osteocalcin may however be working through a coordinated whole-body synergy through other organ systems in the body, such as adipose tissue and the pancreas, to indirectly affect cardiovascular health and disease, which the formats of experiments used in this thesis were not designed to identify.

10. Appendix

Professional internship reflective statement

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

I undertook a 3 month professional internship placement with Artelo Biosciences, a small, virtual, start-up biotech company. This experience played a pivotal role in my career aspirations and broadened my opinions on future career paths more than I could have imagined. I truly appreciated the time with Artelo and how the BBSRC DTP programme allowed this interlude to expand personally and professionally.

Before I started placement I outlined the following aims/questions which I hoped to be able to answer after completing the 3 months:

1. Do I prefer work outside of the lab i.e. desk-based research? Potential as future career?
2. How do I find time management and self-discipline working from home?
3. Do I like the way the company works – 'virtual', small, conference calls, little face to face time?

My first few days introduction to Artelo was an eye-opener to the other side of research away from the lab bench. I sat in on funding bids, meetings for obtaining grants and talks with investors which was overwhelmingly different. It gave me an appreciation of the workload behind funding the research for this company.

My task for the placement was to create an investigator's brochure for a new investigational product. This was a huge endeavour and I was able to appreciate how

the research skills I had gained so far during my PhD were amenable to other areas and applicable to even an entirely new knowledge area. I also gained insight into how much I appreciated and found directly motivating to be working on a relatable clinical project – the making and commercialisation of a drug for the benefit of people – a step beyond the intricate lab work and cell culture I was used to during my PhD.

Getting positive feedback on written work from my placement supervisor gave me a boost of confidence and direction after numerous trials and errors and lack of results at the lab bench before leaving for placement. It gave me a lot of encouragement and I found it uplifting that my skills and organisation that I used in the lab, may in fact be better suited to a more scientific literature review role and delivery of written summaries and reports. Producing the documents directly made my contributions feel worthwhile and meaningful which can be difficult to achieve in the midst of lab experiments. I really enjoyed the writing process and being able to complete a project from beginning to end.

The positive experience at Artelo was motivating and almost a relief to realise the paths outside the lab and academia that I could take after completing my PhD. The experience showed me that you do not know what you may like until you try it. I found working from home and time management perfectly reasonable and had no issues with that.

Another huge development for me during my placement was organising a small conference for the company. This was a significant responsibility I took on independently and which was successful. It gave me confidence in event organisation and to be less reliant on other people, and taking the lead proved well.

A small realisation was that I work better towards deadlines, during lab work I did not realise how much I set my own deadline with cell culture experiments, and not having set deadlines for the writing work I was producing on placement meant things dragged on a bit when perhaps they could have been produced in shorter amounts of time which was interesting to note for myself for future work.

Overall, I am hugely grateful to have had the experience with Artelo. I thoroughly enjoyed the research, learnt more about myself and ways in which I work, and am more open to seeking non-traditional post-PhD roles outside of academia.

A systematic review of cannabidiol dosing in clinical populations

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Statement of personal contribution: substantial contribution to the conception/design of the work along with SOS; performed systematic search and retrieval of articles and data extraction which was conducted independently from NS and ZB; analysis of data with contribution from all authors; preparation of figures and tables; drafting of manuscript with input from all authors.

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SYSTEMATIC REVIEW AND META-ANALYSIS

A systematic review of cannabidiol dosing in clinical populations

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Aims: Cannabidiol (CBD) is a cannabis-derived medicinal product with potential application in a wide-variety of contexts; however, its effective dose in different disease states remains unclear. This review aimed to investigate what doses have been applied in clinical populations, in order to understand the active range of CBD in a variety of medical contexts.

Methods: Publications involving administration of CBD alone were collected by searching PubMed, EMBASE and ClinicalTrials.gov.

Results: A total of 1038 articles were retrieved, of which 35 studies met inclusion criteria covering 13 medical contexts. Twenty-three studies reported a significant improvement in primary outcomes (e.g. psychotic symptoms, anxiety, seizures), with doses ranging between <1 and 50 mg/kg/d. Plasma concentrations were not provided in any publication. CBD was reported as well tolerated and epilepsy was the most frequently studied medical condition, with all 11 studies demonstrating positive effects of CBD on reducing seizure frequency or severity (average 15 mg/kg/d within randomised controlled trials). There was no signal of positive activity of CBD in small randomised controlled trials (range n = 6–62) assessing diabetes, Crohn's disease, ocular hypertension, fatty liver disease or chronic pain. However, low doses (average 2.4 mg/kg/d) were used in these studies.

Conclusion: This review highlights that CBD has a potential wide range of activity in several pathologies. Pharmacokinetic studies as well as conclusive phase III trials to elucidate effective plasma concentrations within medical contexts are severely lacking and highly encouraged.

KEYWORDS

cannabidiol, cannabinoid, dose, dosing, therapeutics

1 | INTRODUCTION

Cannabidiol (CBD) is a non-intoxicating major constituent of the *Cannabis sativa* plant that has been increasing in interest due to its potentially diverse range of therapeutic properties and its favourable safety and tolerability profile.[1] Side effects are generally mild and infrequent, such as sleepiness, diarrhoea or increased temperature. It is also reported that clinically significant drug-interactions pose a

low risk.[2] There is no evidence for dependency or abuse potential with CBD use, as concluded by the World Health Organisation Expert Committee on Drug Dependence.[1] The purported effects of CBD include analgesic, anti-inflammatory, antioxidant, anxiolytic, anticonvulsant and cytotoxic effects, which are mediated through signalling mechanisms including the cannabinoid receptor 1 (weak agonist), the cannabinoid receptor 2 (inverse agonist), the serotonin 1a receptor (5-HT_{1A}), G protein-coupled receptor 55 (GPR55), G protein-coupled receptor 18 (GPR18) and the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptors, amongst others.[3]

Clinically, CBD is being investigated in multiple disease states including neurodegeneration, anxiety disorder, orphan childhood diseases with a prevalence of <5 in 10 000 individuals (e.g. tuberous sclerosis complex) and addiction (ongoing trials in cannabis and cocaine craving).[4-6] Epidiolex has recently become the first Food and Drug Administration-approved CBD medicine, indicated for use in Lennox–Gastaut or Dravet syndrome (childhood epilepsy) by oral administration. Sativex is an oromucosal spray containing both CBD and δ-9-tetrahydrocannibinol, which is licenced in the EU and Canada for the treatment of multiple sclerosis associated spasticity. At the time of writing, there are 49 clinical trials registered on clinicaltrials.gov investigating CBD alone (either not yet recruiting, recruiting or active) and there have been at least a further 100 clinical trials previously registered containing CBD, indicating a significant clinical interest with an ongoing need to ensure that human volunteers engaged in these trials are given doses that are optimised for efficacy and safety. Surprisingly, none of the 49 currently registered trials have explicitly included a study design to investigate the dose-ranging efficacy of CBD.

Hemp-derived CBD is commercially available and is currently used as a health and food supplement commonly for anxiety and pain relief. This market represents a flourishing industry expected to rise financially and globally.[7] However, the blurred lines between CBD as a licensed medicine and CBD as an over-the-counter remedy contribute to the overall lack of understanding of what dose of CBD may be considered *therapeutic*. This is further hampered by the lack of standardisation in over-the-counter CBD products and their unregulated labelled doses.

Despite the prevalence of CBD use and current hype, guidance on dose recommendations has not advanced and is not clear, additionally hampered by the striking lack of accessible pharmacokinetic and bioavailability data of CBD in humans.[8] No published study to date has reported the absolute oral bioavailability of CBD in humans.[8] Limited dose-determination studies have left a paucity in data surrounding desired plasma concentrations to achieve minimum effective doses. Additionally, the lack of information on the role of different formulations and routes of administration on absorption are also apparent. The aim of this review was to comprehensively collate all published data relating to CBD administration in clinical populations to describe the range of CBD doses assessed across different pathological states.

What is already known about this subject

- Due to its favourable toxicity and side effect profile, cannabidiol is under increasing investigation in the commercial and medical industry to treat many clinical indications.

What this study adds

- This study identifies the wide active dosing range of cannabidiol (<1 to 50 mg/kg/d) within a variety of medical conditions including epilepsy, anxiety and graft-*vs*-host disease.
- This review indicates that studies that used higher doses tended to have better therapeutic outcomes compared to lower doses overall.
- This study identifies a strong existing need for dose-ranging clinical studies to be conducted in which plasma concentrations can provide a better indication of the therapeutic range of cannabidiol.

2 | METHODS

2.1 | Search strategy

The systematic review was carried out in accordance with PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. A systematic search of PubMed, EMBASE (including MEDLINE) and clinicaltrials.gov was conducted to retrieve all articles reporting CBD administration in clinical populations using 'CBD or Cannabidiol' as search terms. Searches were restricted to 'humans' and 'clinical trials and case reports' in PubMed and EMBASE, with no restrictions on clinicaltrials.gov. The searches were carried out by 8 August 2018 by 2 independent researchers.

2.2 | Eligibility criteria

The titles and abstracts of retrieved studies were examined by 2 independent researchers, and inappropriate articles were rejected. Inclusion criteria were as follows: an original, peer-reviewed published paper that involved administration of CBD to a clinical population, or reported on clinicaltrials.gov, and included an outcome measurement to assess the efficacy of CBD i.e. improvement in disease. Exclusion criteria were: administration in healthy participants only; CBD administered in combination with other cannabinoids such as with δ-9-tetrahydrocannibinol or as whole cannabis extracts; article not in English; no stated concentration of CBD used; or no statistical results reported. The reference lists of included studies were hand-searched for additional relevant studies.

2.3 | Data acquisition and analysis

The included articles were analysed, and the following data extracted: sample size, clinical population/medical context; study design and length; administration route of CBD; source of CBD; dose of CBD; side effects; and primary outcome results. All data entry was checked by an additional independent researcher. Risk of bias of the 15 randomised controlled trials was assessed using the 2011 Cochrane Collaboration's tool for assessing risk of bias.

As this review included studies of participants of all ages (from infants to adults), dosing is reported in mg/kg of body weight to allow for comparison. Where not available as mg/kg (24 studies), dose was converted for adults using an average adult body weight of 62 kg.[9] In only 1 publication, a case report on a child, an average child weight of 40 kg had to be used to convert reported mg/d dose into mg/kg/d.[10]

A positive effect of CBD was determined by the presence of a significant improvement in primary end points(s) or outcomes reported compared to placebo or baseline. A lack of positive effect was determined if no significant improvements were reported. Mixed findings were reported for example in case reports wherein some patients improved, others did not, or where a primary outcome was not specified (exploratory study) and in which some endpoints improved while others worsened (1 study) or remained unchanged.

3 | RESULTS

The initial search yielded 1038 records, from which 896 abstracts were reviewed, and 35 articles were included in the final analysis, comprising a total number of 1223 participants. A flow chart of article retrieval and selection is presented in Figure 1. Fifteen studies were randomised controlled trials (RCTs), 8 were clinical trials but not both randomised and controlled in design (for example open-label trials), and 12 articles were case reports/series. A description of each study is presented in tables 1–3 according to study design. Results of the risk of bias assessment of the RCTs are presented in Figure 2. A component of blinding was included in 74% of the RCTs . No study was reported with a high risk of selection bias, detection bias, or reporting bias. Overall, most information was from studies at low risk of bias. No study reported plasma concentrations of CBD. All studies reported oral administration of CBD, either as an oral solution (n = 11), capsules (n = 13), spray/sublingual (n = 4), or orally but unspecified (n = 6).

Of the 15 RCTs, the range of doses investigated varied from <1 mg/kg up to 20 mg/kg per day (average 9 mg/kg/d).[11-19,21-25] Seven RCTs reported CBD efficacy (average dose 14 mg/kg/d),[11-13,16,19,20,24] 7 studies describe neutral effects of CBD (average dose 5 mg/kg/d)[14,15,17,21-23,25] and 1 study showed both positive and negative outcomes.[18] In the remaining 8 clinical trials of various

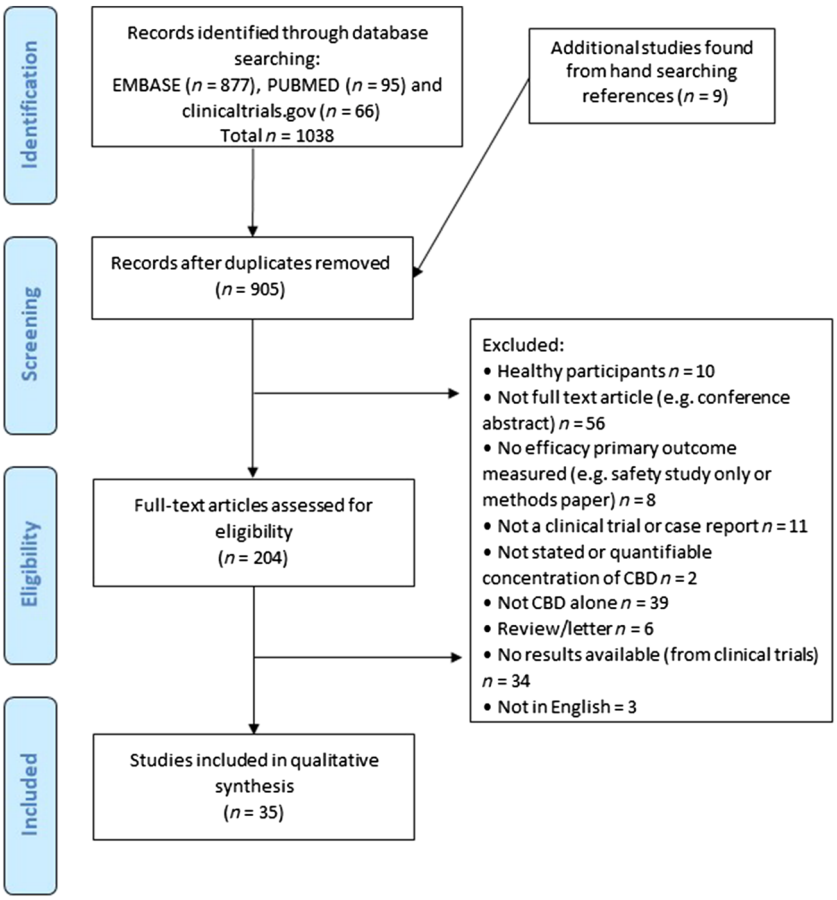

FIGURE 1 Flow chart of study retrieval and selection

TABLE 1 Summary of included studies: randomised controlled trials

Study	Clinical population	Total *n*	Design	Trial length	CBD dose (mg) and approx. mg/kg/d[a]	Route of admin.	CBD source	Results[b]: Primary endpoint(s)	+ effect	Side effects
McGuire, 2018 (NCT02006628)[11]	Schizophrenia, adults	88	Phase II exploratory double-blind, parallel-group, RCT. Add-on therapy to anti-psychotic drugs.	8 wk	1000 mg/d (16.7 mg/kg/d)	Oral solution	GW	Positive psychotic symptoms reduced. Negative, overall and general psychotic symptoms unchanged. Higher proportion of CBD treated patients rated as *improved*. No differences in functionality. No significant improvement in cognitive function except for motor speed. Overall reported as clinically significant improvements with CBD.	Yes	Rates of adverse events similar between CBD and placebo groups
Thiele, 2018 (NCT02224690)[12]	Seizures (Lennox–Gastaut syndrome), ages 2–55 y	171	Double-blind, phase III, RCT. Add-on therapy to AEDs.	14 wk	20 mg/kg/d	Oral solution	GW	Monthly frequency of drop seizures decreased by a median of 43.9% in the CBD group, significantly more than in the placebo group	Yes	Diarrhoea, somnolence, pyrexia, decreased appetite, vomiting
Devinsky, 2018 (NCT02224560)[13]	Lennox–Gastaut syndrome (epilepsy), ages 2–55 y	225	Phase III, double-blind, RCT. Add-on therapy to AEDs.	14 wk	10 or 20 mg/kg/d	Oral solution	GW	Significantly greater reduction in CBD groups in drop seizure frequency than in placebo	Yes	9% taking CBD had elevated liver aminotransferases. Somnolence, decreased appetite, diarrhoea, upper respiratory tract infection, pyrexia, vomiting.

(Continues)

TABLE 1 (Continued)

Study	Clinical population	Total *n*	Design	Trial length	CBD dose (mg) and approx. mg/kg/d[a]	Route of admin.	CBD source	Results[b]: Primary endpoint(s)	+ effect	Side effects
Boggs, 2018 (NCT00588731)[14]	Schizophrenia, adults	36	Double-blind, parallel group, RCT. Add-on therapy to anti-psychotic drugs.	6 wk	600 mg/d (10 mg/kg/d)	Oral capsules	STI	No effect on cognition or symptoms	No	Similar rates between placebo and CBD, with exception of sedation which was higher in CBD group.
Naftali, 2017 (NCT01037322)[15]	Crohn's disease, adults	19	RCT	8 wk	20 mg/d (0.3 mg/kg/d)	Orally, sublingual	On-site	No difference in disease index	No	None observed
Devinsky, 2017 (NCT02091375)[16]	Treatment resistant Dravet syndrome (epilepsy), aged 2–18 y	120	Double-blind, RCT. Add-on therapy to AEDs.	14 wk	20 mg/kg/d	Oral solution	GW	Reduction in frequency of convulsive seizures compared to baseline, significantly greater reduction than with placebo	Yes	Diarrhoea, vomiting, fatigue, pyrexia, somnolence, abnormal results on liver-function: tests were higher in the CBD group than placebo
Jadoon, 2016 (NCT01217112)[17]	Type 2 diabetes patients, adults	62	Double-blind, RCT	13 wk	200 mg/d (3.3 mg/kg/d)	Oral	GW	No change in HDL-cholesterol concentrations or glycaemic control.	No	Well tolerated
Chagas, 2014[18]	Parkinson's disease, adults	21	Double-blind exploratory RCT. Add-on therapy to anti-Parkinson's drugs.	6 wk	75 or 300 mg/d (1.25 or 5 mg/kg/d)	Oral capsules	THC	No effect on motor and general symptoms; 300-mg dose improved well-being and quality of life scores.	Mixed	None reported
Leweke, 2012[19]	Schizophrenia, adults	42	Phase II, double-blind, parallel-group, RCT	4 wk	800 mg/d (max: 13.3 mg/kg/d)	NA	NA	Significant improvement of psychotic symptoms compared to baseline	Yes	Well tolerated
Bergamaschi, 2011[20]	Generalised SAD, adults	24	Double-blind, RCT	Acute	600 mg (10 mg/kg)	Oral capsule	STI and THC	Reduction in anxiety, cognitive impairment, discomfort in speech performance. Alert factors in anticipatory speech were also reduced.	Yes	None reported

(Continues)

TABLE 1 (Continued)

Study	Clinical population	Total *n*	Design	Trial length	CBD dose (mg) and approx. mg/kg/d[a]	Route of admin.	CBD source	Results[b]: Primary endpoint(s)	+ effect	Side effects
Tomida, 2006[21]	Ocular hypertension, adults	6	Double-blind, 4-way cross-over, RCT	Acute	20 or 40 mg (0.3 or 0.7 mg/kg)	Oromucosal spray	GW	20 mg of CBD was ineffective, while 40 mg slightly increased intraocular pressure.	No	Mild—e.g. oral discomfort.
Notcutt, 2004[22]	Chronic pain, adults	24	Double-blind, 4-way cross-over, RCT. Add-on therapy to pain medication.	8 wk	Approx. 9 sprays/d, equivalent of 22.5 mg/d (0.4 mg/kg/d)	Sublingual spray	GW	Symptom control or sleep duration was not improved with CBD; however, sleep quality was.	No	Mid—drowsiness, dry mouth
Consroe, 1991[23]	Huntington's disease, adults	15	Double-blind, cross-over, RCT	6 wk	10 mg/kg/d	Oral capsules	US NIDA	CBD was ineffective	No	Similar between CBD and placebo
Cunha, 1980[24]	Epilepsy, adults	15	Double-blind, RCT study. Add-on therapy to AEDs.	Up to 4.5 months	200–300 mg/d (5 mg/kg/d)	Oral capsules	NA	All but 1 patient improved condition	Yes	Well tolerated
***NCT01284634**[25]	Fatty liver disease, adults	25	Partially-blinded, phase II, RCT	8 wk	200, 400 or 800 mg/d (3.3, 6.7, or 13.3 mg/kg/d)	Oral capsules	GW	No differences in liver triglyceride levels	No	Similar between CBD and placebo

[a]If not supplied, mg/kg/d was calculated based on average adult weight of 62 kg to enable comparisons.

[b]Significant compared to placebo/control ($P < .05$) unless stated otherwise.

*Registered clinical trial identifier: not published in any peer-reviewed journal but results available from clinicaltrials.gov.

AEDs, anti-epileptic drugs; CBD, cannabidiol; GW, GW Pharmaceuticals; HDL, high density lipoprotein; NA, not available; NIDA, National Institute on Drug Abuse; RCT, randomised controlled trial; SAD, social anxiety disorder; STI, STI Pharmaceuticals; THC, THC Pharm.

TABLE 2 Summary of included studies: clinical studies

Study	Clinical population	Total *n*	Design	Trial length	CBD dose (mg) and approx. mg/kg/d[a]	Route of admin.	CBD source	Results[b]: Primary endpoint(s)	+ effect	Side effects
Rosenberg, 2017[26]	Epilepsy, 1–30 y	48	Open label clinical study	12 wk	2–5 mg/kg/d titrated up to 50 mg/kg/d or intolerance	Oral solution or by gastric tube	GW	Improvement in quality of life as well as some cognitive functions (memory and control)	Yes	Somnolence, drowsiness, fatigue
Devinsky, 2016[27]	Drug-resistant epilepsy, ages 1–30 y	137	Prospective, open-label trial	12 wk	2–5 mg/kg/d, up-titrated to 25 or 50 mg/kg/d	Oral solution or gastric tube	GW	Monthly motor seizures reduced by a median of 35.5% from baseline	Yes	Somnolence, fatigue, diarrhoea, decreased appetite, weight loss, status epilepticus (6%).
Hess, 2016[28]	Drug-resistant epilepsy in tuberous sclerosis complex, 2–31 y	18	Prospective study	6–12 months	5 mg/kg/d titrated up to 50 mg/kg/d if tolerated	Oral solution	GW	Decreased seizure frequency	Yes	Drowsiness, ataxia, diarrhoea
Yeshurun, 2015 (NCT01385124)[29]	Cell transplant, (GVHD), adults	48	Prospective, phase II clinical trial	37-day	300 mg/d (5 mg/kg/d)	Oral solution	STI	No patients developed acute GVHD. Significantly reduced risk ratio compared to historical case controls.	Yes	None reported
Crippa, 2011[5]	Generalised SAD, adults	10	Double-blind, placebo-controlled study	Acute	400 mg (6.7 mg/kg)	Oral capsule	THC	Reduced subjective anxiety	Yes	None reported
Hallak, 2010[30]	Schizophrenia, adults	28	Placebo-controlled study	Acute	300 or 600 mg (5 or 10 mg/kg)	Oral capsules	Gift	No beneficial effects on selective attention	No	None reported
Zuardi, 2009[31]	Psychosis in Parkinson's disease, adults	6	Open-label pilot study	4 wk	150 mg/d, increased by 150 mg each week to a total of 400 mg/d (6.7 mg/kg/d)	Oral capsule	THC	Decrease in psychotic symptoms and Parkinson's disease rating compared to baseline	Yes	None reported
Consroe, 1986[32]	Dystonic movement disorder, adults	5	Preliminary open pilot study	6 wk	100–600 mg/d, increased weekly (1.7–10 mg/kg/d)	Oral capsules	NA	Dose-related improvement in dystonia disability	Yes	Mild—drop in standing blood pressure

[a]If not supplied, mg/kg/d was calculated based on average adult weight of 62 kg to enable comparisons.

[b]Significant compared to placebo/control ($P < .05$) unless stated otherwise.

CBD, cannabidiol; GW, GW Pharmaceuticals; GVHD, graft-vs-host disease; STI, STI Pharmaceuticals; SAD, social anxiety disorder; THC, THC Pharm.

TABLE 3 Summary of included studies: case studies

Study	Clinical population	Total *n*	Design	Trial length	CBD dose (mg) and approx. mg/kg/d[a]	Route of admin.	CBD source	Results[b]: Primary endpoint(s)	+ effect	Side effects
Kaplan, 2017[33]	Refractory seizures in Sturge–Weber syndrome, children	5	Case-series	14 wk	5–25 mg/kg/d	Oral solution	GW	Decreases in seizure frequency	Yes	Mild
Warren, 2017[34]	Brain tumour related epilepsy, aged 17–40 y	3	Case series	2–10 mo	10–50 mg/kg/d	Oral	GW	Improvement in seizure frequency (*n* = 2) and severity (*n* = 3)	Yes	Diarrhoea
Gofshteyn, 2017[35]	Febrile infection-related epilepsy syndrome, children	7	Open-label case series	Acute and up to 48 weeks	15–25 mg/kg/d	Oral solution	GW	Improvements in frequency and duration of seizures	Yes	Dizziness, decreased appetite, weight loss
Shannon, 2016[10]	Anxiety and insomnia in PTSD, child	1	Case report	5 mo	25 mg/d (0.6 mg/kg/d)	Oral capsule and spray	CannaVest Corp	Increased sleep quality and duration, and decreased anxiety secondary to PTSD	Yes	None observed
Saade, 2015[36]	Seizures, 10-month old infant	1	Case report	6 mo	25 mg/kg/d	Oral solution	GW	Substantial reductions in seizures	Yes	None reported
Chagas, 2014[37]	RBD in Parkinson's disease, adults	4	Case series	6 wk	75 mg/d (1.25 mg/kg/d)	NA	NA	Substantial reduction in RBD-associated events compared to baseline	Yes	None reported
Crippa, 2013[38]	Cannabis dependency, adult	1	Case report	10 d	300 mg/d increased to 600 mg/d (5–10 mg/kg/d)	Oral capsule	THC	Absence of withdrawal symptoms	Yes	None reported
Zuardi, 2010[39]	Bipolar disorder, adults	2	Case series	30 d	600 mg/d increased to 1200 mg/d (20 mg/kg/d)	Oral	STI and THC	CBD was ineffective for manic episode	No	None observed
Zuardi, 1995[40]	Schizophrenia, adult	1	Case report	4 wk	1500 mg/d (25 mg/kg/d)	Oral capsules	NA	Improvements in psychiatric ratings	Yes	Well tolerated; none reported
Zuardi, 2006[41]	Treatment-resistant schizophrenia, adults	3	Case series	30 d	40 mg/d, increased to 1280 mg/d (21.3 mg/kg/d)	Oral	GW	1 patient showed mild improvement to baseline and discontinuing treatment worsened symptoms	No	Well tolerated; none observed

(Continues)

TABLE 3 (Continued)

Study	Clinical population	Total *n*	Design	Trial length	CBD dose (mg) and approx. mg/kg/d[a]	Route of admin.	CBD source	Results[b]: Primary endpoint(s)	+ effect	Side effects
Snider, 1985[42]	Parkinson's disease, adult	1	Case report	4 wk	100–400 mg/d (3.3 mg/kg/d)	Oral	NA	Improvement of dyskinesia up to 200 mg/d, worsening of Parkinson disease symptoms with 300–400 mg/d	Mixed	Dizziness, drowsiness, increased Parkinson symptoms
Snider, 1984[43]	Meige syndrome, adult	1	Case report	Long-term	Initially 100 mg/d increased to 400 mg/d (6.6 mg/kg/d)	Oral	NA	50% improvement in spasm frequency and severity	Yes	Dry mouth, headache, sedation

[a]If not supplied, mg/kg/d was calculated based on average adult weight of 62 kg to enable comparisons.

[b]Significant compared to placebo/control ($P < .05$) unless stated otherwise.

CBD, cannabidiol; GW, GW Pharmaceuticals; PTSD, post-traumatic stress disorder; RBD, rapid eye movement sleep behaviour disorder; STI, STI Pharmaceuticals; THC, THC Pharm.

FIGURE 2 *Risk of bias* summary of the randomised controlled trials included in the systematic review. Green indicates low-risk bias, red indicates high-risk bias, and yellow indicates intermediate or unclear risk

study design, 7 studies reported CBD positively (average dosing 23 mg/kg/d)[5,26-29,31,32] and 1 study was neutral (8 mg/kg/d).[30] Within the 12 case studies and case series, 9 described positive effects of CBD (average dosing 16 mg/kg/d),[10,33-38,40,43] 2 were neutral (average dosing 21 mg/kg/d)[39,41] and 1 study described mixed results (3 mg/kg/d).[42]

Epilepsy was the most frequently studied medical condition, with all 11 studies describing beneficial effects of CBD in reducing the severity or frequency of seizures.[12,13,16,24,26-28,33-36] Within the 4 conducted RCTs (n = 531), an average dosing of 15 mg/kg/d was used where CBD was administered successfully as an add-on therapy to usual anti-epileptic drugs.[12,13,16,24] Significant improvements were observed compared to placebo as an add-on therapy. Within the other 3 clinical trials of prospective open-label design (n = 203), CBD was administered at an average dosing of 42 mg/kg/d and significant improvements in quality of life and seizure frequency compared to baseline were observed.[26-28] 3 case series and 1 case report (total n = 16) reported beneficial effects of CBD on seizure frequency, duration and severity with an average administered dose of 21 mg/kg/d.[33-36]

Seven studies were conducted in the context of schizophrenia and bipolar disorder. Within the RCTs, 2 conducted with an average dosing

of 15 mg/kg/d over 4 or 8 weeks reported positive reductions in psychotic or psychiatric symptoms and a better side effect profile (n = 130).[11,19] One of these compared CBD against an active control (amisulpride), and the other as an add-on therapy to usual medication compared to placebo as an add-on therapy. However, a third RCT employing CBD as an add-on therapy did not report any improvements in cognition or symptoms of schizophrenia after a lower average dose of 10 mg/kg/d over 6 weeks (n = 36).[14] An acute dose of 5 or 10 mg/kg/d did not improve selective attention in a placebo-controlled trial of 28 schizophrenia patients.[30] A number of case studies have also been conducted by Zuardi and colleagues in this medical context. In 2 patients with bipolar disease, 20 mg/kg/d was ineffective in treating manic episodes.[39] CBD was similarly unable to improve symptoms in 3 schizophrenia patients, although 1 patient described mild improvement.[41] Another case report described improvement in psychiatric ratings following an average dose of 25 mg/kg/d over 4 weeks.[40]

Results are mixed within Parkinson's disease studies. Within an RCT in 21 patients, 1.25 or 5 mg/kg/d CBD had no effect on motor and general symptoms. However, the 5 mg/kg/d dose improved well-being and quality of life scores.[18] The remaining studies are case studies in which CBD decreased psychotic symptoms and Parkinson's disease ratings (n = 6; 7 mg/kg/d),[31] improved rapid eye movement sleep behaviour disorder (n = 4; 1 mg/kg/d),[37] decreased dyskinesia with 2 to 3 mg/kg/d doses (n = 1), but exaggerated Parkinson's disease symptoms with 5 and 7 mg/kg/d doses.[42]

CBD did not change therapeutic outcome variables in a double-blind RCT in Huntington disease patients compared to placebo (n = 15; 10 mg/kg/d for 6 weeks),[23] but improved dystonia disability in an open pilot study (n = 5; 10 mg/kg/d for 6 weeks),[32] and improved spasm frequency and severity in a case report in 1 patient with Meige syndrome (7 mg/kg/d).[43]

Within the RCTs, CBD did not significantly change the primary outcomes in diabetes (n = 62), Crohn's disease (n = 19), ocular hypertension (n = 6), chronic pain (mostly neuropathic; n = 24), or fatty liver disease (n = 25).[15,17,21,22,25] However, an average dose of 2.4 mg/kg/d (range 0.3–13.3 mg/kg/d) was used in these studies, which is very low in the clinical and clinical trial setting compared to other studies. Low doses (10 mg/kg) did, however, produce positive responses in generalised social anxiety disorder (SAD) in a double-blind RCT in 24 patients.[20] Likewise, in another double-blind placebo-controlled study, a dose of 6.7 mg/kg reduced subjective anxiety in 10 adults with generalised SAD.[5] Additionally, in a case report in a child, 0.6 mg/kg/d increased sleep quality and duration, and decreased anxiety secondary to PTSD.[10]

Lastly, it was found that doses of 5 mg/kg/d prevented occurrence of graft-*vs*-host disease in a phase II clinical trial (n = 48) and 5–10 mg/kg/d doses have been shown in a case report to remove withdrawal symptoms from a patient with cannabis dependency.[29,38]

Within studies that compared CBD against a placebo or control (n = 17 publications), only 1 compared CBD against an active control (and a greater clinical improvement and side effect profile was observed with CBD against amisulpride), 8 compared CBD against a placebo (monotherapy), and 8 studies compared CBD as an add-on therapy (adjunctive to antipsychotic medication, antiepileptic medication, anti-Parkinson medication or pain medication) against placebo. Analysis of these data revealed that a greater proportion of studies reported a beneficial effect of CBD in the add-on therapy group compared to the monotherapy group (n = 6 and n = 2 respectively). However, higher doses were used overall within the add-on therapy group compared to the monotherapy group (average 11 and 6 mg/kg/d, respectively) and, due to such a small data set and heterogeneity of studies, we did not perform any further analysis.

4 | DISCUSSION

To our knowledge, this is the first study to compile and compare all publications in which CBD was administered to clinical populations. The aim of this systematic review was to better understand the range of doses of CBD used in clinical studies. In total, 13 medical contexts were included in this review amongst 35 studies including clinical trials and case reports. A positive effect of CBD was reported in 66% of studies, covering disorders including schizophrenia, SAD, epilepsy, cannabis dependency and graft-*vs*-host disease, with doses ranging between <1 and 50 mg/kg/d (i.e. <62–3100 mg/d for an adult). Although we acknowledge that these results mix widely heterogeneous studies, it appears well founded to highlight the differences in average dosing for positive effect studies against those without positive effects, which is confirmed when analysing studies per medical context within each study design format. This suggests that CBD potentially displays a wide therapeutic range, and variable minimum doses are required for effect depending on primary outcomes assessed and the population group. However, it is vital to note that no conclusions can be drawn on the efficacy of CBD as larger phase III and conclusive efficacy trials have not been conducted, with exception of epilepsy. A number of phase III clinical trials are registered on clinicaltrials.gov, which should provide more evidence in the coming years in the contexts of pain, anxiety, Crohn's disease, bipolar disorder, Fragile X syndrome, epilepsy and more.

CBD is increasingly popular, both as a food and health supplement and as a licensed medicine. Within this review, 51% of studies have been published in the last 5 years (since 2013); however, the included articles span over decades, with prominent publications first appearing in the 1980s and early 1990s.[24,40] Despite its long history of sole administration to patients, there is surprisingly little published about the pharmacokinetic properties of CBD, particularly its bioavailability, making it difficult to estimate true effective doses.[8] Historically, there is a striking lack of dose-ranging studies and, looking forward, there are no registered trials on clinicaltrials.gov including specific dose-ranging investigations in their study design. Ideally, this review would have compared plasma concentrations of CBD in order to more accurately estimate therapeutic concentrations, but, due to the lack of reporting, this was not possible.

Different effective plasma concentrations of CBD may be required for achieving different endpoints across clinical populations, which is a

recognised trait in a number of other drugs and diseases. For example, aspirin (acetylsalicylic acid) is used at low doses for antiplatelet therapy, and at higher doses as an analgesic agent.[44,45] With CBD, lower doses may be effective in anxiety relief, while higher doses may be required for effective reduction in epileptic seizures. In studies where there are good rationales for CBD use (e.g. Crohn's disease and chronic pain[46,47]), neutral results may be secondary to subtherapeutic dosing, and dose-escalation trials with embedded pharmacokinetic studies are the next logical step.[15,22] Studies in this review using higher doses concluded that CBD was generally well-tolerated with the most frequent side effects including drowsiness, nausea, somnolence, fatigue and vomiting.

Among the clinical trial records retrieved from clinicaltrials.gov, only 60% of completed trials had results uploaded and available. This may represent a significant publication bias and is suggestive of disregard for the priority of publication of negative results, which is a well-recognised problem.[48] Unfortunately, this may potentially skew the findings presented in this review and so should be interpreted with caution and is acknowledged as a limitation. We also acknowledge that despite all routes of administration being oral, there may be further bias introduced between studies as one dose cannot be directly compared to another due to lack of standardisation of formulations and pharmacokinetic activity, including differences in bioavailability between an oral spray and an oral capsule.

Future studies should also consider the safety of drug interactions with CBD. CBD is a known inhibitor of the cytochrome P450 (CYP) system[49] and can therefore increase plasma concentrations of medicines already in use, in particular antiepileptic drugs. Indeed, this has been reported in a number of publications investigating concomitant use of CBD and antiepileptic drugs.[50] Similarly, CYP inhibitors are predicted to increase CBD plasma concentrations which should be equally monitored. Where possible, further well designed trials with CBD may disentangle whether CBD offers unique therapeutic potential in addition to benefits seen when used as an add-on treatment.

5 | CONCLUSION

Although larger confirmatory and efficacy clinical trials examining dosing in more detail for each medical context is required, this review summarises that CBD appears to offer a wide-range of activity between 1 and 50 mg/kg/d, and there was a tendency of studies with positive outcomes to have used higher doses of CBD. We recommend pharmacokinetic dosing schedules in subsequent trials to consider this range along with safety data and individual patient requirements. Finally, we implore all completed trial results to be made readily available so the research community can progress and learn from equally important positive and negative outcomes for the ultimate benefit of patients.

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COMPETING INTERESTS

A.S.Y. and S.E.O. are paid consultants for Artelo Biosciences and the UK Centre for Medicinal Cannabis. All other authors declare no competing interests.

CONTRIBUTORS

S.E.O. and S.A.M.: substantial contributions to the conception or design of the work. S.M.: writing of the manuscript. S.A.M., Z.D.B. and N.L.S.: database searching and data extraction. All authors: analysis and interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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A systematic review on the pharmacokinetics of cannabidiol in humans

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A Systematic Review on the Pharmacokinetics of Cannabidiol in Humans

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Background: Cannabidiol is being pursued as a therapeutic treatment for multiple conditions, usually by oral delivery. Animal studies suggest oral bioavailability is low, but literature in humans is not sufficient. The aim of this review was to collate published data in this area.

Methods: A systematic search of PubMed and EMBASE (including MEDLINE) was conducted to retrieve all articles reporting pharmacokinetic data of CBD in humans.

Results: Of 792 articles retireved, 24 included pharmacokinetic parameters in humans. The half-life of cannabidiol was reported between 1.4 and 10.9 h after oromucosal spray, 2–5 days after chronic oral administration, 24 h after i.v., and 31 h after smoking. Bioavailability following smoking was 31% however no other studies attempted to report the absolute bioavailability of CBD following other routes in humans, despite i.v formulations being available. The area-under-the-curve and C_{max} increase in dose-dependent manners and are reached quicker following smoking/inhalation compared to oral/oromucosal routes. C_{max} is increased during fed states and in lipid formulations. T_{max} is reached between 0 and 4 h.

Conclusions: This review highlights the paucity in data and some discrepancy in the pharmacokinetics of cannabidiol, despite its widespread use in humans. Analysis and understanding of properties such as bioavailability and half-life is critical to future therapeutic success, and robust data from a variety of formulations is required.

Keywords: pharmacokinetics, endocannabinoid system, bioavailability, CMAX, TMAX, half life, plasma clearance, volume of distribution

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INTRODUCTION

The *Cannabis sativa* plant contains more than a hundred phytocannabinoid compounds, including the non-psychotomimetic compound cannabidiol (CBD) (Izzo et al., 2009). CBD has attracted significant interest due to its anti-inflammatory, anti-oxidative and anti-necrotic protective effects, as well as displaying a favorable safety and tolerability profile in humans (Bergamaschi et al., 2011), making it a promising candidate in many therapeutic avenues including epilepsy, Alzheimer's disease, Parkinson's disease, and multiple sclerosis. GW pharmaceuticals have developed an oral solution of pure CBD (Epidiolex®) for the treatment of severe, orphan, early-onset, treatment-resistant epilepsy syndromes, showing significant reductions in seizure frequency compared to placebo in several trials (Devinsky et al., 2017, 2018a; Thiele et al., 2018). Epidiolex® has recently

received US Food and Drug Administration (FDA) approval (GW Pharmaceuticals, 2018). CBD is also being pursued in clinical trials in Parkinson's disease, Crohn's disease, society anxiety disorder, and schizophrenia (Crippa et al., 2011; Leweke et al., 2012; Chagas et al., 2014; Naftali et al., 2017), showing promise in these areas. Additionally, CBD is widely used as a popular food supplement in a variety of formats for a range of complaints. It is estimated that the CBD market will grow to $2.1 billion in the US market in consumer sales by 2020 (Hemp Business, 2017).

From previous investigations including animal studies, the oral bioavailability of CBD has been shown to be very low (13–19%) (Mechoulam et al., 2002). It undergoes extensive first pass metabolism and its metabolites are mostly excreted via the kidneys (Huestis, 2007). Plasma and brain concentrations are dose-dependent in animals, and bioavailability is increased with various lipid formulations (Zgair et al., 2016). However, despite the breadth of use of CBD in humans, there is little data on its pharmacokinetics (PK). Analysis and understanding of the PK properties of CBD is critical to its future use as a therapeutic compound in a wide range of clinical settings, particularly regarding dosing regimens and routes of administration. Therefore, the aim of this systematic review was to collate and analyse all available CBD PK data recorded in humans and to highlight gaps in the literature.

METHODS

Search Strategy

The systematic review was carried out in accordance with PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (Moher et al., 2009). A systematic search of PubMed and EMBASE (including MEDLINE) was conducted to retrieve all articles reporting pharmacokinetic data of CBD in humans. Search terms included: CBD, cannabidiol, Epidiolex, pharmacokinetics, C_{max}, plasma concentrations, plasma levels, half-life, peak concentrations, absorption, bioavailability, AUC, T_{max}, C_{min}, and apparent volume of distribution. No restrictions were applied to type of study, publication year, or language. The searches were carried out by 14 March 2018 by two independent researchers.

Eligibility Criteria

The titles and abstracts of retrieved studies were examined by two independent researchers, and inappropriate articles were rejected. Inclusion criteria were as follows: an original, peer-reviewed paper that involved administration of CBD to humans, and included at least one pharmacokinetic measurement as listed in the search strategy.

Data Acquisition

The included articles were analyzed, and the following data extracted: sample size, gender, administration route of CBD, source of CBD, dose of CBD, and any pharmacokinetic details. Where available, plasma mean or median C_{max} (ng/mL) were plotted against CBD dose (mg). Similarly, mean or median T_{max} and range, and mean or median area under the curve (AUC_{0-t}) and SD were plotted against CBD dose (mg). The source/supplier of the CBD was also recorded. No further statistical analysis was possible due to sparsity of data and heterogeneity of populations used. All studies were assessed for quality using an amended version of the National Institute for Health (NIH), National Heart, Lung and Blood Institute, Quality Assessment Tool for Before-After (Pre-Post) Studies with No Control Group (National Institute for Health, 2014). A sample size of $\leq$10 was considered poor, between 11 and 19 was considered fair, and $\geq$20 was considered good (Ogungbenro et al., 2006).

Definitions of PK Parameters

T_{max}: Time to the maximum measured plasma concentration.
C_{max}: Maximum measured plasma concentration over the time span specified.
$t_{1/2}$: Final time taken for the plasma concentration to be reduced by half.
AUC_{0-t}: The area under the plasma concentration vs. time curve, from time zero to "t."
AUC_{0-inf}: The area under the plasma concentration vs. time curve from zero to t calculated as AUC_{0-t} plus the extrapolated amount from time t to infinity.
K_{el}: The first-order final elimination rate constant.

RESULTS

In total, 792 records were retrieved from the database searching, 24 of which met the eligibility criteria (**Figure 1**). **Table 1** summarizes each included study. Routes of administration included intravenous (i.v.) ($n = 1$), oromucosal spray ($n = 21$), oral capsules ($n = 13$), oral drops ($n = 2$), oral solutions ($n = 1$), nebuliser ($n = 1$), aerosol ($n = 1$), vaporization ($n = 1$), and smoking ($n = 8$). CBD was administered on its own in 9 publications, and in combination with THC or within a cannabis extract in the remainder. One study was conducted in children with Dravet syndrome, while the remainder were conducted in healthy adult volunteers (Devinsky et al., 2018b). Overall, the included studies were of good quality (**Supplementary Table 1**). However, many studies had small sample sizes. Additionally, not all studies included both males and females, and frequent cannabis smokers were included in a number of studies. Thus, interpretation and extrapolation of these results should be done with caution.

C_{max}, T_{max}, and Area Under the Curve

Within the 25 included studies, C_{max} was reported on 58 occasions (for example within different volunteer groups or doses in a single study), T_{max} on 56 occasions and area under the curve (AUC_{0-t}) on 45 occasions. These data from plasma/blood are presented in **Figures 2A–C**. The AUC_{0-t} and C_{max} of CBD is dose-dependent, and T_{max} occurs between 0 and 5 h, but does not appear to be dose-dependent.

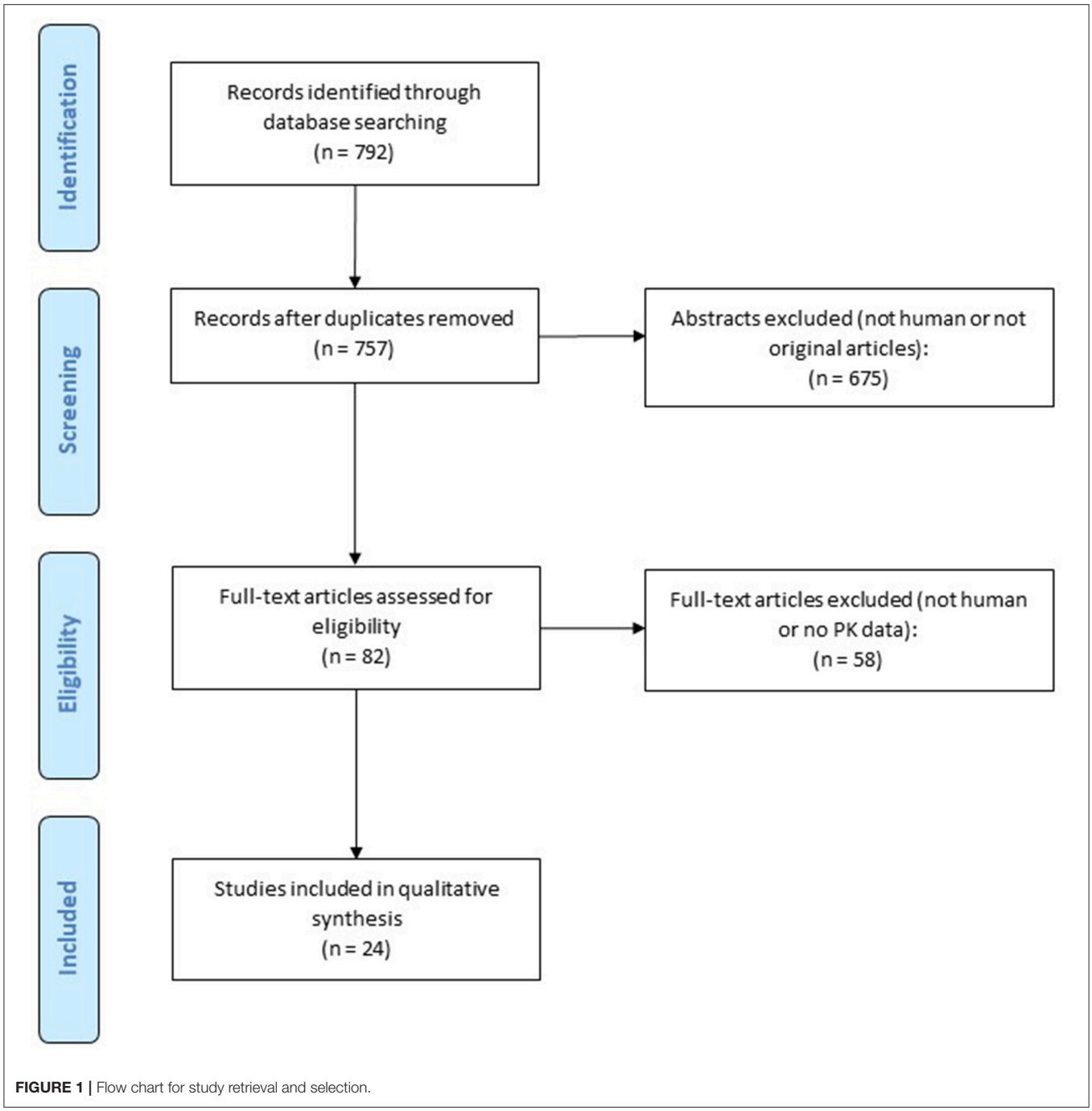

FIGURE 1 | Flow chart for study retrieval and selection.

Oromucosal Drops/Spray

A number of trials in humans were conducted by Guy and colleagues to explore administration route efficiency of sprays, an aerosol, and a nebuliser containing CBD or CBD and THC (CBD dose 10 or 20 mg) (Guy and Flint, 2004; Guy and Robson, 2004a,b). Oromucosal spray, either buccal, sublingual, or oropharyngeal administration, resulted in mean C_{max} between 2.5 and 3.3 ng/mL and mean T_{max} between 1.64 and 4.2 h. Sublingual drops resulted in similar C_{max} of 2.05 and 2.58 ng/mL and T_{max} of 2.17 and 1.67 h, respectively. Other oromucosal single dose studies reported C_{max} and T_{max} values within similar ranges (Karschner et al., 2011; Atsmon et al., 2017b).

Minimal evidence of plasma accumulation has been reported by chronic dosing studies over 5–9 days (Sellers et al., 2013; Stott et al., 2013a). C_{max} appears to be dose-dependent. A dose of 20 mg/day resulted in a mean C_{max} of 1.5 ng/mL and mean AUC_{0-t} of 6.1 h × ng/mL while 60 mg/day equated to a mean C_{max} of 4.8 ng/mL and AUC_{0-t} was 38.9 h × ng/mL (Sellers et al., 2013). In another study, C_{max} increased dose-dependently

TABLE 1 | Human studies reporting pharmacokinetic (PK) parameters for cannabidiol (CBD).

References	Total n, sex	Administration	Source	CBD dose	Plasma[a] PK details							Other
					T_{max} (median, range)[b] hrs	C_{max} (mean, SD)[b] ng/mL	AUC_{0-t} (mean, SD)[b] h × ng/mL	AUC_{0-inf} (mean, SD) h × ng/mL	K_{el} (mean, SD) 1/h	$t_{1/2}$ (mean, SD)[b] h	CL/F (mean, SD) L/h	Mean (SD)
Ohlsson et al., 1986	5, M, infrequent to frequent cannabis smokers	i.v.	In lab	20 mg		686 (239)	ng/ml min × 10^{-3} = 16.67 (3.23)			24 (6)	74.4 (14.4)	Distribution volume: 32.7 (8.6) l/kg.
"		Smoking	In lab	19.2 ± 0.3mg		110 (55)	ng/ml min × 10^{-3} = 4.85 (1.72)			31 (4)		Estimated systemic availability (%) from smoking: 31 (13)
Consroe et al., 1991	15, M/F	Oral capsules	NIDA	10 mg/kg/day daily for 6 weeks						2–5 days		
Guy and Robson, 2004b	12, M/F	Oromucosal spray sublingual (CBD and THC)	GW	10 mg	1.63 (SD 0.68)	2.5 (1.83)	6.81 (4.33)	7.12 (4.31)		1.44 (0.79)		
		Oromucosal spray buccal (CBD and THC)	GW	10 mg	2.79 (SD 1.31)	3.02 (3.15)	6.4 (4.62)	6.8 (4.46)		1.81 (2.05)		
		Oromucosal spray oro-pharyngeal (CBD and THC)	GW	10 mg	2.04 (SD 1.13)	2.61 (1.91)	7.81 (5.13)	8.28 (5.32)		1.76 (0.8)		
		CBME oral capsule (CBD and THC)	GW	10 mg	1.27 (SD 0.84)	2.47 (2.23)	5.76 (4.94)	6.03 (4.97)		1.09 (0.46)		
Guy and Robson, 2004a	24, M	Oromucosal spray sublingual (CBD and THC)	GW	10 mg	4.22	3.33	11.34	11.97		1.81		
Guy and Flint, 2004	6 M/F	Nebuliser (CBD and THC)	GW	20 mg	0.6 (0.08–1)	9.49 (8.01)	9.41 (10.8)	12.11 (10.83)	0.98 (0.58)	1.1 (0.97)		
		Aerosol (with THC)	GW	20 mg	2.35 (0.75–6)	2.6 (1.38)	5.43 (5.88)	13.53 (3.64)	0.43 (0.26)	2.4 (2.02)		
		Sublingual drops (CBD)	GW	20 mg	2.17 (1–4)	2.05 (0.92)	2.60 (3.45)					
		Sublingual drops (CBD and THC)	GW	20 mg	1.67 (1–3)	2.58 (0.68)	3.49 (2.65)	9.65 (4.02)	0.37 (0.114)	1.97 (0.62)		
Nadulski et al., 2005a	24, M/F	Oral capsule (CBD and THC)	Scherer GmbH & Co. KG, Eberbach, Germany	5.4mg once a week for 3 weeks	Mean 0.99 (0.5–2)	0.93 (range 0–2.6)	Mean 4.35, range (2.7–5.6)					

(Continued)

TABLE 1 | Continued

References	Total n, sex	Administration	Source	CBD dose	Plasma[a] PK details							Other
					T_{max} (median, range)[b] hrs	C_{max} (mean, SD)[b] ng/mL	AUC_{0-t} (mean, SD)[b] h × ng/mL	AUC_{0-inf} (mean, SD) h × ng/mL	K_{el} (mean, SD) 1/h	$t_{1/2}$ (mean, SD)[b] h	CL/F (mean, SD) L/h	Mean (SD)
	12, M/F	Oral capsule (CBD and THC) and breakfast consumed 1 hour after	Scherer GmbH & Co. KG, Eberbach, Germany	5.4mg once a week for 3 weeks	Mean 1.07 (0.5–2)	1.13 (range 0.39–1.9)	Mean 4.4 (range 2.5–5.3)					
Nadulski et al., 2005b	24, M/F	Cannabis extract	Sigma	5.4 mg	Mean 1.0 (0.5–2.0)	0.95 (range 0.3–2.57)						
Karschner et al., 2011	9, M/F cannabis smokers	Oromucosal spray (Sativex: CBD and THC)	GW	5 mg	3.6 (1.0–5.5)	Mean (SE): 1.6 (0.4)	4.5 (SE 0.6)					
				15 mg	4.6 (1.2–5.6)	Mean (SE): 6.7 (2.0)	18.1 (SE 3.6)					
Schwope et al., 2011	10, M/F, usual infrequent cannabis smokers	Cannabis cigarette	NIDA	2 mg	0.25 (0.25–0.50 h) whole blood/plasma	Median (range): plasma 2 (<LOQ−3.4)						
Eichler et al., 2012	9, M	Oral capsules (CBD and THC)	Cannapharm AG	Heated CBD (27.8 mg CBD: 0.8 mg CBDA)	0.83 (SD 0.17)	pmol/mL: 0.94 (0.22)	pmol h/moL 3.68 (1.34)					
				Unheated 14.8 mg CBD:10.8 mg CBDA)	1.17 (SD 0.39)	3.95 (0.92) pmoL/mL	pmol h/mol 7.67 (2.06)					
Lee et al., 2012	10, M/F, cannabis smokers	Cannabis cigarette	NIDA	2 mg	Median 0.25 (oral fluid)	0.03 (oral fluid)						
Sellers et al., 2013	60, M/F	Oromucosal spray (CBD and THC)	GW	20 mg, 5 days	1.4 (0, 8.45)	1.5 (0.78)	6.1 (5.76)	14.8 (7.87)				
	51, M/F			90 mg – 60 mg, 5 days	1.5 (0–6.45)	4.8 (3.4)	38.9 (33.75)	60.3 (37.71)				
Stott et al., 2013b	12, M	Oromucosal spray (CBD and THC)	GW	10 mg (fed state)	4.00 (3.02–9.02);	3.66 (2.28)	23.13 (9.29)	20.21 (8.43)	0.155 (0.089)	5.49 (2.17)	533 (318)	
Stott et al., 2013a	24, M	Oromucosal spray (CBD and THC)	GW	5 mg single dose	Mean 1.00 (0.75–1.50)	0.39 (0.08)	0.82 (0.33)	1.66 (0.51)	0.173 (0.084)	5.28 (3.28)	3,252 (1,002)	
				10 mg single dose	Mean 1.39 (0.75–2.25)	1.15 (0.74)	4.53 (3.53)	5.64 (4.09)	0.148 (0.079)	6.39 (4.48)	2,546 (1,333)	
				20 mg single dose	Mean 1.00 (0.75–1.75)	2.17 (1.23)	9.94 (9.02)	13.28 (12.86)	0.123 (0.097)	9.36 (6.81)	3,783 (4,299)	

(Continued)

TABLE 1 | Continued

References	Total n, sex	Administration	Source	CBD dose	Plasma[a] PK details							Other
					T_{max} (median, range)[b] hrs	C_{max} (mean, SD)[b] ng/mL	AUC_{0-t} (mean, SD)[b] h × ng/mL	AUC_{0-inf} (mean, SD) h × ng/mL	K_{el} (mean, SD) 1/h	$t_{1/2}$ (mean, SD)[b] h	CL/F (mean, SD) L/h	Mean (SD)
				5 mg, 9 days	Mean 1.64 (1.00–4.02)	0.49 (0.21)	2.52 (0.73)					
				10 mg, 9 days	Mean 1.27 (0.75–2.52)	1.14 (0.86)	6.66 (3.10)					
				20 mg 9 days	Mean 2.00 (1.02–6.00)	3.22 (1.90)	20.34 (7.29)					
Stott et al., 2013c	36, M	Oromucosal spray (CBD and THC)	GW	10 mg (3 groups)	1.00 (0.50–4.00); 1.38 (0.75–6.00); 1.15 (0.50–3.02)	1.03 (0.81); 0.66 (0.37); 0.63 (0.43)	3.23 (2.13); 1.82 (1.03); 1.83 (1.19)	5.10 (3.06); 3.54 (0.80); 3.00 (1.43)	0.148(0.108); 0.122 (0.111); 0.224 (0.158)	10.86(12.71); 7.81 (3.00); 5.22 (4.51)	2817 (1913); 2998 (896); 4,741 (3,835)	Varea/F (L): 28312 (19355); 31994 (12794); 26298 (14532)
				15 mg	4.5 (1.2–5.6)	Mean (SE): 6.7 (2.0)						
Newmeyer et al., 2014	24, M/F, frequent or occasional cannabis smokers	Cannabis cigarette (frequent smokers)	NIDA	2 ± 0.6 mg	0.5 (0.5–1)	Median (range): 14.8 (1.4–162)	Median (range): 29 (4.7–211)					
		Cannabis cigarette (occasional smokers)		2 ± 0.6 mg	1 (0.5–2)	Median (range): 7 (1.9–111)	Median (range): 11.6 (4.1–185)					
Desrosiers et al., 2014	21, M/F frequent and occasional smokers	Cannabis cigarette (frequent smokers)	NIDA	2 mg	0.5 (0.0–1.1)	1.1 (0.0–1.6)						
		Cannabis cigarette (occasional smokers)		2 mg	0 (0–500)	0 (0–1300)						
Manini et al., 2015	17, M/F	Oral capsules Co-administered with i.v. fentanyl	GW	400 mg	3 and 1.5 (plasma) and 6 and 2 (urine)	Plasma: 181.2 (39.8) and 114.2 (9.5); Urine: 4600 and 2900	704 (283) and 482 (314) mcg*hr/dL					
				800 mg	3 and 4 (plasma) and 4 and 6 (urine)	Plasma: 221 (35.6) and 157.1 (49.0); Urine: 3700 and 2800	867 (304) and 722 (443) mcg*hr/dL					

(Continued)

TABLE 1 | Continued

References	Total n, sex	Administration	Source	CBD dose	Plasma[a] PK details							Other
					T_{max} (median, range)[b] hrs	C_{max} (mean, SD)[b] ng/mL	AUC_{0-t} (mean, SD)[b] h × ng/mL	AUC_{0-inf} (mean, SD) h × ng/mL	K_{el} (mean, SD) 1/h	$t_{1/2}$ (mean, SD)[b] h	CL/F (mean, SD) L/h	Mean (SD)
Haney et al., 2016	8, M/F cannabis smokers	Oral capsules	STI pharmaceuticals	800 mg	Mean 3 (2–6)	77.9 (range 1.6–271.9)						
Cherniakov et al., 2017a	9, M	Oral capsules with piperine pro-nanolipospeheres (CBD and THC)	STI pharmaceuticals	10 mg	1 (0.5–1.5)	2.1 (0.4)	6.9 (1.3)					
		Oromucosal spray (CBD and THC; Sativex®)		10 mg	3 (1–5)	0.5 (0.1)	3.1 (0.4)					
Swortwood et al., 2017	20, M/F Cannabis smokers	Cannabis cigarettes – frequent smokers	NIDA	1.5 mg	Mean 0.29 (0.17–1.5) (oral fluid)	93.3 (range 0.65–350) (oral fluid)						
		Cannabis cigarettes – occasional smokers	NIDA	1.5 mg	Mean 0.17 (oral fluid)	55.9 (range 2.5–291) (oral fluid)						
		Cannabis containing brownie – frequent smokers	NIDA	1.5 mg	Mean 0.53 (0.17–1.5) (oral fluid)	8.0 (range 0.48–26.3) (oral fluid)						
		Cannabis containing brownie – occasional smokers	NIDA	1.5 mg	Mean 0.47 (0.17–1.5) (oral fluid)	5.9 (range 2.1–11.4) (oral fluid)						
		Vaporization – frequent smokers	Volcano® Medic, Storz & Bickel, Tuttlingen, Germany	1.5 mg	Mean 0.29 (0.17–1.5) (oral fluid)	76.3 (range 2.3–339) (oral fluid)						
		Vaporization – occasional smokers	Volcano® Medic, Storz & Bickel, Tuttlingen, Germany	1.5 mg	Mean 0.17 (oral fluid)	28.2 (range 0.23–167) (oral fluid)						
Atsmon et al., 2017b	15, M	CBD extract >93% in a PTL101 formulation (oral gelatin matrix pellet technology) Sublingual/buccal	AiFame-AiLab GmbH (CBD), Gelpell AG (capsules)	10 mg	3.0 (2.0–4.0)	3.22 (1.28)	9.64 (3.99)	10.31 (4.14)		2.95 (2.58)		
				100 mg	3.5 (1.5–5.0)	47.44 (20.14)	149.54 (34.34)	153.04 (34.7)		3.59 (0.26)		

(Continued)

TABLE 1 | Continued

References	Total n, sex	Administration	Source	CBD dose	Plasma[a] PK details						Other
					T_{max} (median, range)[b] hrs	C_{max} (mean, SD)[b] ng/mL	$AUC_{0\text{-}t}$ (mean, SD)[b] h × ng/mL	$AUC_{0\text{-}inf}$ (mean, SD) h × ng/mL	K_{el} (mean, SD) 1/h	$t_{1/2}$ (mean, SD)[b] h	CL/F (mean, SD) L/h	Mean (SD)
		Oromucosal spray (CBD and THC)	GW	10 mg	3.5 (1.0–5.0)	2.05 (1.1)	7.3 (2.86)	7.81 (2.81)	0.33 (0.09)	2.31 (0.72)		
Atsmon et al., 2017a	15, M	CBD and THC in a PTL401 capsule (self-emulsifying oral drug delivery system)	STI pharmaceuticals	10 mg	1.25 (0.5–4.0)	2.94 (0.73)	9.85 (4.47)	10.52 (4.53)	0.29 (0.17)	3.21 (1.62)		
Devinsky et al., 2018b	34, children	Oral solution	GW	2.5 mg			70.23 (mean from 3 groups)					
				5 mg/kg/day			241					
				10 mg/kg/day			722					
				20 mg/kg/day			963					

[a,b] *Unless otherwise stated. PK, pharmacokinetics; CBD, cannabidiol; THC, Tetrahydrocannabinol; M, male; F, female; AUC, area under the curve; Conc., concentration; GW, GW pharmaceuticals; NIDA, US national institute on drug abuse; LOQ, limit of quantification; IV, intravenous; CBME, cannabis based medicine extract; Min(s), min(s).*

from 0.4 to 1.2 and 2.2 ng/mL following 5, 10, and 20 mg single doses, respectively, and from 0.5 to 1.1 and 3.2 ng/mL, respectively following chronic dosing over 9 consecutive days (Stott et al., 2013a). There was a significant increase in time-dependent exposure during the chronic treatment. Mean AUC_{0-t} for the single doses were 0.8, 4.5, 9.9, and 2.5, 6.7, and 20.3 for the chronic dosing schedule, respectively. T_{max} does not appear to be dose-dependent, nor affected by acute or chronic dosing schedules.

Stott et al. reported an increase in CBD bioavailability under fed vs. fasted states in 12 men after a single 10 mg dose of CBD administered through an oromucosal spray which also contained THC (Stott et al., 2013a,b). Mean AUC and C_{max} were 5- and 3-fold higher during fed conditions compared to fasted (AUC_{0-t} 23.1 vs. 4.5; C_{max} 3.7 vs. 1.2 ng/mL). T_{max} was also delayed under the fed state (4.0 vs. 1.4 h).

In children, Devinsky et al. reported mean AUC as 70, 241, 722, and 963 h × ng/mL in groups receiving 2.5, 5, 10, and 20 mg/Kg/day of CBD in oral solution (Devinsky et al., 2018b).

Oral Intake

C_{max} and AUC following oral administration also appears to be dose dependent. A dose of 10 mg CBD resulted in mean C_{max} of 2.47 ng/mL at 1.27 h, and a dose of 400 or 800 mg co-administered with i.v. fentanyl (a highly potent opioid) to examine its safety resulted in a mean C_{max} of 181 ng/mL (at 3.0 h) and 114 ng/mL (at 1.5 h) for 400 mg, and 221 ng/mL (at 3.0 h) and 157 ng/mL (at 4.0 h) for 800 mg, in 2 sessions, respectively (Guy and Robson, 2004b; Manini et al., 2015). A dose of 800 mg oral CBD in a study involving 8 male and female cannabis smokers, reported a mean C_{max} of 77.9 ng/mL and mean T_{max} of 3.0 h (Haney et al., 2016). Although, an increase in dose corresponds with an increase in C_{max}, the C_{max} between the higher doses of CBD does not greatly differ, suggesting a saturation effect (e.g., between 400 and 800 mg).

One hour after oral capsule administration containing 5.4 mg CBD in males and females, mean C_{max} was reported as 0.93 ng/mL (higher for female participants than male) (Nadulski et al., 2005a). A subset ($n = 12$) consumed a standard breakfast meal 1 h after the capsules, which slightly increased mean C_{max} to 1.13 ng/mL. CBD remained detectable for 3–4 h after administration (Nadulski et al., 2005b).

Cherniakov et al. examined the pharmacokinetic differences between an oromucosal spray and an oral capsule with piperine pro-nanolipospheres (PNL) (both 10 mg CBD) in 9 men. The piperine-PNL oral formulation had a 4-fold increase in C_{max} (2.1 ng/mL vs. 0.5 ng/mL), and a 2.2-fold increase in AUC_{0-t} (6.9 vs. 3.1 h × ng/mL), while T_{max} was decreased (1.0 vs. 3.0 h) compared to the oromucosal spray (Cherniakov et al., 2017a). This group further developed self-emulsifying formulations and reported again an increased bioavailability and increased C_{max} within a shorter time compared to a reference spray (Atsmon et al., 2017a,b).

Intravenous Administration

The highest plasma concentrations of CBD were reported by Ohlsson et al. following i.v. administration of 20 mg of

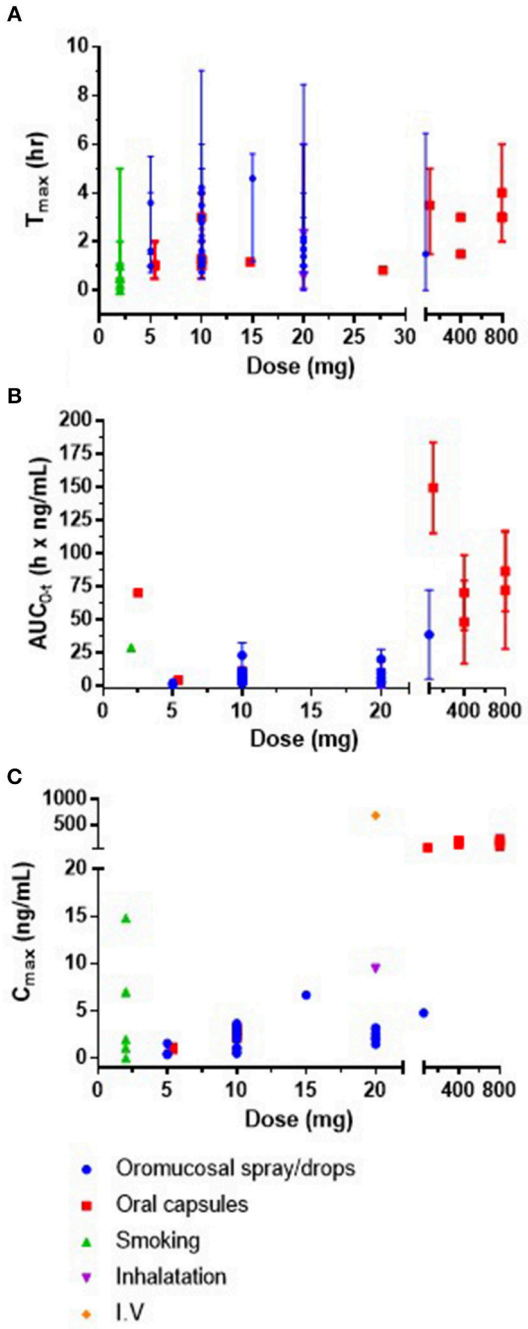

FIGURE 2 | (A) Mean or median Tmax (h) and range against CBD dose (mg) **(B)** mean or median area under the curve (AUC0-t) (h × ng/mL) and SD against CBD dose (mg) and **(C)** plasma mean or median concentration max (Cmax; ng/mL) against CBD dose (mg). It was not possible to present error bars for Cmax as SD and SEM were both reported in the data. IV, intravenous; SD, standard deviation; SEM, standard error of the mean.

deuterium-labeled CBD. Mean plasma CBD concentrations were reported at 686 ng/mL (3 min post-administration), which dropped to 48 ng/mL at 1 h.

Controlled Smoking and Inhalation

After smoking a cigarette containing 19.2 mg of deuterium-labeled CBD, highest plasma concentrations were reported as 110 ng/mL, 3 min post dose, which dropped to 10.2 ng/ml 1 h later (Ohlsson et al., 1986). Average bioavailability by the smoked route was 31% (Ohlsson et al., 1986). A nebuliser resulted in a C_{max} of 9.49 ng/mL which occurred at 0.6 h, whereas aerosol administration produced C_{max} (2.6 ng/mL) at 2.35 h (Guy and Flint, 2004). In 10 male and female usual, infrequent cannabis smokers, C_{max} was 2.0 ng/mL at 0.25 h after smoking a cigarette containing 2 mg of CBD (Schwope et al., 2011). CBD was detected in 60% of whole blood samples and in 80% of plasma samples at observed C_{max}, and no longer detected after 1.0 h. A study in 14 male and female cannabis smokers reported 15.4% detection in frequent smokers with no CBD detected in occasional smokers in whole blood analysis (Desrosiers et al., 2014). In plasma however, there was a 53.8 and 9.1% detection in the frequent and occasional groups, with corresponding C_{max} of 1.1 ng/mL in the frequent group, and below limits of detection in the occasional group.

Half-Life

The mean half-life ($t_{1/2}$) of CBD was reported as 1.1 and 2.4 h following nebuliser and aerosol administration (20 mg) (Guy and Flint, 2004), 1.09 and 1.97 h following single oral administration (10 and 20 mg) (Guy and Flint, 2004; Guy and Robson, 2004b), 2.95 and 3.21 h following 10 mg oral lipid capsules (Atsmon et al., 2017a,b), between 1.44 and 10.86 h after oromucosal spray administration (5–20 mg) (Guy and Robson, 2004b; Sellers et al., 2013; Stott et al., 2013a,b; Atsmon et al., 2017b), 24 h after i.v. infusion, 31 h after smoking (Ohlsson et al., 1986), and 2–5 days after chronic oral administration (Consroe et al., 1991).

Elimination Rate

Mean elimination rate constant (K_{el} [1/h]) has been reported as 0.148 in fasted state, and 0.155 in fed state after 10 mg CBD was administered in an oromucosal spray also containing THC (Stott et al., 2013a,b). After single doses of 5 and 20 mg CBD, mean K_{el} (1/h) was reported as 0.173 and 0.123 (Stott et al., 2013a). Following 20 mg CBD administration through a nebuliser and pressurized aerosol, mean K_{el} was reported as 0.98 and 0.43, respectively, while 20 mg CBD administered as sublingual drops was reported as 0.37 (Guy and Flint, 2004).

Plasma Clearance

Plasma apparent clearance, CL/F (L/h) has been reported to range from 2,546 to 4,741 in a fasted stated following 10 mg CBD administered via oromucosal spray (Stott et al., 2013a,c). This value decreases to 533 following the same concentration in a fed state (Stott et al., 2013b). A plasma apparent clearance of 3,252 and 3,783 was reported following 5 and 20 mg single doses of CBD via oromucosal spray (Stott et al., 2013a). Ohlsson et al. reported plasma apparent clearance as 74.4 L/h following i.v. injection (Ohlsson et al., 1986).

Volume of Distribution

Mean apparent volume of distribution (V/F [L]) was reported as 2,520 L following i.v. administration (Ohlsson et al., 1986). Following single acute doses through oromucosal spray administration, apparent volume of distribution was reported as 26,298, 31,994, and 28,312 L (Stott et al., 2013a).

DISCUSSION

The aim of this study was to review and analyse all available PK data on CBD in humans. Only 8 publications reported PK parameters after administering CBD on its own, and the others were in combination with THC/cannabis. Only 1 study reported the bioavailability of CBD in humans (31% following smoking). From the analysis of these papers, the following observations were made; peak plasma concentrations and area under the curve (AUC) are dose-dependent and show minimal accumulation; C_{max} is increased and reached faster following i.v., smoking or inhalation; C_{max} is increased and reached faster after oral administration in a fed state or in a pro-nanoliposphere formulation; T_{max} does not appear to be dose-dependent; and half-life depends on dose and route of administration. Overall, considerable variation was observed between studies, although they were very heterogeneous, and further work is warranted.

Human studies administering CBD showed that the AUC_{0-t} and C_{max} are dose-dependent, and T_{max} mostly occurred between 1 and 4 h. Animal studies in piglets, mice, and rats also all demonstrate a dose-dependent relationship between CBD and both plasma and brain concentrations (Long et al., 2012; Hammell et al., 2016; Garberg et al., 2017), suggesting that human brain concentrations will also be dose-dependent. Ten publications in this review reported the half-life of CBD which ranged from 1 h to 5 days and varies depending on the dose and route of administration. Very limited data was available for detailed analysis on the elimination rate, apparent clearance or distribution of CBD in humans.

Plasma levels of CBD were increased when CBD was administered with food or in a fed state, or when a meal is consumed post-administration. Oral capsules with piperine pro-nanolipospheres also increased AUC and C_{max}. This is also demonstrated in animal studies; co-administration of lipids with oral CBD increased systemic availability by almost 3-fold in rats (Zgair et al., 2016) and a pro-nanoliposphere formulation increased oral bioavailability by about 6-fold (Cherniakov et al., 2017b). As CBD is a highly lipophilic molecule, it is logical that CBD may dissolve in the fat content of food, increasing its solubility, and absorption and therefore bioavailability as demonstrated by numerous pharmacological drugs (Winter et al., 2013). Thus, it may be advisable to administer CBD orally in a fed state to allow for optimal absorption.

Only one study used intravenous administration of CBD and reported PK details, which could be a beneficial route of administration in some acute indications. Results from other routes such as rectal, transdermal, or intraperitoneal have also not been published in humans, although transdermal CBD gel and topical creams have been demonstrated to be successful in animal studies (Giacoppo et al., 2015; Hammell et al., 2016). Interestingly, intraperitoneal (i.p.) injection of CBD corresponded to higher plasma and brain concentrations than oral administration in mice, however in rats, similar concentrations were observed for both administration routes, and brain concentrations were in fact higher following oral compared to i.p. route (Deiana et al., 2012). No published data exists on the tissue distribution of CBD in humans. Although plasma levels of CBD do not show accumulation with repeated dosing, it is possible that there may be tissue accumulation.

Only one study in this review was conducted in children ($n = 34$) (Devinsky et al., 2018b). Children (4–10 years) with Dravet syndrome were administered an oral solution of CBD and AUC was reported to increase dose-dependently. It is important to emphasize the statement that children are not small adults, and there are many differences in their pharmacokinetic and pharmacodynamic profiles. Absorption, excretion, metabolism, and plasma protein binding are generally reduced in children compared to adults, and apparent volume of distribution is generally increased (Fernandez et al., 2011). These parameters need to be explored fully for CBD in order to understand and advise dose adjustments.

Within the adult studies, inter- and intra-subject variability was observed in studies, and it remains to be seen whether i.v. and other routes of administration that by-pass initial metabolism will alleviate this issue. Interestingly, although each of the subject's weight was taken into account, none of the studies addressed subject fat content as a factor in their exclusion criteria; as muscle can weigh more than the same proportion of fat. It is well-known that cannabinoids are highly lipophilic compounds and accumulate in fatty tissue which can then be released gradually (Gunasekaran et al., 2009). It may be of benefit in future study to either put in place more stringent exclusion criteria and measure subject fat content or assess the possible accumulation of CBD in fatty tissue. Differences in metabolism, distribution and accumulation in fat, and in biliary and renal elimination may be responsible for prolonged elimination half-life and variable pharmacokinetic outcomes. CBD use is widespread and has been recommended for use by the FDA in childhood-onset epilepsy. CBD also displays therapeutic promise in other disorders such as schizophrenia and post-traumatic stress disorder. If we are to understand the actions of CBD in those disorders and increase the success rate for treatment, these groups of patients and their distinct characteristics must be assessed as they may not be comparable to a healthy volunteer population.

A systematic review in 2014 concluded that CBD generally has a low risk of clinically significant drug-interactions (Stout and Cimino, 2014). A few studies in the current review included examination of drug-drug interactions with CBD. GW Pharmaceuticals performed a clinical trial investigating the pharmacokinetic interaction between CBD/THC spray (sativex) and rifampicin (cytochrome P450 inducer), ketoconazole, and omeprazole (cytochrome P450 inhibitors) (Stott et al., 2013c). Authors concluded overall that CBD in combination with the drugs were well-tolerated, but consideration should be noted when co-administering with other drugs using the CYP3A4

pathway. Caution is also advised with concomitant use of CBD and substrates of UDP-glucuronosyltransferases UGT1A9 and UGT2B7, and other drugs metabolized by the CYP2C19 enzyme (Al Saabi et al., 2013; Jiang et al., 2013). Manini et al. co-administered CBD with i.v. fentanyl (a high potency opioid) which was reported as safe and well-tolerated (Manini et al., 2015). In a number of trials with CBD in children with severe epilepsy, clobazam concentrations increased when CBD was co-administered and dosage of clobazam had to be reduced in some patients in one study (Geffrey et al., 2015; Devinsky et al., 2018b). Gaston and colleagues performed a safety study in adults and children in which CBD was administered with commonly-used anti-epileptic drugs (AEDs) (Gaston et al., 2017). Most changes in AED concentrations were within acceptable ranges but abnormal liver function tests were reported in those taking valproate and authors emphasized the importance of continued monitoring of AED concentrations and liver function during treatment with CBD.

Limitations of this review should be acknowledged. Different population types including healthy and patient populations and cannabis naïve or not were all grouped together which may impede generalizability. The proportions of men and women in each study were also not uniform, and it is still being elucidated whether men and women have distinct pharmacokinetic profiles with regards to cannabinoids (Fattore and Fratta, 2010). One study suggested that the PK of CBD was different in their female volunteers (Nadulski et al., 2005a). It should also be mentioned that CBD is currently not an approved product with a pharmacopeia entry so using different sources of CBD that are subject to different polymeric forms, different particle sizes, and different purities may also affect the PK profiles observed. It is important for future work that researchers record the source of the CBD material used so that results have the highest chance of being replicated. Despite a thorough search of the two databases chosen, the addition of more databases may have widened the search to increase the number of results and hence improve the reliability and validity of the findings. However, the review was carried out by two independent reviewers, and searches generated were analyzed separately and then compared.

In conclusion, this review demonstrates the lack of research in this area, particularly in routes of administration other than oral. An absence of studies has led to failure in addressing the bioavailability of CBD despite intravenous formulations being available. This is of critical importance due to the popularity of CBD products and will help interpret other PK values. Standardized and robust formulations of CBD and their PK data are required for both genders, with consideration of other factors such as adiposity, genetic factors that might influence absorption and metabolism, and the effects of disease states.

AUTHOR CONTRIBUTIONS

SM, SO, and AY: substantial contributions to the conception or design of the work. SM: writing of the manuscript. SM and NS: database searching and data extraction. All authors: the analysis and interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01365/full#supplementary-material

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Genetic polymorphisms of the endocannabinoid system

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BRIEF REPORT

Genetic polymorphisms of the endocannabinoid system in obesity and diabetes

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The endocannabinoid system (ECS) is involved in many physiological processes including fertility, pain and energy regulation. The aim of this systematic review was to examine the contribution of single nucleotide polymorphisms (SNPs) of the ECS to adiposity and glucose metabolism. Database searches identified 734 articles, of which 65 were included; these covered 70 SNPs in genes coding for cannabinoid receptors 1 and 2 (CB_1, CB_2), fatty acid amide hydrolase (FAAH) and N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD). No studies included SNPs relating to monoacylglycerol lipase or diacylglycerol lipase. The CB_1 receptor SNP rs1049353 showed 17 associations with lower body mass index (BMI) and fat mass (five studies). It also showed three associations with lower insulin levels (one study). Conversely, the CB_1 receptor SNP rs806368 was associated with increased BMI and waist circumference (two studies). The FAAH SNP rs324420 was associated with increased obesity (three studies). A haplotype of NAPE-PLD was associated with decreased BMI (one study). A total of 60 SNPs showed no association with any measured outcome. This review suggests a complex but important role of ECS SNPs in energy and glucose metabolism.

KEYWORDS

cannabinoid, diabetes, endocannabinoid, obesity, polymorphisms

1 | INTRODUCTION

The endocannabinoid system (ECS) consists of two G-protein coupled receptors (CB_1 and CB_2) and endogenously produced ligands, or endocannabinoids such as anandamide and 2-arachidonoyl glycerol, and the enzymes involved in their synthesis or degradation: fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL), diacylglycerol lipase (DAGL) and N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD). It is well established that CB_1 activation leads to increases in energy storage[1] which occur via increased motivation to consume food and to decreased satiety.

Single nucleotide polymorphisms (SNPs) are naturally occurring variations of a genetic sequence, which often affect protein structure. To date, studies on the effects of endocannabinoid SNPs have focused on central nervous system disorders such as Parkinson's disease and Alzheimer's disease.[2] However, there is accumulating evidence for the role of endocannabinoid SNPs in adiposity[3] and glucose metabolism.[4] Therefore, the aim of this systematic review was to systematically collate the evidence relating to SNPs of the ECS in obese or diabetic phenotypes. By studying amino acid sequence alterations and any resultant residue changes, we hoped to identify important genetic changes that alter the normal physiology of adiposity and glucose metabolism.

2 | MATERIALS AND METHODS

Searches were performed by two independent researchers using PubMed, EMBASE and Web of Science and concluded on January 26, 2018. Additional studies were identified from bibliographies. The search terms used were: Cannabinoid OR endocannabinoid receptor OR CB_1 OR CB_2 OR FAAH OR fatty acid amide hydrolase AND polymorphism AND obesity OR diabetes OR BMI OR monoacylglycerol lipase OR MAGL OR diacylglycerol lipase OR DAGL OR N-acyl phosphatidylethanolamine-specific phospholipase D OR NAPE PLD. A summary of search results and exclusions is given in Supporting Information Figure S1, and a full reference list is available in Appendix S1.

The SNP database dbSNP was used to gather information regarding nucleotide and amino acid changes.[5]

Articles included were original studies relating to polymorphisms of the ECS affecting energy regulation, glucose homeostasis and adiposity. Demographic and clinical parameters included were: body mass index (BMI); waist circumference (WC); waist-to-hip ratio (WHR); body weight; adiposity; type II diabetes mellitus (T2DM); insulin and glucose levels; homeostatic model assessment for insulin resistance ($HOMA_{IR}$); adipokine levels (adiponectin, leptin and resistin); cardiovascular parameters (blood pressure, heart rate); inflammation (levels of interleukin 6 [IL-6], tumour necrosis factor alpha [TNFα] and C-reactive protein [CRP]); and lipid levels (triglycerides, HDL-C and LDL-C). Records excluded were review articles, articles on the ECS not relating to polymorphisms, studies regarding central disorders, studies in non-humans and studies in a language other than English.

Included articles were analysed for significant ($P < 0.05$) positive or negative associations between SNPs and relevant parameters. A "positive" association refers to a higher value of the measured outcome in the presence of the polymorphism, whereas a "negative" association refers to a lower value in the presence of the polymorphism. The absence of a significant association between the measured outcome and the polymorphism is described as a "neutral" association. Risk of bias was assessed using the Cochrane Collaboration's tool for assessing risk of bias.[6]

3 | RESULTS

A total of 65 studies were identified from among 733 full-text articles. Risk of bias in these studies was low overall and is summarized in Supporting Information Figure S2. In total, 38 CB_1, 18 CB_2, 13 FAAH and one NAPE-PLD SNPs were studied. No studies relating to MAGL or DAGL SNPs were found. The most commonly studied SNPs, and those that showed the most significant associations, were CB_1 SNPs rs1049353 and rs806368, and FAAH SNP rs324420. Their associations with body weight and glucose metabolism parameters are presented in Table 1. All SNPs and their associations with measured outcomes are documented in Supporting Information Table S1. A summary of all included studies and their relevant findings is shown in Supporting Information Table S2.

3.1 | BMI and body weight

3.1.1 | CB_1

The rs1049353 mutant allele was associated with lower BMI in six European populations[15–17,19,37] and with decreased fat mass in a Danish population (n = 783).[38] Conversely, homozygosity for the rs1049353 mutant allele was associated with higher WHR and WC in obese men ($P < 0.01$; n = 1064)[7] and with increased childhood obesity in a European population ($P = 0.01$; n = 200).[39] The majority of associations with rs1049353 were neutral (90%) (Table 1). However, negative associations were more common than positive associations (Figure 1), suggesting that this SNP plays a part in a more complex genetic susceptibility to increased adiposity. Male carriers of the rs806368 mutant allele showed greater BMI values in a Japanese cohort ($P = 0.001$) and were more likely to be obese ($P = 0.01$; n = 1452)[25] (Supporting Information Table S1).

3.1.2 | FAAH

FAAH polymorphism rs324420 was positively associated with obesity in four cohorts (n = 18 987).[27–30]

3.1.3 | CB2

The mutant allele of CB_2 SNP rs3123554 was associated with lower total body fat in women but not men, in a European cohort ($P = 0.001$), with lower BMI in individuals at risk of T2DM ($P < 0.01$) and with reduced weight loss ($P < 0.01$; n = 2006).[40]

3.1.4 | NAPE-PLD

In a Norwegian cohort, a haplotype of NAPE-PLD showed an association with increased BMI ($P < 0.05$; n = 5011).[12]

3.2 | Type II diabetes

3.2.1 | CB_1

The mutant allele of CB_1 polymorphism rs1049353 was associated with lower insulin, glucose and $HOMA_{IR}$ levels in Spanish obese women[22] and with lower insulin in two other European cohorts (n = 983).[20,21] CB_1 SNP rs806365 was associated with decreased $HOMA_{IR}$ values and with incidence of T2DM in a North American cohort ($P \leq 0.05$; n = 2411).[4]

3.2.2 | CB_2

The mutant allele of CB_2 polymorphism rs3123554 was associated with raised insulin levels and with $HOMA_{IR}$ values in an obese population (n = 1027)[41] (Figure 1).

3.2.3 | FAAH

The mutant allele of FAAH polymorphism rs324420 was associated with lower insulin levels in two obese populations ($P < 0.05$; n = 165),[32,34] and was also associated with lower $HOMA_{IR}$ levels in obese Spanish females ($P < 0.05$; n = 143).[32]

3.3 | Lipids

Overall, 22 positive associations with lipid levels were seen. The mutant allele of CB_1 SNP rs1049353 was associated with higher HDL and lower TGs in three cohorts,[18,22,23] as well as with lower TGs in two populations (n = 808)[19,22] (Table 1).

FAAH SNPs rs324420 and rs3123554 were associated with higher TG levels in European cohorts ($P < 0.05$; n = 1644)[23,41] (Table 1). FAAH SNP rs324420 was also associated with raised anandamide levels in a Brazilian population ($P < 0.05$; n = 200).[42]

4 | DISCUSSION

The aim of this study was to collate evidence relating to SNPs of the ECS and obese or diabetic phenotypes to identify important genetic

TABLE 1 Associations found between single nucleotide polymorphisms and metabolic and anthropometric parameters

Polymorphism	Gene	Nucleotide change	Nucleotide position	Region of gene	Amino acid change	Amino acid position	Associations
rs1049353	CNR1	G>A	1359	Exon	Thr>Thr	453	**Positive**: • Homozygosity for mutant allele associated with increased WHR and WC in obese men only.[7] • Mutant allele associated with higher fat in post-menopausal women.[8] • Mutant allele associated with increased BMI in T2DM subjects.[9] • Wild-type allele associated with higher $HOMA_{IR}$.[4] • Mutant allele group associated with greater weight loss and decrease in BMI. [10] • Mutant allele associated with childhood obesity.[11] **Negative**: • Mutant allele associated with lower glucose.[10] • Mutant allele associated with lower insulin. [12–14] • Mutant allele with lower BMI.[7,15–18] • Mutant allele with lower $HOMA_{IR}$, TGs.[7,16,17,19–23]
rs806368	CNR1	T>C	4895	Intron	-	-	**Positive:** • Mutant allele associated with increased WHR.[11] • Mutant allele associated with increased TGs.[24] • Mutant allele associated with increased BMI, WC and obesity.[25]
rs324420	FAAH	C>A	385	Exon	Pro>Thr	129	**Positive:** • Mutant allele associated with higher insulin and $HOMA_{IR}$ in patients without MetS.[26] • Homozygosity for mutant allele associated with increased BMI.[27] • Mutant allele associated with obesity.[28,29] • Wild-type allele associated with childhood obesity.[30] • Mutant allele associated with increased TGs.[31] **Negative:** • Mutant allele associated with lower TGs, glucose and $HOMA_{IR}$ levels.[9,32] • Mutant allele associated with better percentage weight loss 9 months and 1 year after bariatric surgery, but not after 3 months.[33] • Lower insulin and $HOMA_{IR}$ in mutant-type group. Mutant allele associated with greater decreases in weight and WC than wild-type following hypocaloric diet. Mutant allele also associated with greater decreases in glucose, $HOMA_{IR}$ and TGs.[34] • Wild-type allele associated with lower WC, BMI, $HOMA_{IR}$ and TGs in subjects with MetS.[35] • Mutant allele associated with lower insulin, glucose and $HOMA_{IR}$ values.[36]

Abbreviations: BMI, body mass index; CNR1, cannabinoid receptor gene 1; FAAH, fatty acid amide hydrolase; $HOMA_{IR}$, homeostatic model assessment of insulin resistance; MetS, metabolic syndrome; T2DM, type 2 diabetes mellitus; TGs, triglycerides; TNF-α, tumour necrosis factor α; WC, waist circumference; WHR, waist-to-hip ratio.

changes that alter metabolism. From among the 65 included articles, 70 polymorphisms were studied. CB_1 SNP rs1049353 showed 17 associations with lower BMI and fat mass. It also showed associations with reduced glucose, insulin and $HOMA_{IR}$ values. CB_1 polymorphism rs806368 showed five associations with increases in BMI, WC and WHR. The FAAH SNP rs324420 showed seven associations with increased incidence of obesity. A total of 60 SNPs showed no association with any measured outcome. These findings suggest an important role of selected SNPs of the ECS in adiposity, although the number of studies showing no associations means that their contribution is probably part of complex interactions.

The SNP rs1049353 occurs at nucleotide position 1359, a region of the CB_1 (CNR1) gene coding for the receptor's intracellular domain or C-terminal. One study showed that replacement of the C-terminal resulted in decreased affinity of the CB_1 agonist CP55940 and increased affinity of the CB_1 antagonist SR141716A.[43] This suggests that the C-terminal is important in receptor signalling. Although rs1049353 is a synonymous SNP and does not result in a change in amino acid residue (Thr>Thr), altered substrate interaction deriving from synonymous SNPs has been observed elsewhere,[44] suggesting that this is a legitimate theory.

The literature showed 13 associations between rs1049353 and reductions in parameters of glucose metabolism[4,7,10,12–14,16,17,19–23] (Figure 1). This suggests that this SNP is important in diabetic phenotypes, probably caused by upregulation of gluconeogenic transcription factors as the result of increased CB_1 receptor activity. It is unclear why many studies (n = 14) showed no association with parameters of glucose metabolism.

The rs324420 SNP reduces FAAH activity and increases the likelihood of the enzyme itself being degraded,[45] leading to cannabinoid

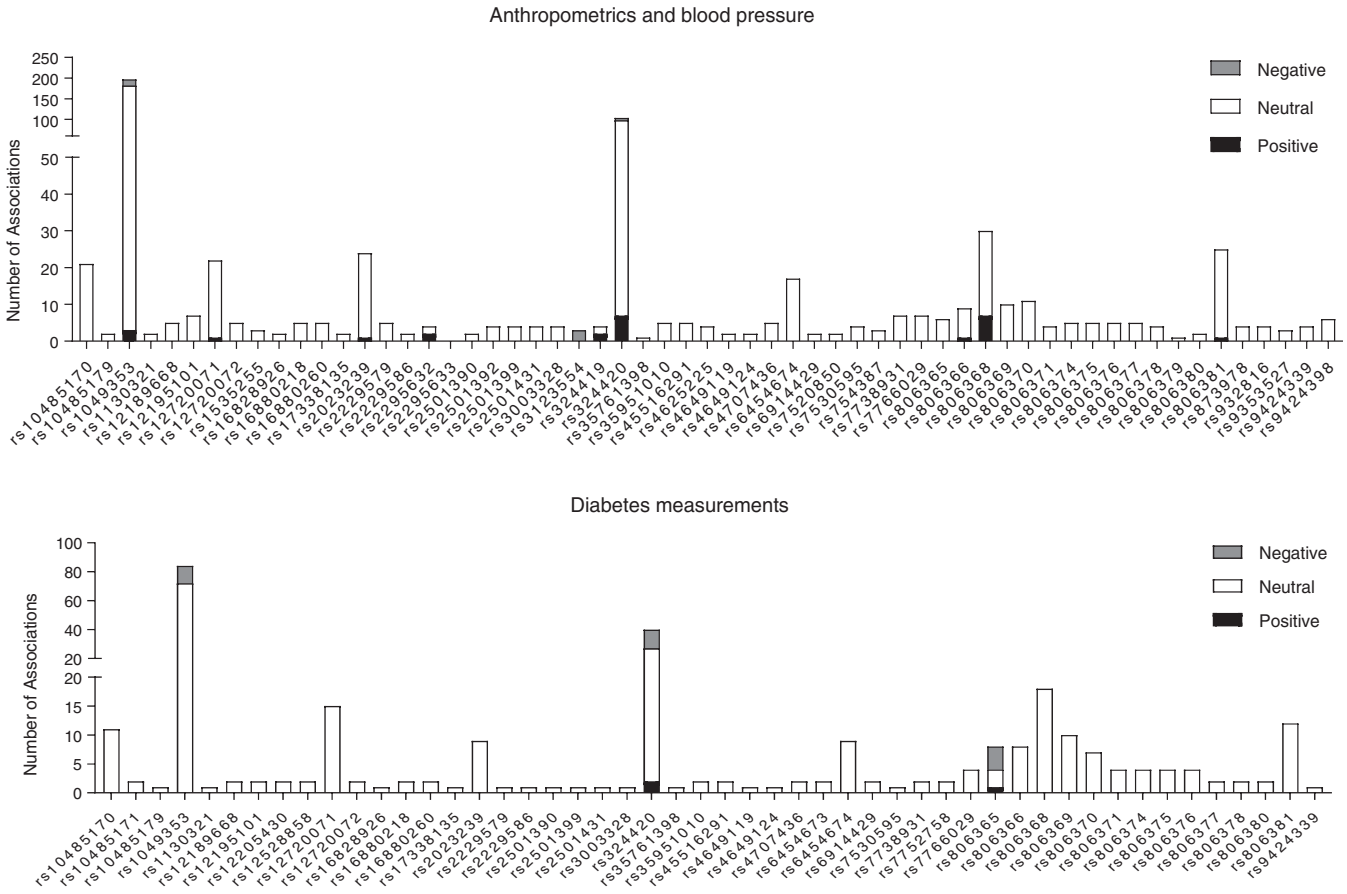

FIGURE 1 Number of associations found between ECS polymorphisms and anthropometric and diabetic parameters

overactivity. Subsequent CB_1 activation leads to adipogenesis and reduced expenditure, all of which contribute to obesity-related phenotypes. Our analysis showed that rs324420 was associated with higher anandamide levels,[42] increased BMI and obesity,[27,30,31] which suggests cannabinoid over-activation and subsequent adiposity and that this SNP, therefore, reduces FAAH activity (Table 1).

The potential contribution of CNR2 polymorphisms to human metabolism is less clear. Fewer studies investigated these SNPs, and the two polymorphisms studied (rs3123554 and rs35761398) showed conflicting associations with body weight parameters and glucose metabolism. As CB_2 receptors are found primarily in the central nervous system and on immune cells, it is likely that they are less involved in the regulation of body fat and, therefore, any alterations in their genetic structure are less relevant here. As no studies were found relating to SNPs of DAGL or MAGL, their contribution to obesity and glucose metabolism remains unclear.

Increasing age may determine the impact of the polymorphism. For instance, associations between SNPs rs2023239 and rs806381 and increased anthropometric measurements were found only in adult subjects.[46,47] Ageing leads to reductions in ligand binding[48] and coupling between the CB_1 receptor and its G-protein,[49] which may account for the delayed onset of increases in body weight parameters in some populations. There may also be an impact of gender on these data. Male carriers of the mutant alleles of CNR1 polymorphisms rs1049353 and rs806368 have an increased likelihood of obesity.[7,25] Similarly, the associations between the CNR2 polymorphism rs3123554 and lower BMI, weight and body fat percentage were reported in women.[40] Gender differences in feeding behaviour have been observed previously in animal models.[13] This may be explained by the action of oestrogen, which uncouples CB receptors from their effector systems in synaptic terminals, thus reducing the effect of cannabinoids.[50] Higher oestrogen levels in non-pregnant females may therefore contribute to these gender-specific findings.

In conclusion, associations between the mutant allele of the CB_1 SNP rs1049353 and decreased fat mass, weight and BMI indicate that this SNP is an important contributor to alterations in metabolism. Evidence indicates that decreased receptor functionality affects normal pathways of adipogenesis and energy regulation. Its effects also extend to improvements in lipid levels and parameters of glucose metabolism. The mutant allele of FAAH polymorphism rs324420 was associated with increased BMI and triglyceride levels, possibly caused by decreased enzyme activity and overactivation of the ECS. Other SNPs had varying associations, but results were often conflicting. These findings represent therapeutic targets for the management of obesity and hyperlipidaemia, and assessment of patients for these genetic changes would provide an opportunity to provide personalised treatment for a proportion of patients. Further studies in populations of varying demographics are needed, to investigate the role that other SNPs play in adiposity and glucose metabolism, as well as genetic studies to determine the molecular changes of the SNPs responsible for alterations in function.

Conflict of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contributions

J. M. D. was responsible for search terms, conducting initial literature searches, gathering relevant data from articles, writing the majority of the manuscript, construction of the tables and figures and performing referencing throughout. S. A. M. performed a second literature search, confirmed the findings, helped in writing and editing the manuscript, produced graphs using data gathered and contributed to referencing. I. I. helped in editing the manuscript, suggested alterations to tables and provided assistance in the publishing and proofing process. S. E. O. suggested search terms and databases to use, provided guidance, edited the manuscript and assisted in the publishing process.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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ORIGINAL RESEARCH
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An Analysis of Endocannabinoid Concentrations and Mood Following Singing and Exercise in Healthy Volunteers

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The euphoric feeling described after running is, at least in part, due to increased circulating endocannabinoids (eCBs). eCBs are lipid signaling molecules involved in reward, appetite, mood, memory and neuroprotection. The aim of this study was to investigate whether activities other than running can increase circulating eCBs. Nine healthy female volunteers (mean 61 years) were recruited from a local choir. Circulating eCBs, haemodynamics, mood and hunger ratings were measured before and immediately after 30 min of dance, reading, singing or cycling in a fasted state. Singing increased plasma levels of anandamide (AEA) by 42% ($P < 0.05$), palmitoylethanolamine (PEA) by 53% ($P < 0.01$) and oleoylethanolamine (OEA) by 34% ($P < 0.05$) and improved positive mood and emotions ($P < 0.01$), without affecting hunger scores. Dancing did not affect eCB levels or hunger ratings, but decreased negative mood and emotions ($P < 0.01$). Cycling increased OEA levels by 26% ($P < 0.05$) and tended to decrease how hungry volunteers felt, without affecting mood. Reading increased OEA levels by 28% ($P < 0.01$) and increased the desire to eat. Plasma AEA levels were positively correlated with how full participants felt ($P < 0.05$). Plasma OEA levels were positively correlated with positive mood and emotions ($P < 0.01$). All three ethanolamines were positively correlated with heart rate (HR; $P < 0.0001$). These data suggest that activities other than running can increase plasma eCBs associated with changes in mood or appetite. Increases in eCBs may underlie the rewarding and pleasurable effects of singing and exercise and ultimately some of the long-term beneficial effects on mental health, cognition and memory.

Keywords: endocannabinoids, anandamide, human, clinical, high, mood, singing and dancing

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Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anandamide; BBB, blood brain barrier; BDNF, brain derived neurotrophic factor; eCBs, endocannabinoids; LC-ESI-MS-MS, electrospray ionization liquid chromatography/mass spectrometry; OEA, oleoylethanolamine; PEA, palmitoylethanolamine.

INTRODUCTION

The classic "runners high" is described as the sense of well-being and mood elevation associated with moderate distance running. Other typical indicators include a decrease in anxious thinking (anxiolytic), positive emotions/mood (euphoria), reduced pain perception (analgesia) and a feeling of increased endurance (Sparling et al., 2003; Dietrich and McDaniel, 2004; Tsatsoulis and Fountoulakis, 2006; Raichlen et al., 2012). To explain these positive effects post-exercise, attention was directed to the endocannabinoid (eCB) system, and a number of groups have found significant correlations between physical activity, mood and elevated eCB levels. Interestingly, the majority of studies have only observed significant rises in the first identified eCB, anandamide (AEA; Sparling et al., 2003; Heyman et al., 2012; Raichlen et al., 2013), whilst the reports analyzing 2-arachidonylglycerol (2-AG) levels post-exercise have been less clear. Heyman et al. (2012) reported no change in circulating 2-AG levels after cycling. However, Brellenthin et al. (2017) showed that 2-AG and AEA were significantly increased in a study analyzing the effects of preferred (self-selected) and prescribed (70%–75% of max) exercise on eCB levels and mood.

The eCB system consists of the cannabinoid receptors 1 and 2 (CB_1 and CB_2), eCBs, and the enzymes that are responsible for their synthesis and breakdown (Devane et al., 1992; Mechoulam et al., 1995; De Petrocellis and Di Marzo, 2009). AEA and 2-AG are partial agonists of CB_1 and CB_2, whilst palmitoylethanolamine (PEA) and oleoylethanolamine (OEA) share similar synthesis and degradation mechanisms, without directly interacting with these receptors themselves (Hansen et al., 2000; Okamoto et al., 2004). Instead, these molecules interact with other receptors, primarily peroxisome proliferator-activated receptor alpha (PPAR-α) and transient receptor potential cation channel subfamily V member 1 (TRPV1; Ahern, 2003; Fu et al., 2003; Lo Verme et al., 2005a,b; Karwad et al., 2017). eCB signaling mediates a number of physiological and psychological processes including emotional responses, cognition, memory, motor behavior, feeding and energy consumption (Berger and Motl, 2000; Cota et al., 2003; Cota, 2007; Brellenthin et al., 2017). Studies have also established prominent roles of eCB signaling in the positive reinforcement in reward driven activities such as masturbation, arousal, binge-eating and social interactions in humans (Klein et al., 2012; Monteleone et al., 2015, 2017; Fuss et al., 2017).

Singing and dancing, especially as a group activity, are associated with positive mood in humans (Zajenkowski et al., 2015; Pearce et al., 2016; Tarr et al., 2016; Schladt et al., 2017). However, little has been studied to elucidate how these positive emotions are mediated. Recently, Hahn et al. (2017) studied the relationship between song practice and the eCB system in European starlings. They found a significant positive correlation between conditioned place preference (a measure of reward and song production), the number of songs a bird produced and the expression of CB_1 in areas of the brain associated with reward, primarily the ventral tegmental area. Therefore suggesting a role for eCB signaling in singing and reward (Hahn et al., 2017; Riters et al., 2017). In humans, singing has been studied as a therapy for long-term disorders such as Alzheimer's (to improve cognition, memory and long-term pain), chronic obstructive pulmonary disease, as well as to improve mood in conditions such as anxiety and depression (Reagon et al., 2016; Kang et al., 2017). Similarly, dancing has been explored as a potential therapy for cognitive and emotional dysfunction in conditions such as depression, dementia and Parkinson's. In a systematic review of 11 studies, Kiepe et al. (2012) found that depression and psychological distress were reduced by dance therapy in patients suffering from Parkinson's, diabetes, breast cancer or heart failure. Dance therapy in a group of 60 students also significantly reduced depression over a period of 12 weeks (Akandere and Demir, 2011). To date, no study has assessed singing or dancing and whether they modulate eCB levels in humans and whether that correlates to an improved mood. Given that mood is central in the measure of overall psychological well-being, low intensity activities that can positively modulate mood could be useful therapeutic tools in numerous conditions such as depression, anxiety and stress, especially if a patient cannot undertake moderate/higher intensity exercise.

The purpose of this study was to investigate whether activities other than running can give you a measurable "high" through changes in circulating eCBs levels. We examined activities that are associated with euphoria (singing and dancing) as well as an exercise regime other than running (cycling), with the hypothesis that these activities would increase plasma eCB levels. Quiet reading was used as a control condition. A secondary objective of this study was to establish whether there was a link between cycling, dancing, singing and reading with regards to mood and hunger ratings.

MATERIALS AND METHODS

Participants

All procedures were approved by the University of Nottingham Faculty of Health Sciences ethics committee, and were carried out according to the declaration of Helsinki. Nine healthy post-menopausal female volunteers (age range 55–67, mean 61 years) were recruited from a local choir as people who enjoyed singing and exercise. The inclusion criteria were that volunteers be non-smokers, in good physical health, accustomed to singing in a group, and also enjoy exercise. Volunteers gave written informed consent prior to participation. The medications taken included antihypertensives (n = 2), antacids (n = 2), antidepressants/anti-anxiety medication (n = 2), HRT (n = 1), and an inhaler for asthma (n = 1).

Subjects arrived fasted (feeding affects plasma eCB levels; Monteleone et al., 2012) with no consumption of caffeine and this was verbally confirmed on arrival at the study facility. Participants were also asked to refrain from any exercise prior to attending the laboratory. Volunteers were unaware of the activity they were to perform on a given day until all baseline measurements were made to avoid any anticipatory effects.

Study Days

Subjects came to the test site on four occasions between 8 am and 10 am in loose fitting sportswear. Each day,

individuals were asked to complete two questionnaires before and after completing the activity. A visual analog scale (VAS) questionnaire was used to assess how hungry subjects were feeling on a scale of 1–10, using the questions "how hungry do you feel?", "how full are you?", "how much food could you eat?" and "how strong is your desire to eat?". A positive and negative affect schedule (PANAS) questionnaire was used to assess subject's mood before and after each activity using the following scoring system: 1 = "very slightly or not at all," 2 = "a little," 2 = "moderately," 4 = "quite a bit" and 5 = "extremely;" Watson et al., 1988; Crawford and Henry, 2004). Positive affect score was calculated by adding the positive emotional responses and the negative affect score was calculated based on the addition of the negative affect scores.

Blood pressure was measured by oscillometry with the participant seated according to the British Hypertension Society guidelines, and heart rate (HR) was taken prior to commencing the activity and immediately after finishing the activity. Blood pressure and HR measurements were taken as the average over three (pre-activity) or 2 (post-activity) measurements. Blood draws (approximately 5 mL) were taken before commencing the activity and immediately after finishing the activity into pre-chilled K2-EDTA (Ethylenediaminetetraacetic acid) tubes and immediately placed on ice. After collection, blood was centrifuged at 2,000 *g* for 15 min at 4°C, plasma was removed and aliquoted, and immediately snap frozen in liquid nitrogen. Samples were stored at −80°C until subsequent analysis.

After the baseline measurements were made, volunteers were informed of the activity they were to perform. On day 1, volunteers did a supervised 30 min dance exercise class preceded by a 5 min warm up, to upbeat music. On day 2, volunteers did 30 min of supervised quiet reading (of boiler and dishwasher catalogs) to classical music. On day 3, volunteers for 30 min choir practice led by their choral director. On day 4, volunteers did a 30 min spin class (cycling) with a qualified instructor from the University of Nottingham Sports facility, with a 5 min warm up to upbeat music. All activities were performed as a group.

eCB Quantification

eCB analysis was based on the method as described by Richardson et al. (2007). Samples were thawed and 100 μL of internal standard of 2-AG-d8 (10 μM) and 15 μL of AEA-d8 (28 μM) were added to a 0.4 mL aliquot of each plasma sample or blank sample (0.4 mL water) vortexed briefly. Ethyl acetate:hexane (9:1 v/v) was added to each sample and subjected for a slow vortex (10 min) and centrifuged for 13,000 rpm, 10 min, 4°C. The supernatants were transferred and the procedure was repeated. Supernatants were then pooled and evaporated using a centrifugal evaporator. Prior to analysis, each sample extract was reconstituted in 100 μL of acetonitrile (ACN). Standards for AEA, 2-AG, PEA, OEA, *N*-(2-hydroxyethyl)-9Z-octadecenamide), arachidonyl ethanolamide-d8 (*N*-(2-Hydroxyethyl)-5Z, 8Z, 11Z, 14Z-eicosatetraenamide-d8, AEA-d8) and 2-arachidonyl glycerol-d8 (2-AG-d8, (5Z, 8Z, 11Z, 14Z)-5, 8, 11, 14-Eicosatetraenoic acid-d8, 2-hydroxy-1-(hydroxymethyl)ethyl ester-d8) were purchased from Cambridge BioSciences, UK.

Following sample preparation, 10 μL of final sample extract was analyzed using liquid chromatography electrospray ionisation mass spectrometry (LC-ESI-MS/MS). The HPLC system used was a modular Shimadzu Vp series LC (Shimadzu, Milton Keynes, UK), with pumps, chilled autosampler and column oven. The HPLC column used was an ACE 3 C8 (100 × 2.1 mm, 3 mm) with guard column. The mobile phase A was water with 1 g/L ammonium acetate and 0.1% formic acid and mobile phase B was ACN with 1 g/L ammonium acetate and 0.1% formic acid pre-dissolved in 10% H_2O. The flow rate was 300 μL/min. The MS system used was a SCIEX 4000 QTrap triple quadrupole mass spectrometer (Sciex, Warrington, UK) operated in electrospray positive multiple reaction monitoring mode. Quantification was performed using Analyst 1.6 and identification of each compound in plasma was confirmed by LC retention times of each standard and precursor and product ion *m/z* ratios. The peak area of each analyte is compared to a known amount of standard to determine the amount of target compound present.

2-AG in these samples were below the limit of quantification with our methodology in the plasma samples and the data has not been reported.

Statistical Analysis

Data is presented as a scatter plot with mean ± SEM. Data sets were compared by paired Student's *t*-test pre and post-activity. Correlations between plasma eCBs levels and hunger ratings, cardiovascular parameters or mood pre and post-activities were analyzed by linear regression. A quality control check was performed by a separate researcher on data entry.

RESULTS

All but one of the participants completed the study in full; one participant was unable to finish the cycling activity and did not have a final blood draw or complete the surveys. Thus nine participates were in the final comparison, except for the cycling activity where $n = 8$.

Haemodynamics

Thirty minutes of dancing significantly increased HR ($t_{(8)} = 4.894$, $P < 0.01$, **Figure 1A**) and decreased diastolic blood pressure ($t_{(8)} = 2.764$, $P < 0.05$, **Figure 1I**). Thirty minutes of reading caused a small but significant reduction in HR ($t_{(8)} = 3.736$, $P < 0.01$, **Figure 1B**). Thirty minutes of singing increased systolic blood pressure ($t_{(8)} = 5.66$, $P < 0.001$, **Figure 1G**). Thirty minutes of cycling significantly increased HR ($t_{(7)} = 7.314$, $P < 0.001$, **Figure 1D**) and decreased diastolic blood pressure ($t_{(7)} = 2.567$, $P < 0.05$, **Figure 1L**).

Hunger Scores

The only significant change in hunger and appetite scores were observed after 30 min of reading when volunteers reported a significantly higher desire to eat (**Figure 2N**). Volunteers tended to have reduced hunger ratings after dancing, singing and cycling (**Figure 2**), but this only reached near significance for

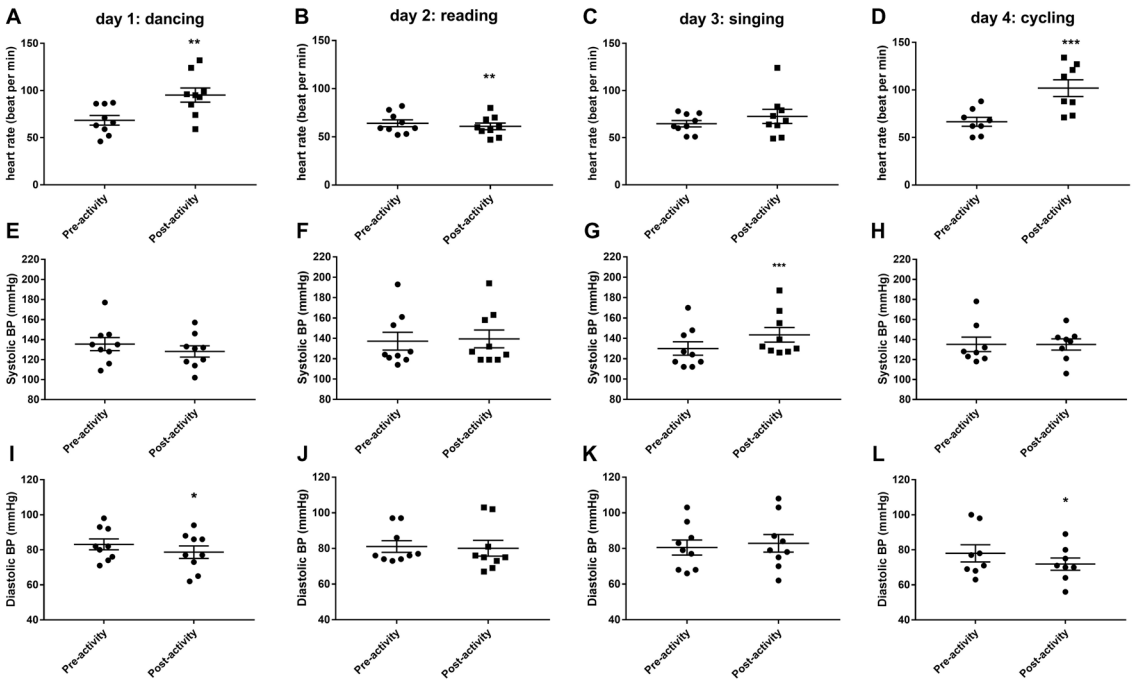

FIGURE 1 | Changes in cardiovascular parameters (heart rate, HR; **A–D**), systolic blood pressure **(E–H)** and diastolic blood pressure **(I–L)** before and after 30 min activity (dancing, reading, singing or cycling) in nine healthy female volunteers. Data is presented as a scatter plot with mean ± SEM. Data sets were compared by paired Student's *t*-test pre and post-activity (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

the question "how hungry do you feel?" immediate post-cycling ($t_{(7)} = 2.348$, $P = 0.0512$, **Figure 2D**).

Mood Scores

Dancing decreased negative mood and emotions ($t_{(8)} = 3.671$, $P < 0.01$, **Figure 3E**), while reading decreased positive mood and emotions ($t_{(8)} = 5.751$, $P < 0.001$, **Figure 3B**). Only singing was found to significantly improve positive mood and emotions ($t_{(8)} = 4.951$, $P < 0.01$, **Figure 3C**) and also tended to decrease negative mood and emotions (eight out of nine volunteers reported a lower NAS post-singing, **Figure 3E**). Cycling has no effect on mood ratings.

Plasma Levels of Endocannabinoids

Dancing had no effect on circulating levels of eCBs measured immediately the activity, although there was a trend for AEA and OEA levels to be increased (**Figures 4A,E**). Thirty minutes of reading significantly increased plasma OEA levels ($t_{(8)} = 4.586$, $P < 0.01$, **Figure 4F**) and tended to increase PEA levels ($t_{(8)} = 2.02$, $P = 0.078$, **Figure 4J**). Singing significantly increased the plasma levels of all eCBs measurable; AEA ($t_{(8)} = 3.049$, $P < 0.05$, **Figure 4C**), OEA ($t_{(8)} = 4.81$, $P < 0.01$, **Figure 4G**) and PEA ($t_{(8)} = 3.319$, $P < 0.05$, **Figure 4K**). OEA levels were also increased after 30 min cycling ($t_{(6)} = 3.594$, $P < 0.05$, **Figure 4H**).

At baseline (before activities started) across all 4 days, there was a significant positive correlation between plasma OEA levels and the rating for "how much food could you eat?" ($r^2 = 0.2226$, $F = 9.16$, $P < 0.01$) and positive mood and emotions ($r^2 = 0.1355$, $F = 5.172$, $P < 0.05$). Resting HR was positively correlated with both plasma AEA ($r^2 = 0.3363$, $F = 16.72$, $P < 0.001$) and PEA ($r^2 = 0.169$, $F = 6.711$, $P < 0.05$) levels.

Across all days and time points (pre- and post-activity), plasma AEA levels were positively correlated with the rating for "how full are you?" ($r^2 = 0.0626$, $F = 4.472$, $P < 0.05$, **Figure 5A**), and plasma OEA levels tended to be positively correlated with the rating for "how much food could you eat?" ($r^2 = 0.0404$, $F = 2.821$, $P = 0.097$, **Figure 5B**) and "how strong is your desire to eat?" ($r^2 = 0.04624$, $F = 3.248$, $P = 0.076$, **Figure 5C**) and with increased ratings for positive mood and emotion ($r^2 = 0.1269$, $F = 9.879$, $P < 0.01$, **Figure 5D**). All three ethanolamines were positively correlated with HR (AEA: $r^2 = 0.4394$, $F = 53.3$, $P < 0.0001$, **Figure 5E**; OEA: $r^2 = 0.2639$, $F = 24.37$, $P < 0.0001$, **Figure 5F** and PEA: $r^2 = 0.2093$, $F = 18$, $P < 0.0001$, **Figure 5G**).

DISCUSSION

It is well reported that running is correlated with mood elevation. These positive effects have been attributed to an evolutionary trait, where positive re-enforcement ultimately led to increased food foraging, survival and subsequent passing of relevant genes to offspring and have recently been attributed, at least in part, to increases in eCBs (Bramble and Lieberman, 2004; Raichlen et al., 2012). Our study aimed to examine whether activities other than running also increase eCBs and enhance mood. We have shown for the first time that singing significantly increases levels of AEA, OEA and PEA in healthy post-menopausal females and

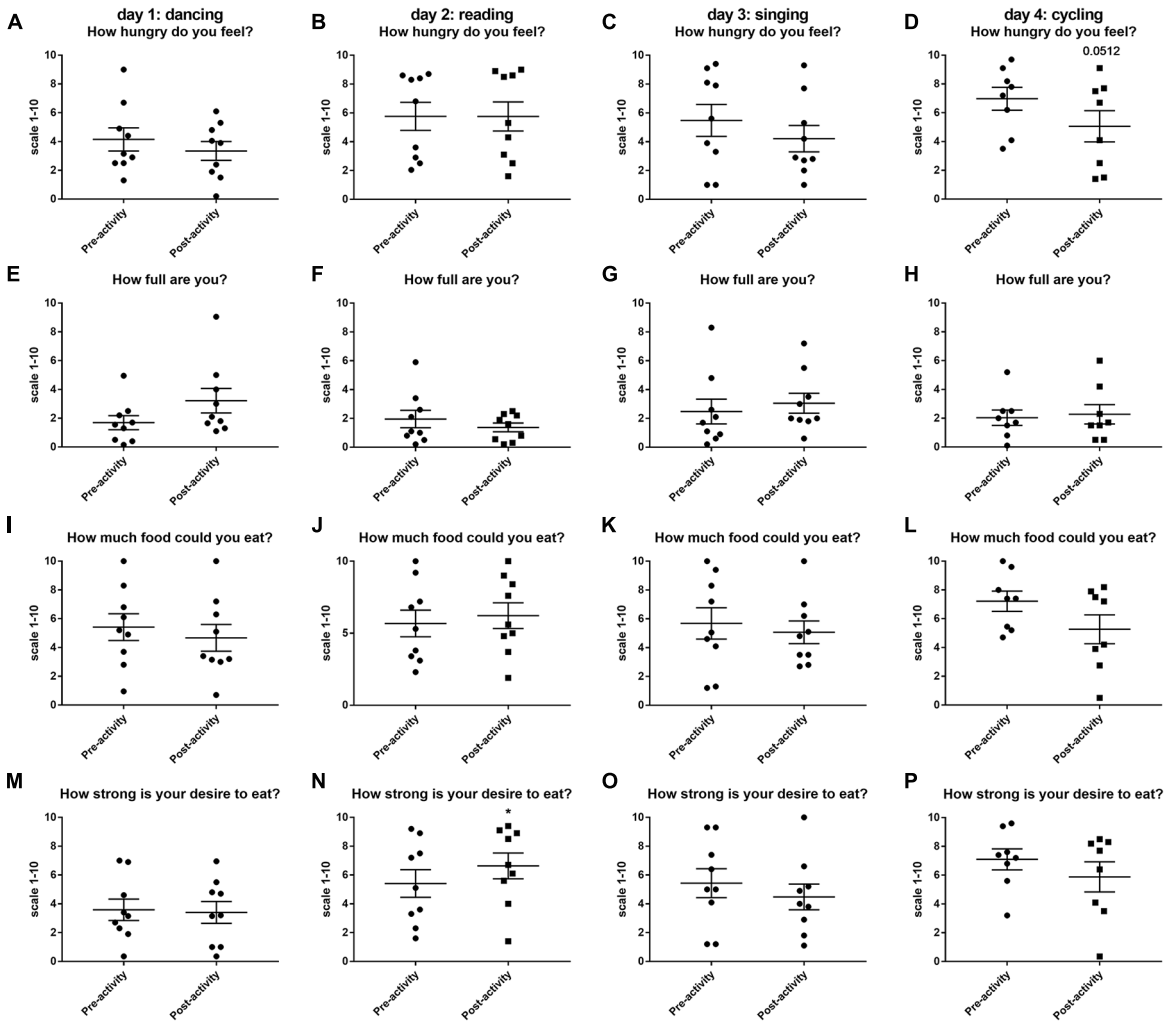

FIGURE 2 | Changes in hunger and appetite scores as assessed using a visual analog scale (VAS) (1–10) before and after 30 min activity (dancing **(A,E,L,M)**, reading **(B,F,J,N)**, singing **(C,G,K,O)** or cycling **(D,H,L,P)** in nine healthy female volunteers. Data is presented as a scatter plot with mean ± SEM. Data sets were compared by paired Student's *t*-test pre and post-activity (*$P < 0.05$).

enhanced mood. Dancing (on mood) and cycling (on eCBs) also had positive effects in this group. Although singing was the most beneficial activity in this study, this is likely to reflect the fact that the volunteers were recruited from local choirs and already find this an enjoyable activity. These data provide biochemical evidence of an increase in novel signaling messengers known to improve mood, reduce stress and anxiety, enhance memory, protect brain function and reduce pain.

Singing, in particular group singing, has been associated with an increase in positive mood and improved immune function in humans (Kreutz et al., 2004; Schladt et al., 2017). Choir singing also enables social interactions, exhibiting a greater benefit to mood than singing alone (Schladt et al., 2017). Our results also demonstrate that singing increases mood, and also for the first time that singing increasing circulating levels of AEA, OEA and PEA. As AEA is a partial agonist of CB_1 and has full agonist activity at TRPV1, an increase in the levels of AEA post activity could therefore facilitate increases in positive emotions, as well as anxiolytic and analgesic effects (Chapman et al., 2009; Starowicz et al., 2012). Levels of OEA post activity were correlated with a decrease in hunger and desire to eat. This supports previous data that OEA attenuates food consumption and increase lipolysis and energy expenditure (Lo Verme et al., 2005a,b). *In vivo* studies conducted in mice have also suggested beneficial neuroprotective effects of OEA, this protective effect could potentially be translated to humans and warrants further study (Galan-Rodriguez et al., 2009; Zhou et al., 2012; Yang et al., 2015). An abundance of evidence has supported PEA as a potential therapy for neurological and inflammatory disorders, particularly those associated with pain (Costa et al., 2008; Keppel Hesselink, 2012; Esposito and Cuzzocrea, 2013). PEA has also been taken into clinical trials, whereby 600 mg of PEA was shown to be effective in various pain states, without exhibiting any safety issues

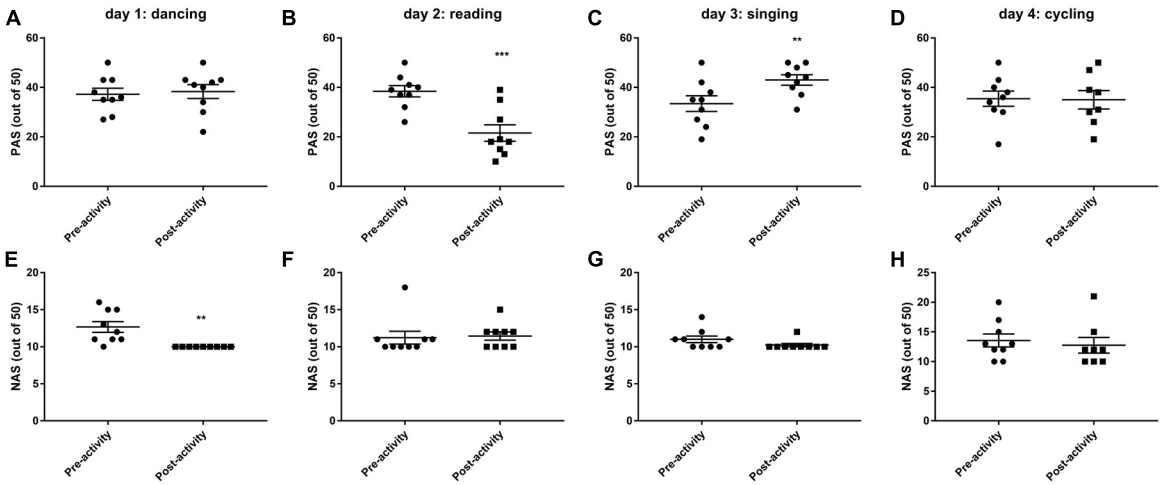

FIGURE 3 | Changes in postive (PAS, **A–D**) and negative (NAS, **E–H**) mood and emotions before and after 30 min activity (dancing, reading, singing or cycling) in nine healthy female volunteers. Data is presented as a scatter plot with mean ± SEM. Data sets were compared by paired Student's *t*-test pre and post-activity (**$P < 0.01$, ***$P < 0.001$).

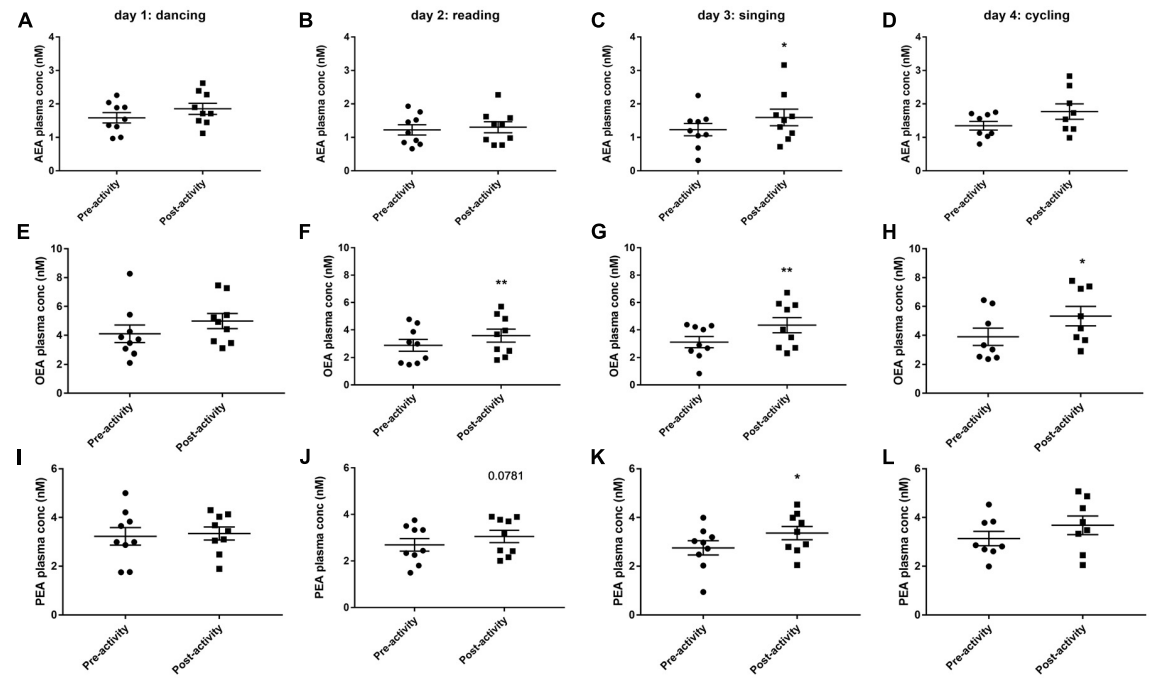

FIGURE 4 | Plasma endocannabinoid levels (AEA, anandamide, **A–D**; OEA, oleoylethanolamine, **E–H**; PEA, palmitoylethanolamine, **I–L**) before and after 30 min activity (dancing, reading, singing or cycling) in nine healthy female volunteers. Data is presented as a scatter plot with mean ± SEM. Data sets were compared by paired Student's *t*-test pre and post-activity (*$P < 0.05$, **$P < 0.01$).

(Hesselink and Hekker, 2012). Therefore, it could be beneficial to increase levels of PEA via activities such as singing, to promote neuroprotection, analgesia and reduce inflammation. It is also important to note that increasing OEA and PEA can indirectly increase AEA responses by the entourage effect by competitive inhibition of AEA degradation by fatty acid amide hydrolase (FAAH; Di Marzo et al., 2001; Costa et al., 2008; Ho et al., 2008). Overall, singing could be a valuable activity in patient populations that suffer with dysfunctions in psychological well-being and struggle to participate in aerobic/moderate intensity exercise.

Cycling resulted in a significant increase in OEA, and in a trend for increases in both AEA and PEA. These changes corresponded with a decrease in participants desire to eat and

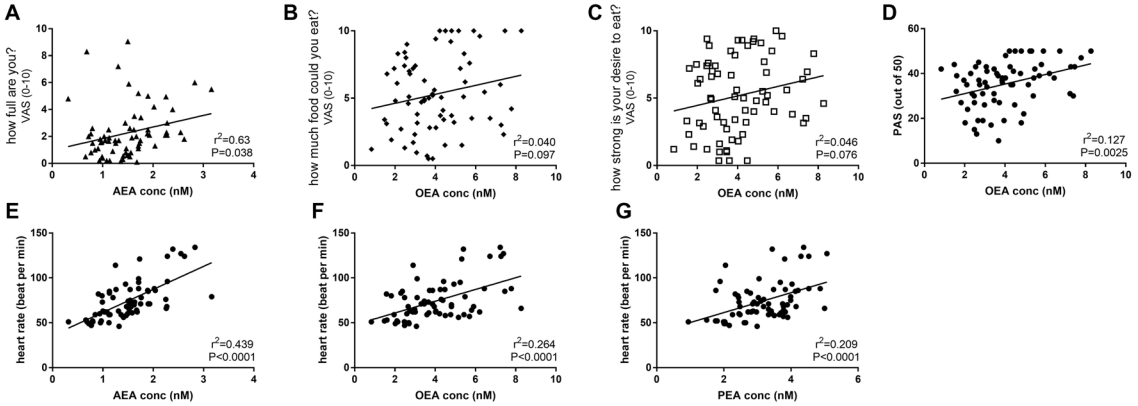

FIGURE 5 | Correlations between plasma eCBs levels and appetite **(A–C)**, mood **(D)** and HR **(E–G)** in healthy female volunteers when measured pre or post-activities and analyzed by linear regression.

how hungry they felt. These data are consistent with results from a previous study where plasma OEA levels were significantly increased after 30 min of cycling in 16 male non-smokers with a mean age of 22.9 years (Cedernaes et al., 2016). Our data did not show that cycling positively affected mood (no increase in PAS or decrease in NAS). Brellenthin et al. (2017) showed that acute aerobic exercise (both prescribed and preferred) resulted in positive mood outcomes in individuals capable of a range of levels of physical activity, as well as showing modulation of the eCB system. Interestingly, the group that undertook their "preferred exercise" had the best effect in reducing anxiety and improving mood. In the present study, singing by participants recruited from a choir support these observations, suggesting that the eCB system is not only responsible for the motivation for exercise (i.e., reward driven), but also the pleasure associated with an activity that an individual enjoys. It would also have been interesting if another group had been included (i.e. not recruited from a choir) to directly assess the concept of preferred vs. prescribed activity and to confirm that carrying out an activity that is "pleasurable" to an individual is an important factor in the psychological benefits of exercise and other related activities. Subjects were also not asked to rate how much they enjoyed each of the activities, this would have been an interesting endpoint to assess to what degree the participant's moods were influenced by how much they liked a particular activity and should be considered in future study. These factors would also have provided further evidence to why individuals in this study failed to experience positive mood changes or significant increases in AEA post cycling; compared with the study by Heyman et al. (2012) where increases in AEA were seen in well-trained cyclists, who presumably enjoy cycling.

Exercise intensity may be another factor explaining the lack of AEA increases in our participants. Brellenthin et al. (2017) showed that the greatest increases in 2-AG and AEA were seen in the higher intensity exercise group. Sparling et al. (2003) also showed significant increases in AEA when participants reached 70%–80% max HR. According to Gulati et al. (2010), maximum HR for women is calculated as 206−(0.88 × age of patient). As the average age of our participants was 61 years, their average maximum HR (max HR) is approximately 154 bpm, meaning their 70%–80% max HR should be 107–123 bpm. Cycling was the only activity that almost reached this (average 102 bpm immediately post exercise) and dancing resulted in an average HR of 95 bpm (immediately post exercise). This could suggest that our activities may not have been intense enough to elicit significant changes in circulating eCBs.

A number of studies have shown that dance is an effective therapy in improving mood (including mild depression), enhancing social interactions, boosting self-confidence, as well as improving physical activity (Akandere and Demir, 2011; Kiepe et al., 2012; Meekums et al., 2015). In one study, dancing caused an increase in plasma serotonin levels and a decrease in negative psychological symptoms in a group of 20 female adolescents with mild depression, compared to 20 control subjects (Jeong et al., 2005). We found post activity that there was a significant decrease in negative emotions following 30 min of dancing. It should be acknowledged that the decrease in negative emotions could also be because this was the activity undertaken on day 1 and participants had higher NAS scores before starting the study. Although there was a trend in increasing levels of AEA and OEA levels post-activity, this did not reach significance. Our results suggest that dancing did not effectively increase eCB levels or improve mood, however this could be because they were unfamiliar with the class, therefore not finding it as enjoyable as singing as this was more familiar to them, or that the class was not at a high enough intensity to produce changes in eCB levels. It should also be noted that our participants were older than those previously studied, and there could be an age-related decline in the eCB response to exercise.

Reading was used as a control activity to assess baseline eCB levels and mood. We found that reading was the only activity that increased participants desire to eat but had little impact on overall fullness or actual hunger and was correlated to increases in OEA post activity. Reading also decreased the ratings for positive mood and emotions. In hindsight, because subjects were unaware of the task, the activity set-up looked

like they were about to take an exam, which may have resulted in unforeseen heightened anxiety levels. Recent studies have implicated the eCB system as a possible mediator of hedonic vs. homeostatic eating response to the consumption of food (as a reward) as well as acute stress and anxiety (Matias et al., 2006; Monteleone et al., 2015, 2017). Dlugos et al. (2012) showed that AEA, PEA and OEA were all increased in serum in response to stress. They also found that higher levels of AEA at baseline, associated with decreased levels of anxiety. Furthermore, a common phenomenon is that typically negative emotions, particularly boredom, stress and depressive emotions increase our desire to eat in order to increase positive emotions (Koball et al., 2012; Yau and Potenza, 2013; Moynihan et al., 2015). These factors could explain the elevated levels of OEA post activity and lower PAS scores.

A limitation of our study is that participants already had very low negative affect scores and high positive affect scores. This suggests that the individuals that took part in the study were generally happy and positive and there was therefore little room for mood to be further improved. It would therefore be interesting in future work to see the effects of these same activities on individuals that exhibit depressive, or anxious behavior in order to see greater differences in negative emotional responses. Intensity of a physical activity has also been shown to influence exercise induced increases in eCB levels. Raichlen et al. (2013) built on previous work showing that eCBs follow a U-shaped curve, with moderate level activity resulting in the biggest increase in eCB levels (Berger and Motl, 2000). This trend in eCB levels is also correlated with mood as the positive emotional state post exercise is not experienced at very low or very high intensities (Berger and Motl, 2000). As all the participants were unfamiliar to the activities they carried out, a lot of their focus would have been on "mastering" the class rather than actually enjoying it in the moment.

It can also not be overlooked that this study only recruited healthy female volunteers. Evidence from animal studies has already shown distinct sexual dimorphism in the eCB system, particularly in CB_1 expression and activation (Reich et al., 2009; Mateos et al., 2011; Dias-Rocha et al., 2018). Limited preliminary evidence from human studies have also shown variations in the eCB system between males and females (Cupini et al., 2006; Hill et al., 2008). Thus future study should look to establish whether the effects observed in this study translate to male participants as well as females.

In conclusion, we found that activities other than running (singing, dancing and cycling) can increase plasma eCB levels. Singing was the only activity to increase plasma levels of AEA and improve positive mood outcomes, suggesting that singing in this group of volunteers was able to produce an endogenous "high." This is interesting as the participants were recruited from a choir, suggesting that the enjoyment of an activity may influence their feeling of reward and the eCB response. This preliminary evidence suggests that activities like singing could be recommended to individuals suffering from mood disorders such as anxiety and depression, as well as a potential therapy for neurological and inflammatory disorders. Future research should consider an individual's preference to a particular activity, as this could be an important factor in influencing the eCB system, as well as being a factor in deciding appropriate therapy.

AUTHOR CONTRIBUTIONS

NS and SO'S wrote the article with contributions from all the other authors. SO'S, SM and NS carried out cardiovascular measurements, surveys and blood processing. SO'S processed the study data and performed the statistical analysis. CO and DB performed the eCB analysis on the plasma samples. PH carried out the blood draws from the subjects. VM developed the study with SO'S.

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A systematic review and meta-analysis of the *in vivo* haemodynamic effects of D9-tetrahydrocannabinol

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Review

A Systematic Review and Meta-Analysis of the In Vivo Haemodynamic Effects of Δ^9-Tetrahydrocannabinol

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Abstract: Δ^9-Tetrahydrocannabinol (THC) has complex effects on the cardiovascular system. We aimed to systematically review studies of THC and haemodynamic alterations. PubMed, Medline, and EMBASE were searched for relevant studies. Changes in blood pressure (BP), heart rate (HR), and blood flow (BF) were analysed using the Cochrane Review Manager Software. Thirty-one studies met the eligibility criteria. Fourteen publications assessed BP (number, n = 541), 22 HR (n = 567), and 3 BF (n = 45). Acute THC dosing reduced BP and HR in anaesthetised animals (BP, mean difference (MD) −19.7 mmHg, $p < 0.00001$; HR, MD −53.49 bpm, $p < 0.00001$), conscious animals (BP, MD −12.3 mmHg, $p = 0.0007$; HR, MD −30.05 bpm, $p < 0.00001$), and animal models of stress or hypertension (BP, MD −61.37 mmHg, $p = 0.03$) and increased cerebral BF in murine stroke models (MD 32.35%, $p < 0.00001$). Chronic dosing increased BF in large arteries in anaesthetised animals (MD 21.95 mL/min, $p = 0.05$) and reduced BP in models of stress or hypertension (MD −22.09 mmHg, $p < 0.00001$). In humans, acute administration increased HR (MD 8.16 bpm, $p < 0.00001$). THC acts differently according to species and experimental conditions, causing bradycardia, hypotension and increased BF in animals; and causing increased HR in humans. Data is limited, and further studies assessing THC-induced haemodynamic changes in humans should be considered.

Keywords: Δ^9-Tetrahydrocannabinol; THC; cardiovascular system; blood pressure; heart rate; blood flow

1. Introduction

Δ^9-Tetrahydrocannabinol (THC) is the most abundant and widely studied phytocannabinoid, first discovered in 1964 [1]. THC is a partial agonist of both cannabinoid receptors CB_1 and CB_2 and other targets including G protein-coupled receptors GPR55 and GPR18 [2–4]. THC possesses interesting therapeutic potential as an antiemetic, appetite stimulant, and analgesic, and for the treatment of glaucoma, epilepsy, Parkinson's disease, and multiple sclerosis [5–7]. THC has been shown to be effective against refractory nausea and vomiting in cancer patients undergoing chemotherapy [8]. However, its use as a therapeutic agent is limited by its recognised psychogenic side effects including hallucinations, euphoria, dizziness, mood changes, nausea, and fatigue [8–10].

THC has numerous cardiovascular effects in animals and humans. In vitro studies have shown that THC causes endothelium-independent vasorelaxation of rabbit superior mesenteric arteries [11] and vasorelaxation of the rat mesenteric artery through sensory nerves via a CB_1 and CB_2 receptor-independent mechanism [12]. Other studies have found THC to activate a G

protein-coupled receptor, inhibit calcium channels, and activate potassium channels in the rat mesenteric vasculature [13] and to cause endothelium-dependent and time-dependent vasorelaxation in the rat aorta [14,15]. In contrast, other studies have shown that THC causes vasoconstriction in guinea pig pulmonary arteries [16], rat mesenteric arteries and aorta [14,17], and rabbit ear arteries [18].

In vivo studies have reported different haemodynamic responses post-THC. An acute administration of THC caused hypotension and bradycardia in anesthetised dogs (intravenously; i.v.), conscious bats (intraperitoneal; i.p.), and humans (oral) [19–21]. In contrast, tachycardia and hypertension were reported in rats after i.p. administration of THC [22,23]. More complex effects on BP were induced by THC in anaesthetised rats [24]. The available evidence to date suggests that THC alters the haemodynamics in animals and humans, albeit with conflicting results variable with species, route of administration, and experimental conditions. Therefore, the aim this study was to systematically review and meta-analyse the in vivo literature assessing the effects of THC on the cardiovascular system in all species under different conditions.

2. Results

From the initial 2743 search results, 1935 relevant publications were identified and evaluated from three databases (Medline, EMBASE, and PubMed). Of these, 30 articles met the inclusion criteria and 1 article was added manually (Figure 1). A summary of the data extracted from the included studies is shown in Table 1.

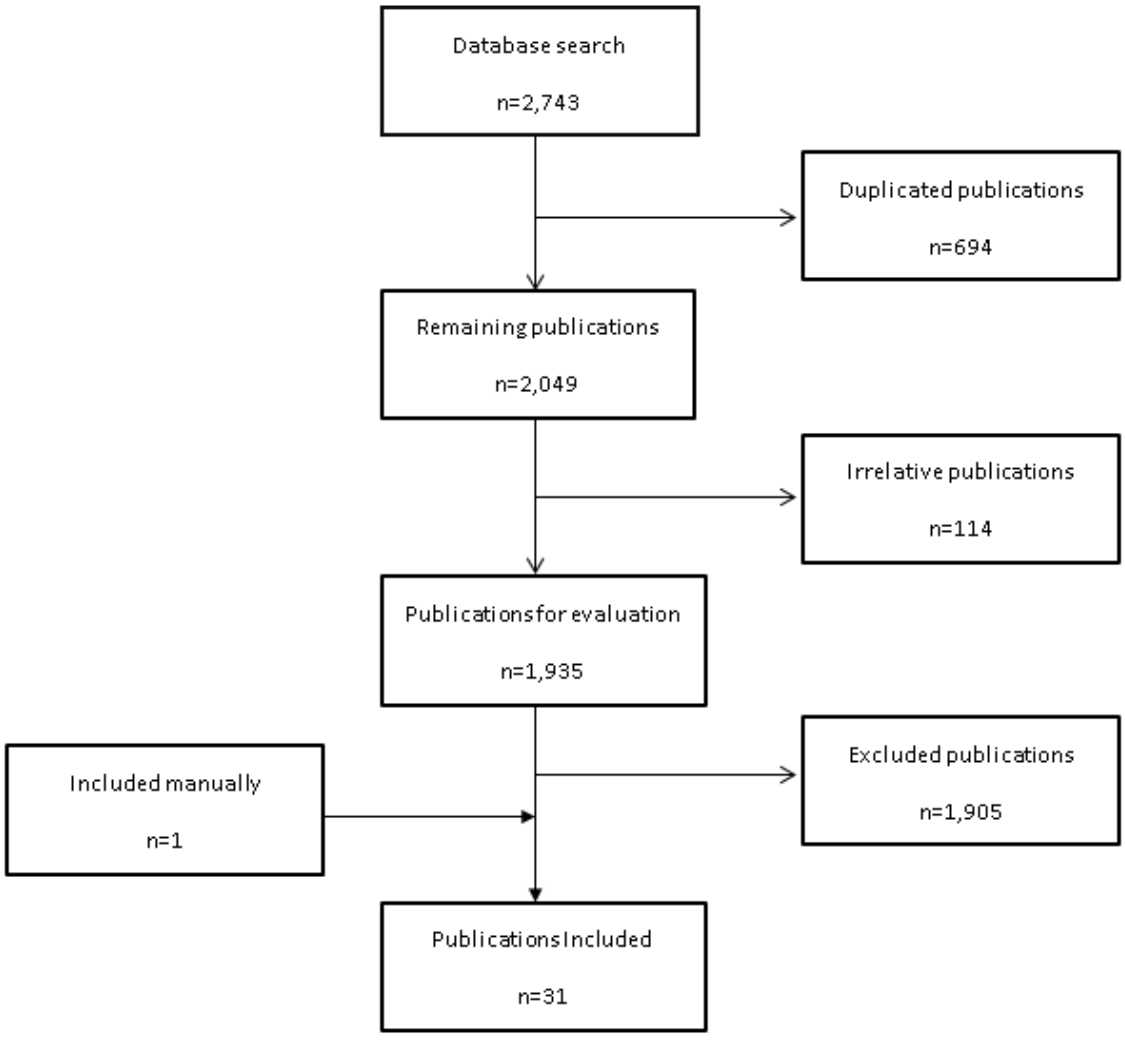


Figure 1. Flow chart for study retrieval and selection.

Table 1. Summary of the included studies divided according to the experimental conditions.

Author & Year	Study Description	Species, Model (Anaesthetic & Route)	Sample Size	THC Dose	THC Route	Time of THC Administration	Time of Haemodynamic Measurements	Basal Parameters *	Outcomes and Comments
Anaesthetised animals									
Cavero 1972 [25]	Investigate the haemodynamic effects of THC	Dogs Anaesthetised (pentobarbital, iv)	11	2.5 mg/kg	i.v.	Post-anaesthesia	Continues for 30 m post-drug	-	THC altered distribution of regional BF, and reduced HR and BP.
Cavero 1973a [26]	Investigate the haemodynamic effects of THC	Dogs Anaesthetised (pentobarbital, iv)	23	39 µg/kg–2.5 mg/kg	i.v.	Post-anaesthesia	Continues for 2 h post-drug	C: HR:169, BP:91.7; T: HR:165.7, BP:93.5	THC caused reduction in HR and BP mediated via central nervous system.
Cavero 1973b [27]	Characterise the mechanism of action of THC on HR	Dogs Anaesthetised (pentobarbital, iv)	29	39 µg/kg–5 mg/kg	i.v.	Post-anaesthesia	Continues for 140 m post-drug	-	THC induced reduction in HR through alteration of autonomic innervation to myocardium.
Cavero 1974 [19]	Investigate the effect of THC on venous return	Dogs (heart bypass) Anaesthetised (dibucaine, spinal)	8	2.5 mg/kg	i.v.	Post-anaesthesia	Pre-drug and continues for 30 m post-drug	C: HR:156, BP:85.8; T: HR:147, BP:85.	THC caused reduction in HR and BP, and reduced venous return.
Daskalopoulos 1975 [28]	Investigate the mechanism of THC on CV system	Cats Anaesthetised (urethane, iv)	40	30–300 µg/kg	i.v.	Post-anaesthesia	20 m post-drug	-	THC reduced HR and BP mediated via central nervous system.
Adams 1976 [29]	Examined the CV effects of THC	Rats Anaesthetised (urethane, ip)	72	0.1–3 mg/kg	i.v.	Post-anaesthesia	Continues for 30 min post-drug	C: HR:316.2, BP:76.2; T: HR:314.8, BP:73.5.	THC caused reduction in HR and biphasic BP response (↑ BP followed by ↓ BP), suggesting that THC depressed CV reflex functions.
Jandhyala 1976 [30]	Evaluated possible interaction with THC on HR	Dogs Anaesthetised (pentobarbital)	12	1 mg/kg	s.c.	Twice/day for 7 days Pre-anaesthesia	On the 7th day post-anaesthesia	-	Chronic THC antagonised the elevation in HR induced by the anaesthetic agent via vagal stimulation.
Jandhyala 1977 [31]	Determined chronic administration of THC on CV function	Dogs Anaesthetised (pentobarbital)	16	1 mg/kg	s.c.	Twice/day for 7 days Pre-anaesthesia	On the 7th day post-anaesthesia	-	Chronic THC had no effect on haemodynamics.
Jandhyala 1978 [32]	Investigated prolonged THC effects on CV system	Dogs Anaesthetised (pentobarbital)	16	2 mg/kg	s.c.	Single dose per day for 35 days	On the 35th day post-anaesthesia	-	Chronic THC increased BF in femoral and mesenteric arteries with no effect on HR or BP.
McConnell 1978 [33]	Examined the effects of THC on salivary flow	Cats Anaesthetised (urethane & pentobarbital, ip)	20	0.1–2 mg/kg	i.v.	Post-anaesthesia	Continues for 1 h post-drug	-	THC had no effect in stimulated salivary flow of cats. THC caused a reduction in HR and BP.

Table 1. *Cont.*

Author & Year	Study Description	Species, Model (Anaesthetic & Route)	Sample Size	THC Dose	THC Route	Time of THC Administration	Time of Haemodynamic Measurements	Basal Parameters *	Outcomes and Comments
Anaesthetised animals									
Siqueira 1979 [24]	Clarify the triple BP response post-THC	Rats Anaesthetised (urethane, ip)	50	1–10 mg/kg	i.v.	Post-anaesthesia	Continues for 70 m post-drug	-	THC induced triphasic BP response (↓ BP via vagal stimulation, then ↑ BP not dependent on sympathetic activity followed by ↓ BP due to central decrease in sympathetic tone).
Kawasaki 1980 [23]	Investigated the effect of THC on the CV system and behavior changes	Rats Anaesthetised (urethane, ip)	29	1–5 mg/kg	i.v.	Post-anaesthesia	Continues for 70 m post-drug	-	THC induced CV effects (↓ HR and ↑ BP) through vagal activity, and influence behavior changes to brain stimulation.
Schmeling 1981 [34]	Investigated the effect of THC on hypothalamus	Cats Anaesthetised (urethane, ip)	12	2 mg/kg	i.v.	Post-anaesthesia	Continues for 30 m post-drug	-	THC produced significant reductions in HR and BP and attenuated the pressor response threshold suggesting that THC reduces sympathetic activity.
Estrada 1987 [35]	Investigated the CV effects of THC	Rats Anaesthetised (pentobarbital, ip)	28	0.078–5 mg/kg	i.v.	Post-anaesthesia	3-12 min post-drug	-	THC produced adverse effects on the CV system (↓ HR and ↓ BP)
Krowicki 1999 [36]	Investigated whether CB_1 activation by THC inhibits gastric motor function	Rats Anaesthetised (ketamine and xylazine)	36	0.02–2 mg/kg	i.v.	Post-anaesthesia	Continues for 10 m post-drug	-	THC decreased gastric motor function, HR, and BP via autonomic effects mediated by CB_1.
Conscious animals									
Kaymakcalan 1974 [37]	Investigated chronic effects of THC on HR	Rats Conscious	20	10 mg/kg	s.c.	Single dose per day for 16 days	Hourly interval to 6 h on the 1st, 4th, 8th and 16th days	-	THC produced marked reduction in HR
Borgen 1974 [38]	Examined possible interaction of CBD on THC effects	Rabbits Conscious	8	3 mg/kg	i.v.	Pre-test	Pre-drug and hourly interval to 7 h post-drug	C: HR:264; T: HR:276	CBD reduced the hypothermic effect of THC and attenuated the depressant effects of THC on respiration, rectal temperature and HR
Brown 1974 [20]	Investigated CV response to THC	Bats Conscious	12	100 and 200 mg/kg	i.p.	Pre-test	Pre-drug and continues for 145 m post-drug	C: HR:436, BP:101; T: HR:390, BP:114	THC induced hypothermia and reduction in HR and BP.

Table 1. *Cont.*

Author & Year	Study Description	Species, Model (Anaesthetic & Route)	Sample Size	THC Dose	THC Route	Time of THC Administration	Time of Haemodynamic Measurements	Basal Parameters *	Outcomes and Comments
Conscious animals									
Osgood 1977 [22]	Investigated THC effects on HR	Rats Conscious	18	0.5 mg/kg	i.p.	Pre-test	Continues for 30 m post-drug	-	THC had minimal effect on BP and caused an increase in HR, which may be related to central mediation release of epinephrine from adrenal gland.
Kawasaki 1980 [23]	Investigated the effects of THC on the CV system and behavior changes	Rats Conscious	21	4–8 mg/kg	i.p.	Pre-test	Continues for 2 h post-drug	-	THC induced CV effects (↓ HR and ↑ BP) through vagal activity, and influenced behavior changes to brain stimulation.
Matsuzaki 1987 [39]	Examined the effects of THC on EEG, body temperature, and HR	Monkeys Conscious	6	0.4–4 mg/kg	i.p.	Pre-test	Continues for 5 h post-drug	-	THC induced reduction in HR and hypothermia and induced responses of EGG along with behavioral depression and alertness.
Hayakawa 2007a [40]	Investigated CBD and THC effects on ischemic brain damage	Stroke Mice Conscious	17	10 mg/kg	i.p.	Pre-, 3 and 4 h post-occlusion, and 1 and 2 h post-reperfusion	BP and HR: pre-reperfusion. CBF: continued 4 h post-occlusion and 1 post-reperfusion	-	Pre and post-ischemic treatment with CBD induced neuroprotection, whereas only preischemic treatment with THC induced neuroprotection. THC increased CBF with no effects on BP or HR
Hayakawa 2007b [41]	Explored the development of tolerance of THC and CBD neuroprotection	Stroke Mice Conscious	7	10 mg/kg	i.p.	Pre-occlusion and 3 h post-occlusion. Single dose per day for 14 days	During 4 h and on day 14 post-occlusion	-	Repeated treatment with CBD, but not THC, induced neuroprotection with development of tolerance. THC increased CBF on day 1 only with no effects on BP or HR.
Stress and hypertensive animal models									
Williams 1973 [42]	Studied the effects of THC on BP	Rats Stress	30	20 mg/kg	s.c.	Single dose per day for 4 days	Pre-drug, 4 h, 48 and 96 h post-drug	C: BP:128; T: BP:129	THC reduced BP
Birmingham 1973 [43]	Studies the effects of THC on BP	Rats Hypertensive	10	3 mg/kg	i.p.	Single dose per day for 7 days	Hourly to 5 h for 7 days	-	THC reduced BP
Kosersky 1978 [44]	Examined the antihypertensive effects of THC	Rats Hypertensive	12	25 mg/kg	Oral	Single dose per day for 10 days	4 h and every day for 14 days post-drug	-	THC effectively reduced BP to the same degree over the treatment period.

Table 1. *Cont.*

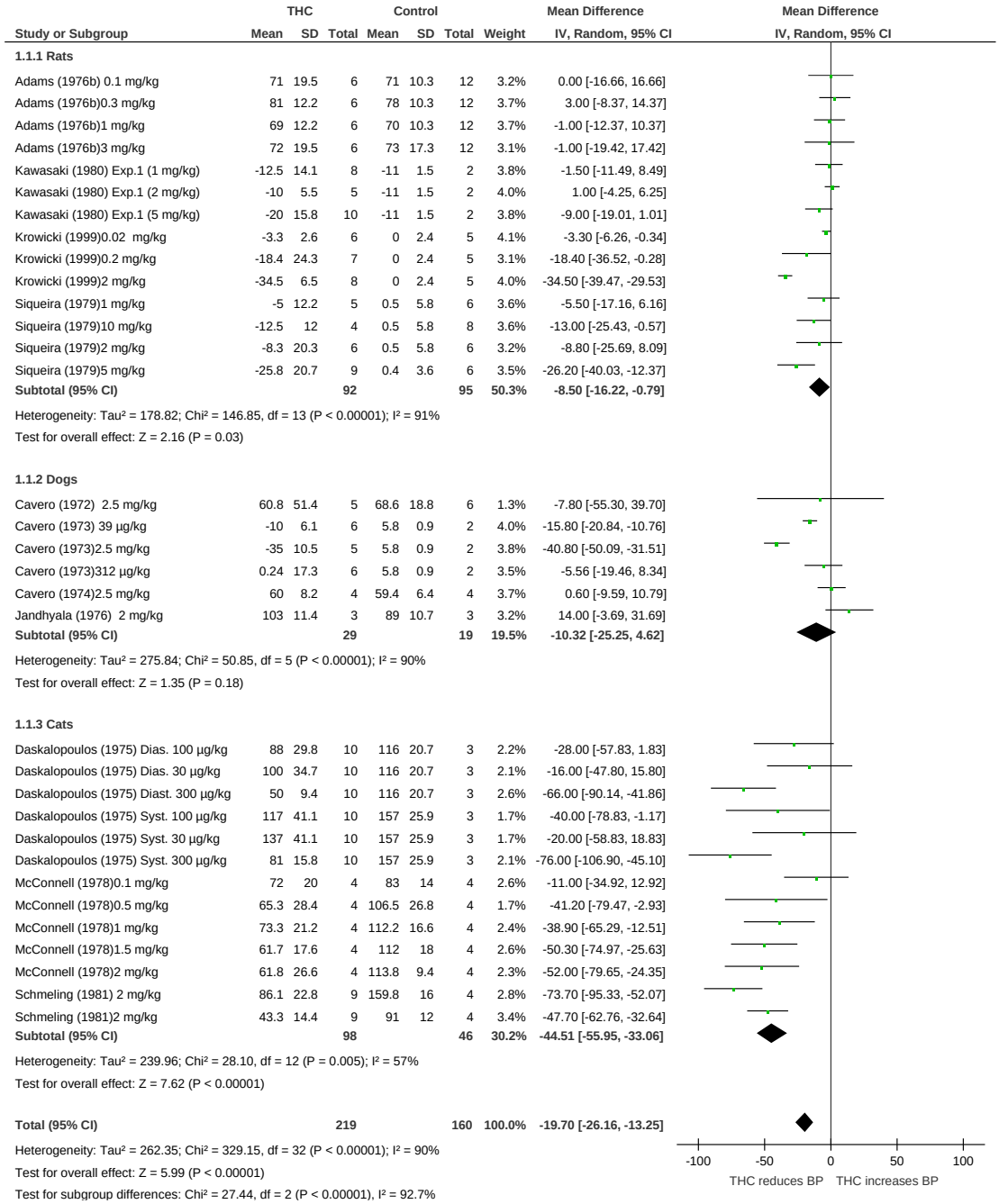
Author & Year	Study Description	Species, Model (Anaesthetic & Route)	Sample Size	THC Dose	THC Route	Time of THC Administration	Time of Haemodynamic Measurements	Basal Parameters *	Outcomes and Comments
				Humans					
Karniol 1973 [45]	Compared the effects of 8-THC and 9-THC	Human Healthy	21	5–20 mg	Inhale	Pre-test	Avrg. of 20 m post-drug	C: HR:82; T: HR:85	9-THC was twice as active as 8-THC in increasing HR and caused more subjective symptoms.
Karniol 1975 [46]	Examined the interaction between THC and CBN	Human Healthy	5 (M)	25 mg	Oral	Pre-test	50, 70 and 160 m post-drug	-	THC induced increase in HR and psychological effects. No change on THC effects when combined with CBN
Zimmer 1976 [47]	Examined changes of somatic parameters post-THC	Human Healthy	36	250 μg/kg	Oral	Pre-test	Pre-drug and 4 h post-drug	C: HR:87.9, BP:127.5; T: HR:89, BP:123	THC raised HR with no changes on other parameters including BP
Haney 2007 [48]	Determined the effects of naltrexone in combination with THC	Human Healthy	21 (11 M & 10 F)	2.5–10 mg	Oral	Pre-test	Continues for 6 h post-drug	-	Naltrexone enhanced intoxication effects of THC; THC increased HR
Beaumont 2009 [21]	Evaluated whether THC has inhibitory effect on transient esophageal sphincter	Human Healthy	18 (M)	10 and 20 mg	Oral	Pre-test	Continues for 4 h post-drug	C: HR:59; T: HR:59	THC inhibited the increased induced meal transient esophageal sphincter relaxation. THC increased HR and decreased BP
Klooker 2011 [49]	Assessed the effect of THC on rectal sensation	Human Healthy and IBD	10 and 12	5 and 10 mg	Oral	Pre-test	Continues for 105 m post-drug	-	THC had no effect on rectal perception to distension. THC increased HR with no effect on BP

Abbreviations: BP: blood pressure, BF: Blood flow, C: control group, CB_1: cannabinoid receptor 1, CBD: Cannabidiol, CBF: cerebral blood flow, CBN: cannabinol, CV: cardiovascular, D: THC treated group, F: females, G: gender, h: hour(s), HR: heart rate, , IBD: inflammatory bowel disease i.p.: intraperitoneal, i.v.: intravenous, M: males, m: minute(s), s.c.: Subcutaneous, T: treatment group, THC: Δ^9-Tetrahydrocannabinol. ↑: increased, ↓: decreased. * Basal parameters values before intervention (i.e., anaesthetic agents or THC). The units of the parameters are HR: beats/m, BP: mmHg, BF: mL/m.

2.1. Effect of THC Treatment on Haemodynamics

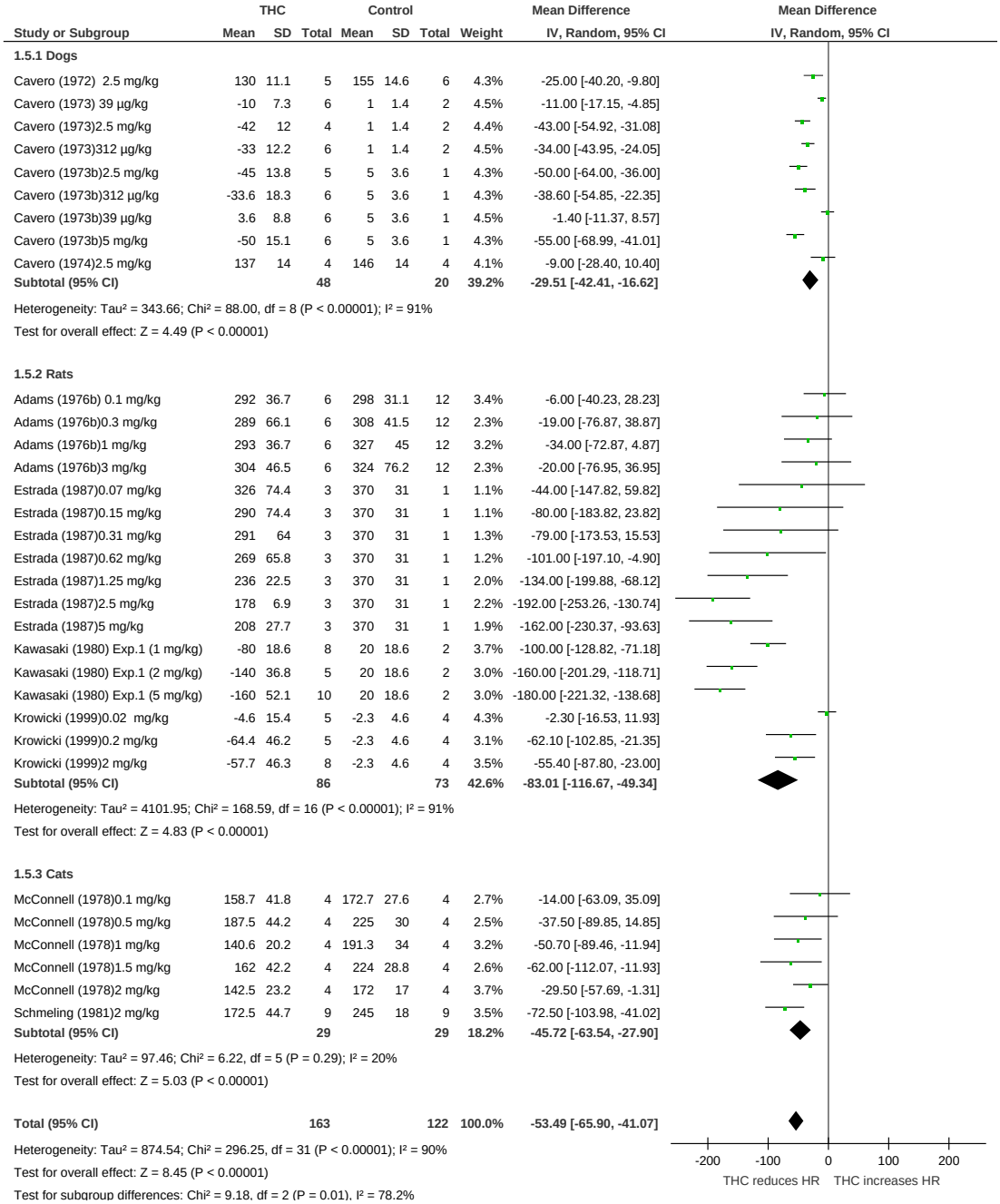
2.1.1. Anaesthetised Animals

Fifteen publications [19,23–36] assessed the effect of THC administration in three anaesthetised species (rats, dogs, and cats, $n = 664$). THC significantly reduced BP and HR after acute dosing (BP, MD −19.7 mmHg, 95%CI −26.16, −13.25, $p < 0.00001$; HR, MD −53.49 bpm, 95%CI −65.9, −41.07, $p < 0.00001$, Figure 2A,B). A cross-species analysis revealed that THC responses in the three species were significantly different in both BP ($p < 0.00001$) and HR ($p = 0.01$) (Figure 2A,B), and acute THC significantly reduced BP in rats and cats, but not in anesthetised dogs ($p = 0.18$, Figure 2A).



(**A**)

Figure 2. *Cont.*



(**B**)

Figure 2. Changes in (**A**) BP and (**B**) HR induced by acute THC dosing in anaesthetised animals.

Chronic THC administration (7–35 days) tended to increase mesenteric, femoral, and renal BF ($p = 0.05$, Figure 3C) with no significant effect on HR or BP. Heterogeneity was statistically significant for BP and HR measurements after acute THC dosing ($p < 0.00001$; $I^2 = 90\%$) and for BP after chronic THC dosing (BP, $p = 0.03$, $I^2 = 72\%$).

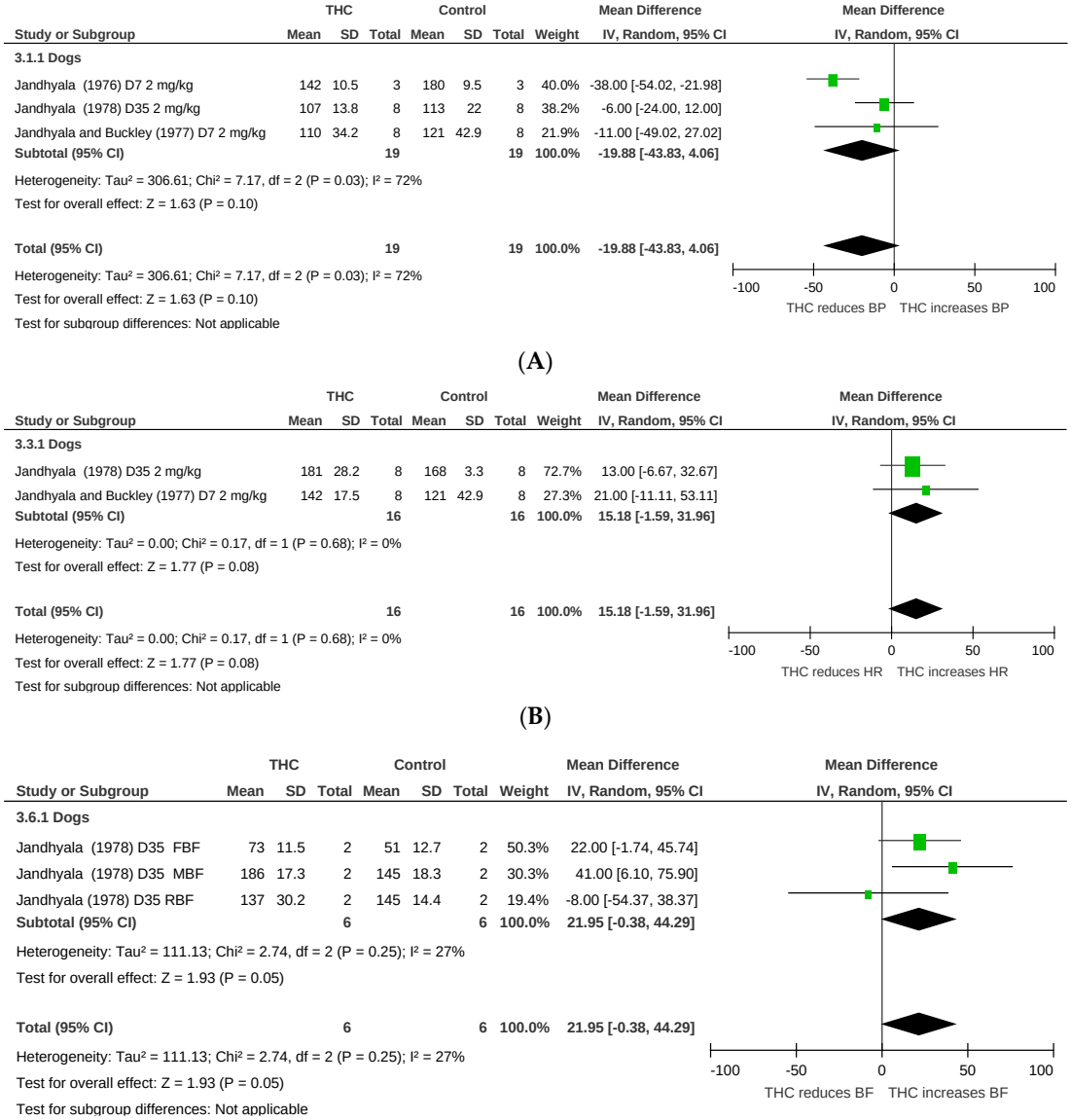


Figure 3. Changes in (**A**) blood pressure, (**B**) heart rate, and (**C**) blood flow (BF) induced by chronic THC dosing in anaesthetised animals.

2.1.2. Conscious Animals

Eight publications [20,22,23,37–41] assessed the effect of THC administration in five conscious species, including rats, bats, mice, rabbits, and monkeys (n = 170). THC significantly reduced BP and HR after acute dosing (BP, MD −12.3 mmHg, 95%CI −19.42, −5.18, p = 0.0007; HR, MD −30.05 bpm, 95%CI −38.47, −21.64, p < 0.00001, Figure 4A,B), and significantly increased CBF in murine models of stroke (BF, MD 32.35%, 95%CI 23.81, 40.88, p < 0.00001, Figure 4C). A cross-species analysis revealed that acute THC did not affect BP in bats (p = 0.36) and rats (p = 0.11) (Figure 4B). Heterogeneity was statistically significant for BP and HR measurements after acute THC dosing (BP, p < 0.00001, I^2 = 83%; HR, p < 0.00001, I^2 = 87%), but not in BF (p = 0.5, I^2 = 0%).

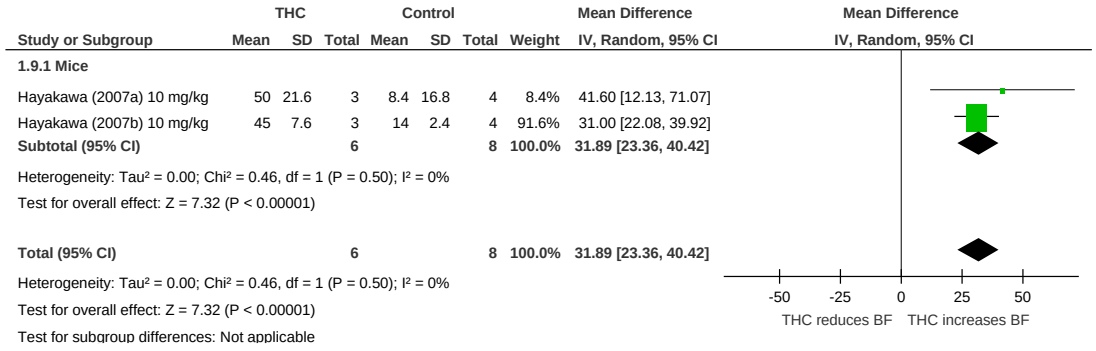
2.1.3. Conscious Animal Models of Stress or Hypertension

Two publications [43,44] assessed the effect of THC administration on BP in hypertensive rats (n = 22), and one [42] in a rat model of stress (n = 30). Acute and chronic (4–10 days) THC dosing

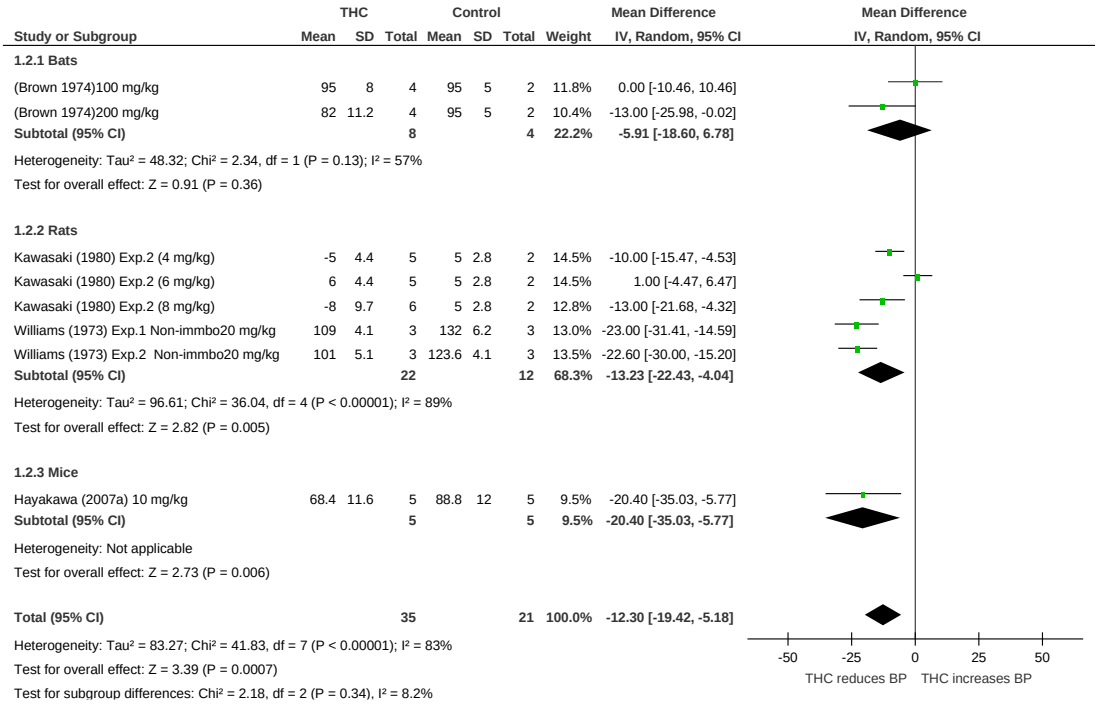
significantly reduced BP (acute THC, MD −61.37 mmHg, 95% CI –117.56, −5.17, $p = 0.03$, Figure 5A; chronic THC, MD −22.09 mmHg, 95% CI −30.61, −13.58, $p < 0.00001$, Figure 5B). Heterogeneity was statistically significant after acute dosing ($p < 0.00001$, $I^2 = 99\%$), but not after chronic dosing ($p = 0.69$, $I^2 = 0\%$).

2.1.4. Human Studies

Six publications [21,45–49] assessed the acute effect of THC administration on HR in humans ($n = 150$), no studies examined BP or BF. THC significantly increased HR after acute dosing (HR, MD 8.16 bpm, 95% CI 4.99, 11.33, $p < 0.00001$, Figure 6). Heterogeneity was statistically significant ($p < 0.00001$; $I^2 = 76\%$).

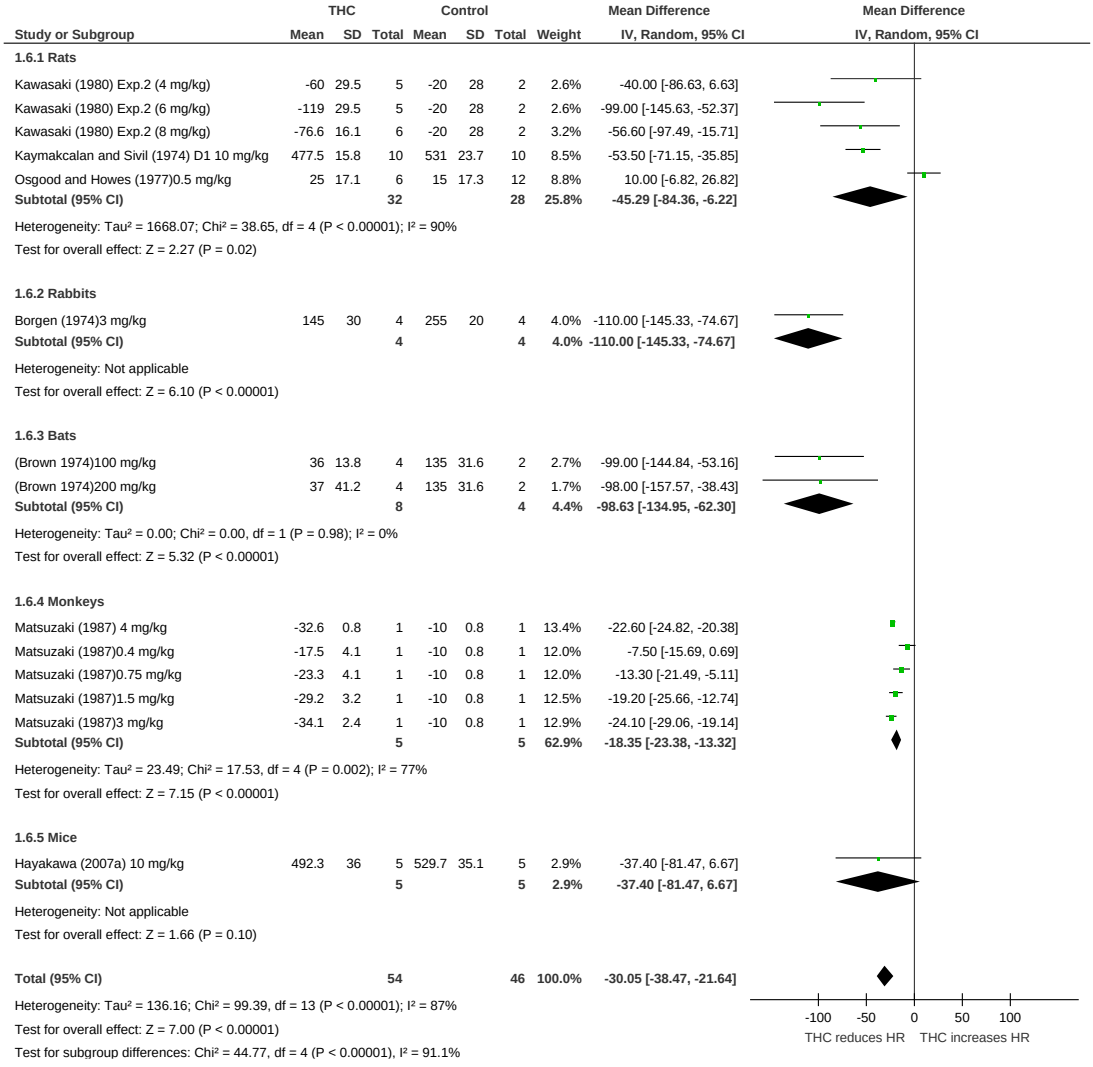


(**A**)



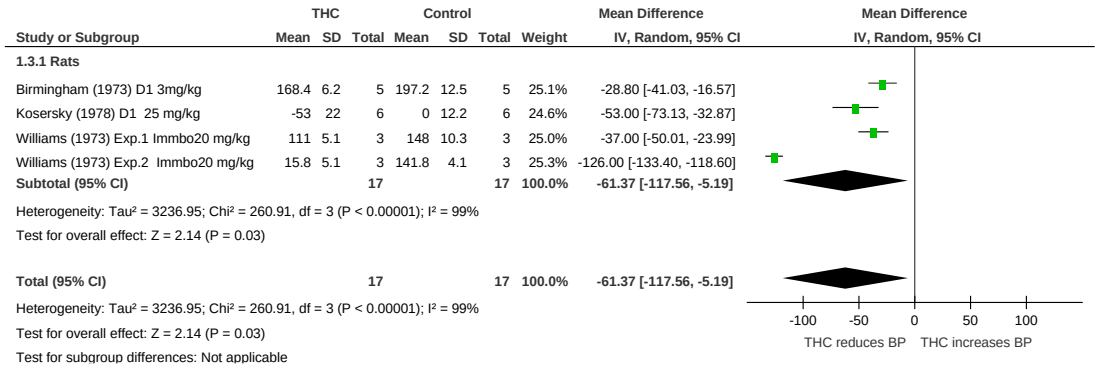
(**B**)

Figure 4. *Cont.*



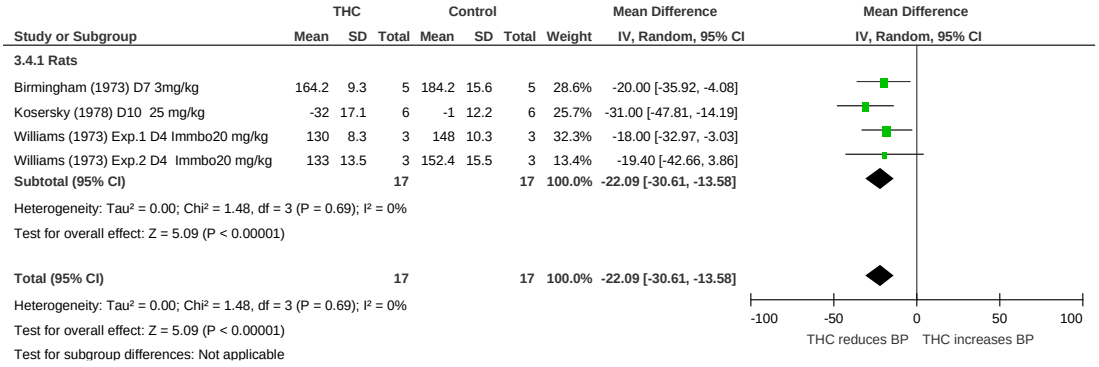
(**C**)

Figure 4. Changes in (**A**) BP, (**B**) HR, and (**C**) blood flow induced by acute THC dosing in conscious animals.



(**A**)

Figure 5. *Cont.*



(**B**)

Figure 5. Changes in BP induced by (**A**) acute and (**B**) chronic THC dosing in animal models of stress or hypertension.

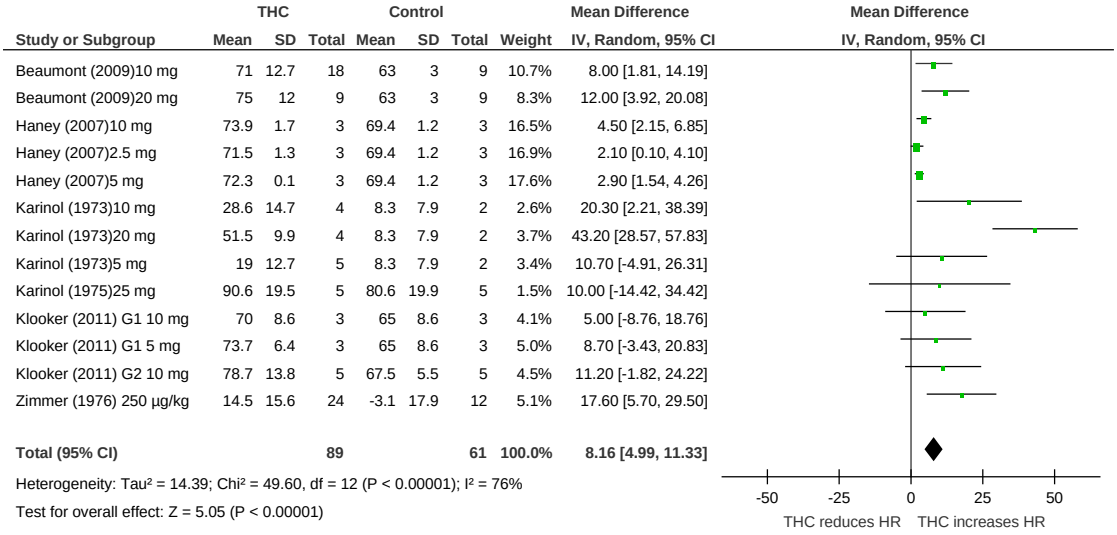


Figure 6. Changes in HR induced by acute THC dosing in humans.

2.2. Dose–Response to THC

Doses ranging from 0.0003 to 770 mg were used in different species. The animal analyses showed a trend in the reduction of BP with higher THC doses ($p = 0.07$), with no change in HR. In humans, THC caused dose-dependent tachycardia ($p = 0.01$) (Figure 7).

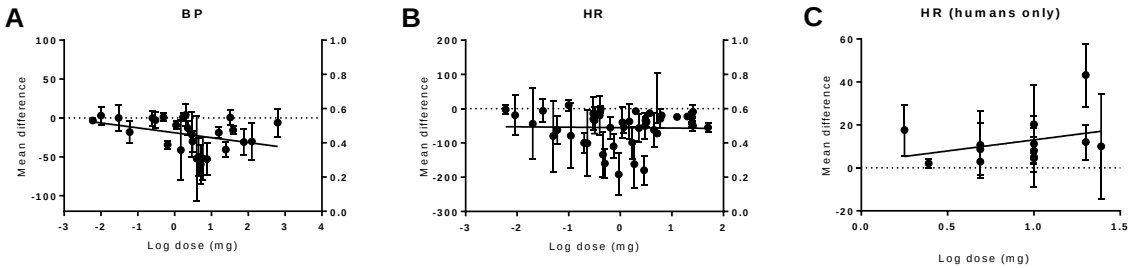


Figure 7. The effect of different THC doses on haemodynamic responses in vivo. The mean difference (MD) in animals' blood pressure (BP, (**A**)), animals' heart rate (HR, (**B**)), or heart rate (in humans only) ($p = 0.01$) (HR, (**C**)) is plotted against the log dose (mg) for each study. Error bars represent 95% confidence intervals (CI). Near-significant and significant dose-dependent effects on the blood pressure in animals ($p = 0.07$) and on the HR in humans ($p = 0.01$).

2.3. Quality

Among the 31 included publications, 6 publications used randomisation in their design and reported blinding assessment of outcome and measurements. Twenty publications assessed more than one outcome, 19 conducted dose–response relationships, 26 assessed a time window for intervention, 11 measured outcomes >24 h post-drug, and no publications provided incomplete data. There was no significant relationship between the quality score and any outcome (Spearman's rho coefficient of BP 0.22, $p = 0.09$; HR 0.27, $p = 0.07$ and BF 0.58, $p = 0.3$).

2.4. Publication Bias

Egger's test showed that bias was present in all studies except in studies in anaesthetised animals, conscious animals ($p = 0.001$), animal models of stress or hypertension (C) ($p = 0.001$), and humans (D) ($p < 0.0001$) (Appendix A, Figure A1).

3. Discussion

The aim of this study was to determine the effect of THC on haemodynamics in vivo in animals and cannabis-naïve humans. Our analysis has shown that an acute dosing of THC reduced BP and HR, and increased BF in animals of different models. Chronic dosing of THC tended to increase BF in anaesthetised animals and reduced BP in animal models of stress or hypertension. The data concerning the effects of THC in humans was limited to HR only, revealing a dose-dependent increase, suggesting further work is required to determine the full haemodynamic effects of acute and chronic THC administration in humans, especially given the different effects of THC on HR observed across species.

Our meta-analysis showed that acute THC dosing in anaesthetised animals reduced BP and HR, while a subgroup analysis revealed that there was no effect on BP or HR of anaesthetised dogs. However, Cavero et al. (1972, 1973, 1974) reported that intravenous administration of THC induced hypotension and bradycardia in dogs anaesthetised with pentobarbital caused by a reduction in the cardiac output and venous return mediated by the autonomic system [19,25–27]. Similarly, Schmeling reported that the reduction in sympathetic activity induced by THC in cats may cause hypotension and bradycardia [34]. It is suggested that the vagus nerve and the sympathetic outflow play a role in these effects induced by THC [36] and can be inhibited by the administration of a CB_1 antagonist [50]. The administration of THC for seven days subcutaneously reduced the increase in HR induced by pentobarbital anaesthetic agent in dogs, suggesting that THC antagonises the pentobarbital effect on the parasympathetic system (inhibiting the vagal tone) [30]. In rats anesthetised with pentobarbital, hypotension was reported after the administration of THC [35]; on the contrary, hypertension was reported in rats anesthetised with urethane post-THC [36], suggesting that THC may act differently with different anaesthetic agents. These studies suggest that the effects of THC in anaesthetised animals (hypotension and bradycardia) are induced through a central mechanism via the activation of CB_1 receptors.

In conscious animals under normal conditions, THC caused a variety of effects: hypotension was observed in bats, an effect which may be related to a change in venous activity [20], whereas another study in rats reported that THC induced tachycardia and hypertension, which are centrally mediated by increasing the level of adrenaline in the circulation [22]. However, studies in rat models of stress and hypertension, showed that THC lowered BP effectively [42–44]. The mechanism of the antihypertensive effect of THC in these models still needs to be studied.

Our meta-analysis in cannabis-naïve humans highlighted the limited number of studies investigating the effect of THC in humans (6 publications, $n = 123$ participants) with insufficient data to meta-analyse BP or regional BF. Studies in cannabis-naïve volunteers showed that the administration of THC orally or by inhalation caused tachycardia [46–49,51]. Tachycardia is also reported in humans after smoking cannabis [52–54] which may indicate that tachycardia induced post-cannabis smoking is caused by THC. The increase in HR caused by THC can be inhibited by CB_1 antagonism [55], suggesting

that CB_1 activation may play a role in the haemodynamic effect of THC in humans. A greater number of studies investigating the haemodynamic effect of THC and its mechanisms under normal and pathological conditions in humans are required.

Several studies have reported that phytocannabinoids such as cannabidiol (CBD) may alter the effect of THC. For example, Borgen and Davis suggested that CBD may act as a potential antagonist of the THC effect on HR in rabbits and rats [38] and protects against some of the negative effects of THC in humans with potentially opposite effects on regional brain functions [56,57]. The combination of CBD and THC such as in Sativex®, a licenced agent for the symptomatic treatment of spasticity in multiple sclerosis, has shown that CBD inhibits the tachycardia effect induced by THC in humans [58].

Dose–response analyses showed a relationship between THC dose and effect size on BP, but not HR, in different animal models, and on human HR. Dose-dependent effects on BP were also observed post-THC in anaesthetized rats [24,36], cats [28], and dogs [26]. A dose of 100 and 200 mg caused a dose-dependent reduction on the BP of conscious bats, but not on HR [20]. HR dose-dependent reduction was reported in anaesthetized dogs [26,27] and conscious monkeys [39]. In human studies, doses between 2.5 and 25 mg were used. A dose-dependent increase in HR was observed in humans after oral THC administration of 5, 10, and 20 mg [21,49]. Over-intoxication has been reported after 20 mg of oral administration of THC in 5 of 21 healthy volunteers [48].

There are a number of limitations to consider in this analysis. First, the principal intention of 10 of the included studies was not to assess the cardiovascular effects of THC administration; therefore, the data extracted through secondary haemodynamic outcomes in this meta-analysis is for hypothesis-generating purposes. Second, the results should be interpreted with caution because of the heterogeneity between studies in terms of THC dose, time, and route of administration; the responses to THC will clearly be dependent upon peak plasma concentration, which are not easily comparable across studies. Indeed, a significant statistical heterogeneity was observed in the majority of the meta-analyses. Third, only 6 out of 31 articles used randomisation and described a masked assessment of outcomes, factors that can influence the reported outcomes. However, we found no significant correlation between study quality and effect size in this review.

In conclusion, this study has summarised the in vivo cardiovascular effects of THC administration. Our analysis demonstrates that THC acts differently according to species, causing tachycardia in humans, and bradycardia, hypotension, and an increase in regional BF in animals under different conditions. THC may be a potential future treatment for cardiovascular disorders, though its use as a single agent will be limited by CB_1 mediated psychogenic side effects, events that could be counterbalanced with other agents such as CBD. Data from human studies using THC alone is limited to heart rate only, thereby further good quality, randomised, blinded studies investigating the haemodynamic effects of THC in humans should be considered.

4. Materials and Methods

4.1. Search Strategy

All studies investigating the haemodynamic effects of THC (including BP, HR, and BF) were searched for (until April 2017) in Medline, EMBASE, and PubMed. Search keywords included: Δ^9-Tetrahydrocannabinol, Tetrahydrocannabinol, THC, Dronabinol, Marinol, Nabilone, Namisol, cardiovascular, blood pressure, systolic, diastolic, hypertension, hypotension, heart rate, tachycardia, bradycardia, blood flow, haemodynamic, vasodilation, vasorelaxation, and vasoconstriction. References from the included studies were also hand-searched.

Prespecified inclusion and exclusion criteria were used to prevent bias; the studies had to be in vivo, assess haemodynamics (BP, HR or BF), be original articles, be controlled studies, and use cannabis-naïve participants. Therefore, the exclusion criteria were: *in vitro* studies, mixtures of Δ^9-THC with other cannabis extracts, studies investigating the interaction of THC with other drugs or

cannabinoids, studies not assessing haemodynamics (BP, HR, or BF), review articles, editorials, and uncontrolled studies.

4.2. Data Acquisition

Data on BP (mmHg), HR (beats per minute, bpm), and BF (% change from baseline or mL/min) were extracted from the included papers, and the changes in haemodynamics 2 h post-drug after acute THC dosing were used for the analyses. This time point was selected as the peak plasma time is between 30 min and 4 h after oral administration and it was the most common time point when haemodynamics were measured throughout the articles. If there were no measurements taken at this time point (2 h post-drug), the closest time point to 2 h was used for the analyses. In chronic studies, the measurements taken at the end of the studies were used for the analyses. If the exact number of animals used in each drug group was not available, the lowest number of animals within the range given was used for the experimental group (THC), and the highest number was used for the control group. If a crossover design was used in a study, the total number of humans was distributed equally to the two groups. Articles were excluded if data were not available. Grab application (version 1.5) was used to extract values from the figures given in published articles if no values were stated within the text. If the published articles used multiple groups (e.g., to assess dose-dependent effects) with one control group, then the number of humans or animals per control group was divided into the number of comparison groups. For the dose–response analysis, the total dose of the drug administrated up to the time when the haemodynamics was measured was used.

4.3. Quality

Eight-point criteria derived from Stroke Therapy Academic Industry Recommendations (STAIR) [59–61] and the Cochrane collaborations tool [62] were used to identify the risk of bias. Each of the following criteria was equal to 1 point: randomisation, blinding of outcome assessment, blinding of personnel and participant, assessment of more than one outcome, dose–response relationship, therapeutic time window, assessment of outcome >24 h, and incomplete outcome data.

4.4. Data Analysis

The studies were divided into acute and chronic groups. The data from human and animal studies were analysed separately. The animals were divided into two groups, anaesthetised and conscious, as the autonomic nervous system may respond differently in the two conditions [63], then grouped before the analysis in normal and abnormal (i.e., models of stress or hypertension) models and then subgrouped by species (mice, rats, dogs, etc.). For the THC dose–response analysis, the data were grouped according to the endpoint (BP, HR, or BF), and then subgrouped according to the dose. The data from each group were analysed as forest plots using the Cochrane Review Manager software (Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014), and as funnel plots using Stata (StataCorp. 2009. Stata Statistical Software: Release 11. College Station, TX, USA). Funnel plot asymmetry (publication bias) was assessed by Egger's test [64]. Stata was also used for meta-regression that described the relationship between THC dose and effect size. PRISM 7 (GraphPad, Software, La Jolla, CA, USA) was used to produce the figures of dose–response. Since heterogeneity was expected between the study protocols (different species, models, dose, and time) random-effect models were used. The results of continuous data are expressed as mean difference (MD) with 95% confidence intervals (CIs). The studies were weighted by sample size, and statistical significance was set at $p < 0.05$.

Author Contributions: T.J.E. and S.E.O. conceived and designed the experiments; S.R.S. and S.A.M. collected and analyzed the data; all authors wrote and revised the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

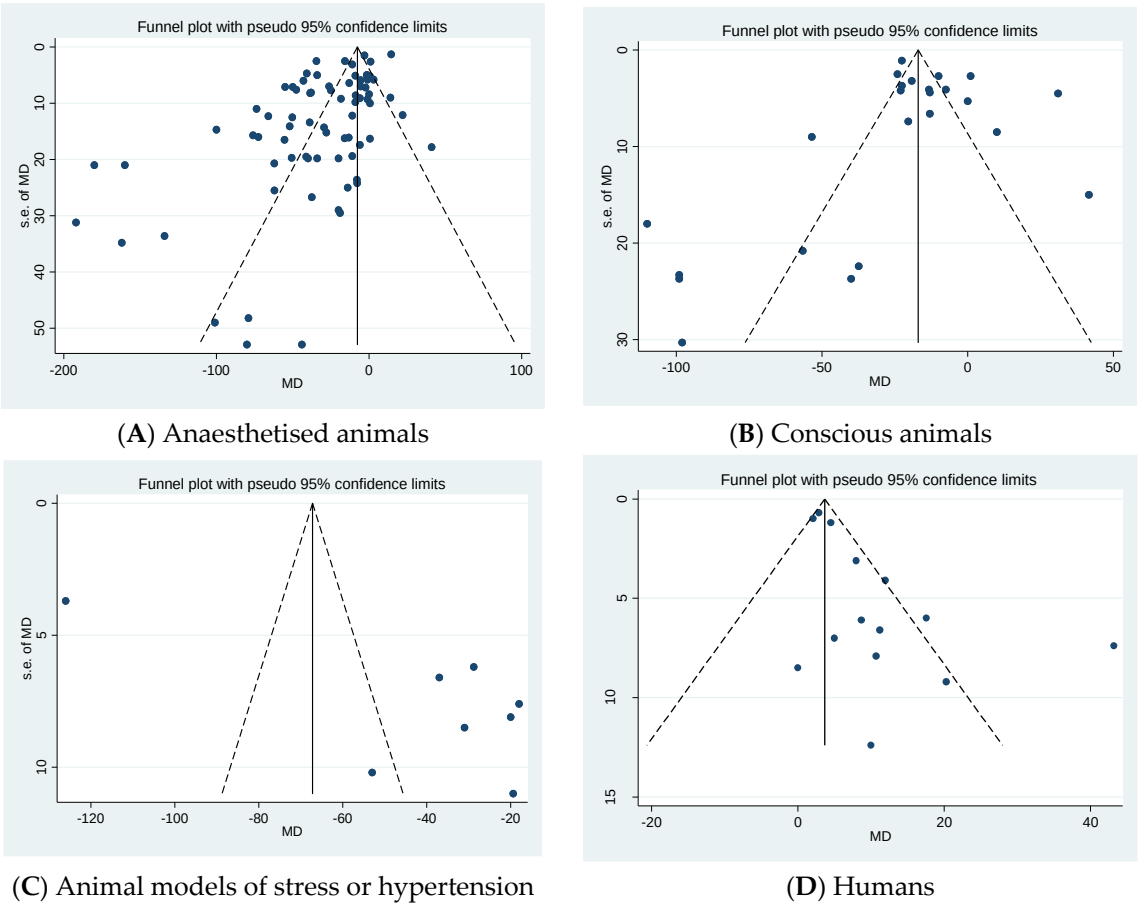


Figure A1. Funnel plots for each outcome evaluating the publication bias. The standard error (SE) of the mean difference (MD) in haemodynamics (MD, *y* axis) for each study is plotted against its effect size (*horizontal* axis). There was significant bias in conscious animals (**B**) ($p = 0.001$), animal models of stress or hypertension (**C**) ($p = 0.001$), and humans (**D**) ($p < 0.0001$). No significant bias in anaesthetised animals (**A**).

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A systematic review and meta-analysis of the haemodynamic effects of cannabidiol

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A Systematic Review and Meta-Analysis of the Haemodynamic Effects of Cannabidiol

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Despite cannabidiol (CBD) having numerous cardiovascular effects *in vitro*, its haemodynamic effects *in vivo* are unclear. Nonetheless, the clinical use of CBD (Epidiolex) is becoming more widespread. The aim of this systematic review was to establish whether CBD is associated with changes in haemodynamics *in vivo*. Twenty-five studies that assessed the haemodynamic effects of CBD (from PubMed, Medline and EMBASE) were systematically reviewed and meta-analyzed. Data on blood pressure (BP), heart rate (HR), and blood flow (BF) were extracted and analyzed using random effects models. Twenty-two publications assessed BP and HR among 6 species (BP $n = 344$ and HR $n = 395$), and 5 publications assessed BF in 3 species ($n = 56$) after acute dosing of CBD. Chronic dosing was assessed in 4 publications in 3 species (total subjects BP, $n = 6$; HR, $n = 27$; BF, $n = 3$). Acute CBD dosing had no effect on BP or HR under control conditions. Similarly, chronic dosing with CBD had no effect on HR. In models of stress, acute CBD administration significantly reduced the increase in BP and HR induced by stress (BP, mean difference (MD) −3.54, 95% CI −5.19, −1.9, $p < 0.0001$; HR, MD −16.23, 95% CI −26.44, −6.02, $p = 0.002$). In mouse models of stroke, CBD significantly increased cerebral blood flow (CBF, standardized mean difference (SMD) 1.62, 95% CI 0.41, 2.83, $p = 0.009$). Heterogeneity among the studies was present, there was no publication bias except in HR of control and stressful conditions after acute CBD dosing, and median study quality was 5 out of 9 (ranging from 1 to 8). From the limited data available, we conclude that acute and chronic administration of CBD had no effect on BP or HR under control conditions, but reduces BP and HR in stressful conditions, and increases cerebral blood flow (CBF) in mouse models of stroke. Further studies are required to fully understand the potential haemodynamic effects of CBD in humans under normal and pathological conditions.

Keywords: cannabidiol, Epidiolex, CBD, cardiovascular system, haemodynamic, blood pressure, heart rate, blood flow

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INTRODUCTION

Cannabidiol (CBD) is the second most abundant phytocannabinoid, after Δ^9-tetrahydrocannabinol (THC) (Pertwee, 2006; Tambaro and Bortolato, 2012) and was first isolated from the cannabis extract in 1940 (Adams et al., 1940). The pharmacological actions of CBD are complex; CBD has a low affinity to the cannabinoid receptor 1 (CB_1) and cannabinoid

receptor 2 (CB_2) (Thomas et al., 2007; Pertwee, 2008), and targets a wide range of other receptors including 5-hydroxytryptamine ($5\text{-}HT_{1a}$), transient receptor potential vanilloid receptor 1 (TRPV1), peroxisome proliferator-activated receptors (PPARs) and G protein-coupled receptor 55 (GPR55) (Pertwee, 2008; Stanley et al., 2013a). It is suggested that CBD may have therapeutic effects in a variety of disorders including diabetes, gastrointestinal disturbances, cancer, oxidative stress, inflammation and in cardiovascular disease (Russo and Guy, 2006; Capasso et al., 2008; Zuardi, 2008; Iuvone et al., 2009; Booz, 2011). CBD may also have desirable effects in multiple neurological and psychological disorders, including dystonia, schizophrenia, epilepsy and Parkinson's disease (Consroe et al., 1986; Leweke et al., 2012; Chagas et al., 2014; Devinsky et al., 2016). In a recent open-label trial investigating the effects of CBD (Epidiolex, 2–50 mg/kg) on 214 young patients with treatment-resistant epilepsy, CBD reduced seizure frequency and demonstrated an acceptable safety profile (Devinsky et al., 2016). Epidiolex was also safe and well tolerated in refractory epilepsy secondary to tuberous sclerosis and for epileptic spasms, highlighting its potential as a new treatment for refractory epilepsy (Abati et al., 2015; Geffrey et al., 2015).

Pre-clinical studies on the effects of CBD on the cardiovascular system have shown that CBD causes endothelium- and nitric oxide-dependent vasorelaxation of isolated human mesenteric arteries (Stanley et al., 2015) and PPARγ-dependent vasorelaxation of the rat aorta (O'Sullivan et al., 2009). CBD also improves vasorelaxation in the femoral arteries of Zucker diabetic fatty rats via enhanced production of vasodilator COX-1/2-derived products acting at EP4 receptors (Stanley et al., 2013b; Wheal et al., 2014). CBD also decreases myocardial infract size in a rat model of ischaemia/reperfusion injury (Durst et al., 2007), attenuates myocardial dysfunction and inflammation in an animal model of diabetes (Rajesh et al., 2007), and attenuates inflammatory and oxidative stress changes induced by high glucose in human coronary artery cells (Rajesh et al., 2010). CBD also reduces cerebral vascular inflammation and associated dilatation induced by lipopolysaccharide in mice (Ruiz-Valdepenas et al., 2011), infarct size in animal models of stroke (England et al., 2015) and reduced blood brain barrier permeability (Hind et al., 2016). These effects on the cerebral vasculature appear to involve $5HT_{1A}$ and PPARγ. Together, these pre-clinical studies might suggest that the cardiovascular system is a therapeutic target for CBD (Stanley et al., 2013a). However, despite these many vascular effects of CBD, it is not yet clear whether CBD administration alters haemodynamics under control or pathological situations.

Given the increasing clinical use of CBD, and the numerous effects of CBD in the cardiovascular system, the aim of the present study was to systematically review and analyse *in vivo* studies evaluating the effects of CBD on alterations in haemodynamics.

MATERIALS AND METHODS

Search Strategy

All studies potentially investigating the haemodynamic effect of CBD (including BP, HR, and BF) were searched (until November 2016) in Medline, EMBASE, and PubMed. Search keywords included: Cannabidiol, Epidiolex, cardiovascular, blood pressure (BP), systolic, diastolic, hypertension, hypotension, heart rate (HR), tachycardia, bradycardia, blood flow (BF), haemodynamic, vasodilatation, vasorelaxation, and vasoconstriction. References from included studies were also hand searched. Initially, the National Institute for Health and Excellent Care platform was used in which two databases (EMBASE and Medline) were used for searching. Then, a separate search was conducted using PubMed. Pre-specified inclusion and exclusion criteria were used to prevent bias; studies had to be *in vivo*, assess haemodynamics (BP, HR, or BF), be original articles, and be a controlled study. The exclusion criteria were: *in vitro* studies, mixtures of CBD with other cannabis extracts, studies not assessing haemodynamics (BP, HR, or BF), review articles and editorials, or uncontrolled studies.

Data Acquisition

Data on BP, HR, and BF were extracted from the included papers, and the changes in haemodynamics at 2 h post-drug after acute CBD dosing were used for analysis. A standardized time point of 2 h was decided as this was commonly available throughout the articles and CBD has been previously shown to peak at 2 h in plasma (Nadulski et al., 2005a,b). If there were no measurements taken at this time point (2 h post-drug) the closest time point to 2 h was used for analysis. In chronic studies, the mean of total measurements or measurements taken at the end of the study were used for analysis depending on data provided. If the exact number of animals used in each drug group were not available, the authors were contacted. If the authors were not able to provide the necessary information, the lowest number of animals within the range given was used for the experimental group CBD, and the highest number was used for the control group. If a crossover design was used in a study, the total number of humans was distributed equally to the drug groups. Grab application (version 1.5) was used to extract values from figures given in published articles if no values were stated within the text. If published articles used multiple groups (e.g., to assess dose-dependent effects) with one control group, then the number of humans or animals per control group was divided into the number of comparison groups. For the dose-response analysis, the total dose of the drug administrated to species up to the time in which the haemodynamics were measured was used.

Quality

The methodological quality was assessed to identify risk of bias using six-point criteria derived from the Cochrane collaborations tool for assessing risk of bias (Higgins et al., 2011) and Stroke Therapy Academic Industry Recommendations (STAIR) (Stroke Therapy Academic Industry Roundtable, 1999). Each of the following criteria was equal to 1 point: randomisation, allocation concealment, blinding of outcome assessment, blinding of personnel and participant, assessment of more than one outcome, dose-response relationship, therapeutic time window, assessment of outcome >24 h and incomplete outcome data.

Data Analysis

Studies were divided into two groups (i.e., acute and chronic). Data were grouped before analysis according to model (non-stress and stress), and then sub-grouped by species (human, mice, rats, etc.). For the CBD dose-response analysis, data were grouped according to endpoint (BP, HR, or BF), and then sub-grouped according to dose. Data from each group were analyzed as forest plots using the Cochrane Review Manager software (Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014), and as funnel plots using Stata (StataCorp. 2009. Stata Statistical Software: Release 11. College Station, TX, USA). Funnel plot asymmetry (publication bias) was tested by Egger's test (Egger et al., 1997). Stata was also used for meta-regression that described the relationship between CBD dose and effect size. PRISM 7 (GraphPad, Software, La Jolla, CA, USA) was used to produce figures of dose-response. Since heterogeneity was expected between study protocols (different species, models, dose and time) random-effect models were used. The results of continuous data on BP and HR are expressed as mean difference (MD), and as standardized mean difference on BF with 95% confidence intervals (CIs) due to the different scales used in assessing BF. Studies were weighted by sample size and statistical significance was set at $p < 0.05$.

RESULTS

From the initial 1016 search results, 277 relevant publications were identified from three databases (Medline, EMBASE, and PubMed). Of these, 25 articles met the inclusion criteria (see **Figure 1**). A summary of the data extracted from included studies is shown in **Table 1**.

Blood Pressure and Heart Rate

Ten publications assessed the acute effect of CBD administration on BP, and 15 publications assessed the effect of CBD administration on HR in 5 species, including humans, mice,

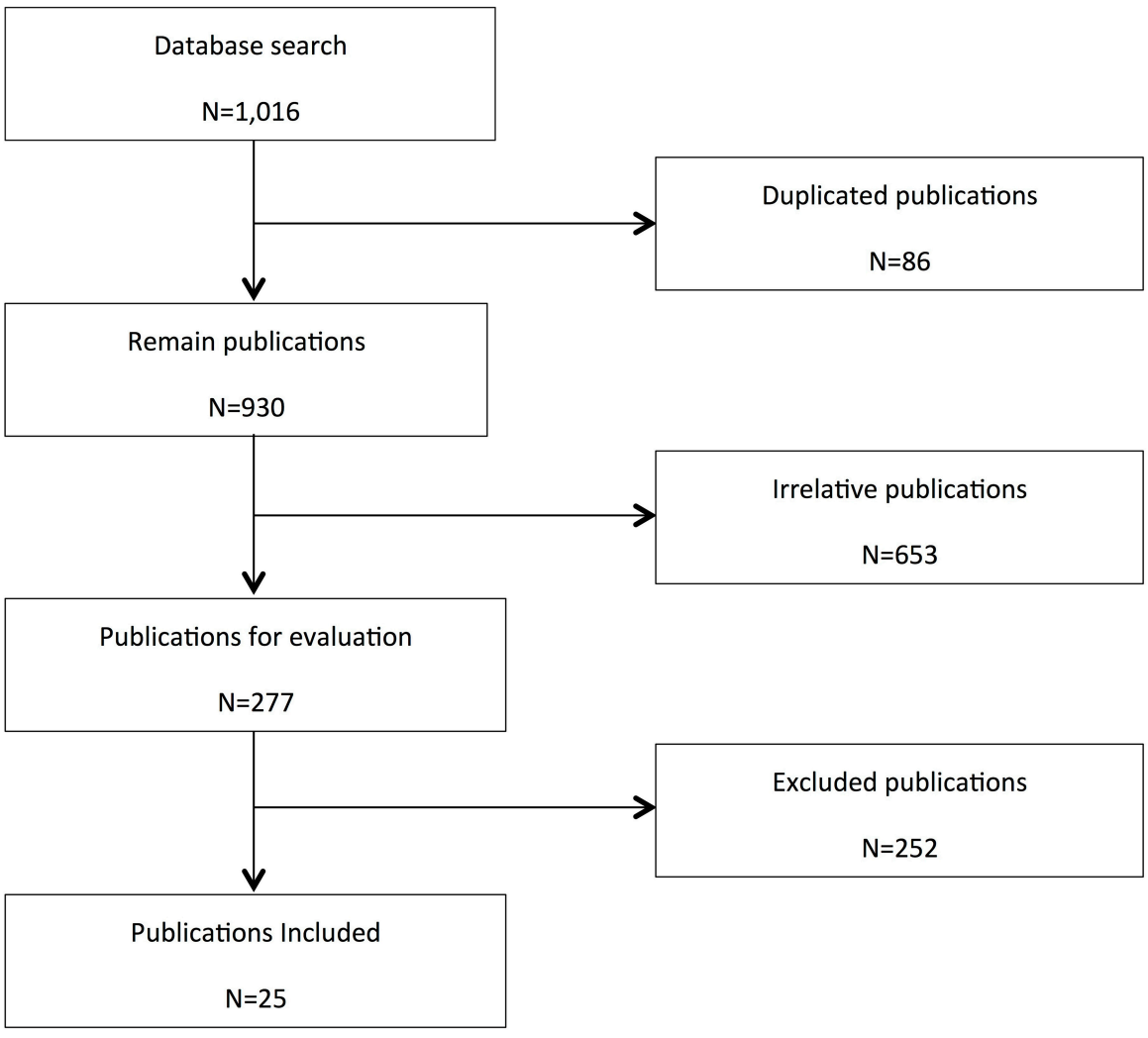

FIGURE 1 | Flow chart for study retrieval and selection. *In vitro* studies, interaction studies including mixtures of CBD with other cannabis extracts, studies not assessing haemodynamics (BP, HR, or BF), review articles and editorials, or uncontrolled studies were excluded.

TABLE 1 | Summary of included studies in chronological order.

Reference	Species	Model	Dose (CBD)	Route	Time of CBD administration	Time of haemodynamic measurements	Finding
Borgen and Davis, 1974	Rabbits	Anaesthetised	25 mg/kg	i.v.	Pre-test	Pre-drug and hourly interval to 7 h post-drug	No changes on HR
Bright et al., 1975	Dogs	Anaesthetised	0.5 or 1 mg/kg	i.v.	Pre-test	During 30 min post-drug.	↑MBP and HR
Belgrave et al., 1979	Humans	Healthy volunteers	320 μg/kg	Oral	Pre-test	Pre-drug and at 1.5, 2.5 and 3.5 h post-drug	No changes on HR
Gong et al., 1984	Human	Healthy volunteers	100, 600 or 1200 mg	Oral	Pre-test	Hourly interval to 6 h post-dru	No changes on SBP, DBP or HR
			1200 mg		Pre-test	Hourly interval to 6 h post-drug	
			1200 mg		Single dose per day for 20 days	Hourly interval to 6 h post-drug and on days 5, 12 and 19	
Consroe et al., 1991	Human	Patients with Huntington disease	10 mg/kg	Oral	Single dose per day for 6 weeks	Pre-drug, during and post-drug	No changes on MBP or HR
Zuardi et al., 1993	Human	Healthy volunteers	300 mg	Oral	Pre-test	Pre-drug and Post drug at 80 min (pre-stress), 85 min (during stress) and 100 min (post-stress)	No changes on SBP or HR
Mishima et al., 2005	Mice	MCAO	3 mg/kg	i.p.	Pre and 3 h post-occlusion	MBP and HR: 2 h post-occlusion	No changes on MBP or HR
						CBF: during 4 h post-occlusion	↑CBF
Resstel et al., 2006	Rats	Stress (fear)	10 mg/kg	i.p.	Pre-test	Pre-stress and during 10 min post-stress	↓MBP and HR post-stress
Hayakawa et al., 2007a	Mice	MCAO	3 mg/kg	i.p.	Pre, 3 and 4 h post-occlusion, and 1 and 2 h post-reperfusion	MBP and HR: pre-reperfusio	No changes on MBP or HR
						CBF: during 4 h of occlusion and post-reperfusion	↑CBF
Hayakawa et al., 2007b	Mice	MCAO	3 mg/kg	i.p.	Pre-occlusion and 3 h post-occlusion and Single dose per day for 14 days	During 4 h and on day 14 post-occlusion	↑CBF
Durst et al., 2007	Rats	Myocardial infarction	5 mg/kg for 7 days	i.p.	Pre-ischaemia and post-ischaemia for 7 days	On day 1 and 7 post-ischaemia	No changes on HR
Alvarez et al., 2008	Piglets	Carotid occlusion and hypoxic ischaemia	0.1 mg/kg	i.v.	15 min and 4 h post-procedure	MBP and HR: pre-procedure and at 3 and 6 h post-procedure	Maintain MBP and HR after the fall post- hypoxic ischaemia
						CBF: pre-procedure and during 6 h post-procedure	↑CBF
Hayakawa et al., 2008	Mice	MCAO	3 mg/kg	i.p.	Pre-occlusion and 3 h post-occlusion	During 4 h post-occlusion	No changes on MBP or HR
Resstel et al., 2009	Rats	Stress (restraint)	1, 10, or 20 mg/kg	i.p.	Pre-stress	Pre-stress and during 1 hr post-stress	↓MBP and HR post-stress
Alves et al., 2010	Rats	Conscious	60 nmol	BNST	Post-ACSF	During 60 min post-drug	No changes on MBP or HR
Walsh et al., 2010	Rats	Myocardial infarction	10 or 50 μg/kg	i.v.	Pre-ischaemia and pre-reperfusion	Pre- ischaemia during 2.30 h post-ischaemia,	↓MBP
Granjeiro et al., 2011	Rats	Stress (restraint)	15, 30 or 60 nmol	intracisternal	Pre-stress	Pre-stress and during 1 hr post-stress	No change in MBP or HR
Hallak et al., 2011	Human	Healthy volunteers	600 mg	Oral	Pre-test	Pre-drug and at 30 min interval for 2.30 h post-drug	No change in SBP, DBP or HR

(Continued)

TABLE 1 | Continued

Reference	Species	Model	Dose (CBD)	Route	Time of CBD administration	Time of haemodynamic measurements	Finding
Gomes et al., 2012	Rats	Stress (fear)	15, 30 or 60 nmol	BNST	Pre-stress	Pre-stress and during 10 min post-stress	30 and 60 nmol: ↓MBP and HR
Martin-Santos et al., 2012	Human	Healthy volunteers	600 mg	Oral	Pre-test	Pre-drug and Hourly interval for 3 h post-drug	No changes on BP or HR
Gomes et al., 2013	Rats	Stress (restraint)	15, 30 or 60 nmol	BNST	Pre-stress	Pre-stress and during 60 min post-stress	Enhanced the HR increase post-stress No changes on MBP
Pazos et al., 2013	Piglets	Hypoxic ischaemia	1 mg/kg	i.v.	Post-HI	Pre-drug, at 30 and 90 min post-HI	No changes on MBP
Gonca and Darici, 2015	Rats	Myocardial infarction	50 μg/kg	i.v.	Pre-ischaemia	Pre-ishaemia At 1, 5 and 11 min post-ischaemia	No changes on BP or HR
Feng et al., 2015	Rabbits	Myocardial infarction	100 μg/kg	i.v.	Pre-reperfusion	At 15, 30 and 45 min post-drug	↑ BF
Garberg et al., 2016	Piglets	Hypoxic ischaemia	1 mg/kg	i.v.	Post-HI	Immediately post HI and at 30, 210 and 570 min post-HI	No changes on BP or HR

ACSF, Artificial cerebrospinal fluid; BP, blood pressure; BNST, bed nucleus of the stria terminalis; CBD, Cannabidiol; HI, Hypoxic ischemia; HR, heart rate; h, hours; i.p., Intraperitoneal; i.v., intravenous; MBP, mean blood pressure; MCAO, middle cerebral artery occlusion; min, minutes; SBP, systolic blood pressure.

dogs, rats, piglets, and rabbits ($n = 403$). Chronic dosing was assessed in 3 publications in 2 species, including humans and rats (BP: one study, $n = 6$; HR: 3 studies, $n = 27$). CBD had no effect on BP or HR after acute dosing (BP, MD 3, 95% CI −1.81, 7.8, $p = 0.22$; HR, MD −0.05, 95% CI-2.68, 2.57, $p = 0.97$, **Figures 2A,B**) or chronic dosing (HR MD 0.3, 95% CI −3.97, 4.57, $p = 0.89$, **Figure 3**). Within species analysis revealed that acute CBD dosing significantly increased HR in rats ($p = 0.004$, **Figure 2B**). Heterogeneity was statistically significant for BP measurements after acute CBD dosing ($p = 0.0006$; $I^2 = 65\%$) and HR measurements after chronic CBD dosing ($p = 0.05$; $I^2 = 55\%$; **Figures 2**, **3**).

Six publications assessed the effect of CBD administration on BP and HR in models of stress in rats and humans ($n = 336$). Overall, CBD administration significantly reduced the increase in BP (BP, MD: −3.54, 95% CI −5.19, −1.9, $p < 0.0001$, **Figure 4A**) and HR (HR, MD: −16.23, 95% CI −26.44, −6.02, $p = 0.002$, **Figure 4B**) induced by stress. Heterogeneity was statistically significant in both BP ($p < 0.00001$; $I^2 = 73\%$) and HR ($p < 0.00001$; $I^2 = 92\%$; **Figure 4**).

Blood Flow

Changes in BF after acute CBD dosing were assessed in 5 publications examining the effects of CBD in models of stroke or myocardial infarction in 3 species (mice, piglets and rabbits, $n = 56$). Overall, CBD had no effects on BF (SMD: 0.58, 95% CI −0.1, 1.26, $p = 0.1$). However, subgroup analysis showed that CBD significantly increased cerebral blood flow (CBF) in mouse models of stroke ($p = 0.009$, **Figure 5**); heterogeneity was not statistically significant ($p = 0.27$; $I^2 = 21\%$). As only one study assessed BF after chronic dosing, a meta-analysis was not applicable.

Route of Administration

We compared differences between local and systemic administration of CBD on haemodynamics. Local (intracisternal or intracerebral) administration of CBD was only used in studies on rats (4 out of 9 studies; 1 under control conditions and 3 under stressful situations). After systemic administration, there was a significant reduction in HR ($p < 0.0001$; 2 studies), but not after local (intracisternal or intracerebral) administration of CBD ($p = 0.11$; 3 studies).

CBD Dose-Response on Haemodynamics

The dose-response to CBD was analyzed to establish if there is a relationship between CBD dose and effect size. Doses ranging from 0.003 to 22800 mg were used in different species of different models. Overall, there was no relationship between drug dose and the size of the effect on haemodynamics (BP $p = 0.81$, HR $p = 0.97$, BF $p = 0.97$; **Figure 6**).

Quality

Among the 25 included publications, 9 publications used randomisation and allocation concealment in their design, 6 reported blinding assessment of outcome and blinding of outcome measurements, 20 publications assessed more than one outcome, 12 conducted dose-response relationships, 19 assessed a time window for intervention, 4 measured outcomes >24 h post-drug and 2 publications provided incomplete data due to subject withdrawal There was no significant relationship between quality score and any outcome except in BF (Spearman's rho coefficient of BP, −0.1, $p = 0.54$, HR, 0.06, $p = 0.66$ and BF, 0.87, $p = 0.01$).

Publication Bias

Egger's test showed no bias present except in studies assessing for changes in HR in either control or stressful conditions after acute

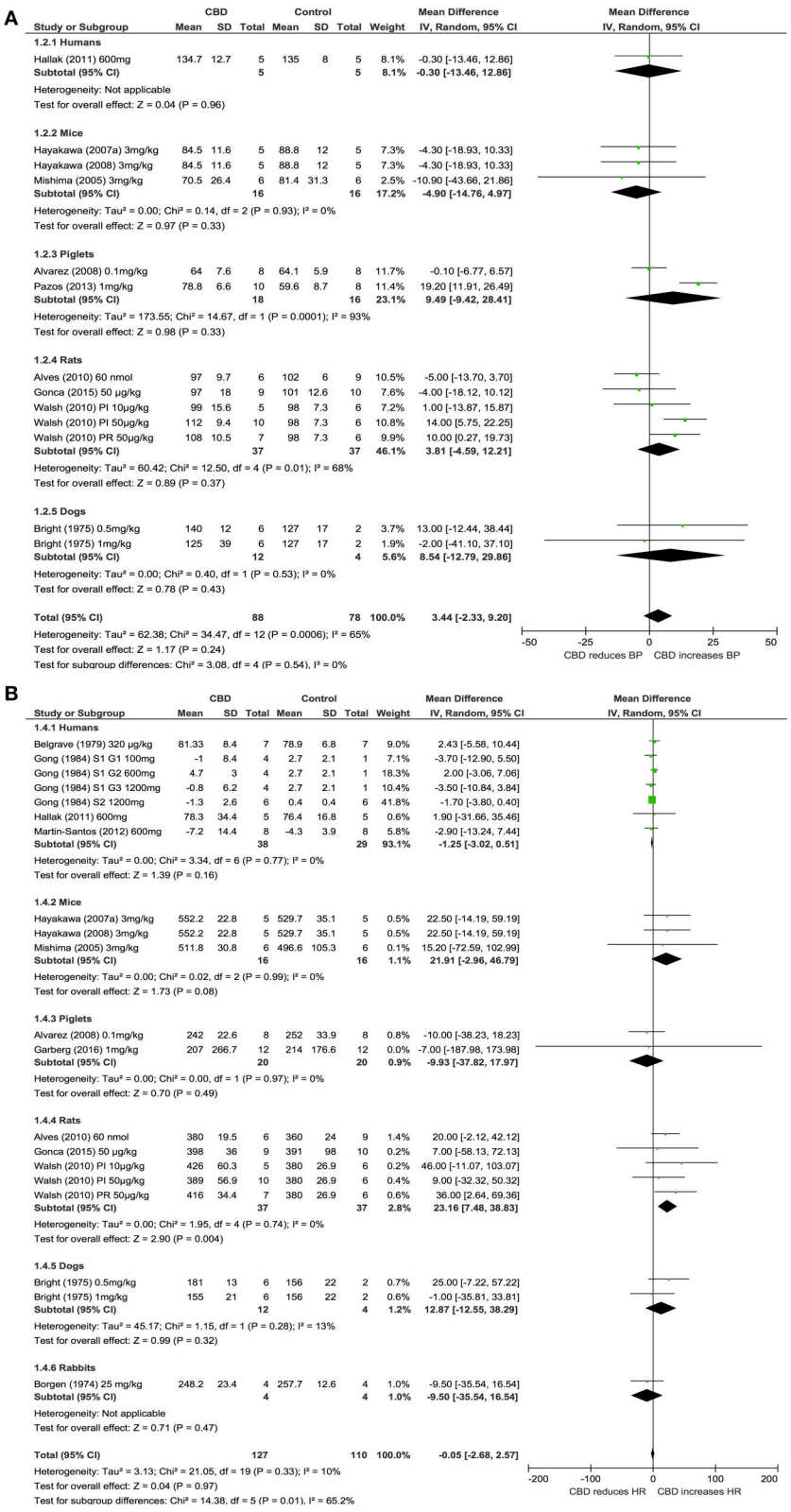

FIGURE 2 | Changes in BP (A) and HR **(B)** induced by acute CBD dosing.

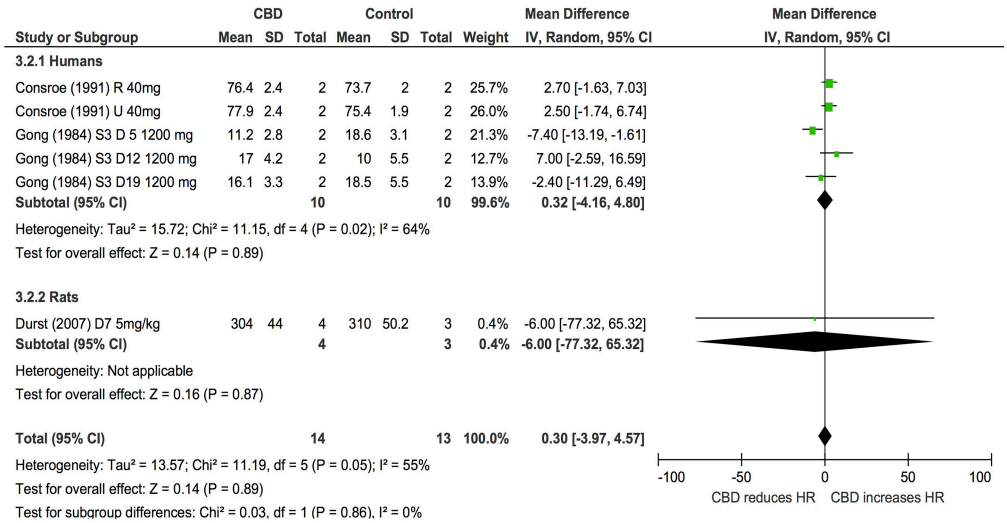

FIGURE 3 | Changes in HR induced by chronic CBD dosing.

administration of CBD (HR control $p = 0.01$; HR stress $p = 0.049$; **Figure 7**).

DISCUSSION

The aim of this study was to determine whether CBD alters haemodynamics *in vivo*. Our analysis has shown that acute and chronic dosing of CBD had no effect on BP, HR, or BF under control conditions. However, in stressful situations, CBD reduces the increase in MBP and HR observed in rats. Subgroup analysis revealed that acute CBD administration increases HR in mice and rats, and increases CBF in mouse models of stroke. Our analysis has highlighted the limited amount of human research carried out to date, and suggests that further work is required to assess the haemodynamic and regional BF impact of acute and chronic CBD administration in healthy volunteers and patients.

Overall, our meta-analysis showed that acute and chronic dosing of CBD had no effect on BP, HR, or BF under control conditions. However, there was significant heterogeneity with regards to species and model, dose and route of administration, and method and time of endpoint measurement (see **Table 1**) which makes it difficult to compare studies. It is possible that species differences may play a role. For example, Bright et al. found a significant increase in HR and BP with CBD (0.5 and 1 mg/kg) in anesthetized dogs (Bright et al., 1974, 1975), however, no changes were seen with CBD (25 mg /kg) in the HR of anesthetized rabbits (Borgen and Davis, 1974). It is also worth noting that most of the human data reviewed did not show any significant effects of CBD while significant effects were observed in animal studies. The time of cardiovascular measurements is also very important. For example, in a study that examined the cardioprotective effect of CBD (50 μg/kg i.v.) in rats showed that BP was significantly reduced post-CBD treatment compared to control two and a half hours post-ischaemia (Walsh et al., 2010). However, another study assessing CBD at the same dose and route of administration on cardiac arrhythmia (Gonca and Darici, 2015) found no change in BP, although this was only measured 11 min post-ischaemia, thus any potential later changes in haemodynamics are not reported. A greater number of homogenous studies are required to assess the haemodynamic effects of CBD under control conditions.

Our systematic review has highlighted that there are a limited number of studies examining changes in regional BF with CBD, with studies to date only examining changes in cerebral or myocardial BF. From the limited studies available, our analysis showed there were no significant changes in BF overall post-CBD administration. However, in mice and piglet models of stroke, either intraperitoneal or intravenous administration of CBD (3 mg/kg or 0.1 mg/kg, respectively) significantly reduced the infarct volume and increased the CBF after acute and chronic dosing (Mishima et al., 2005; Hayakawa et al., 2007a,b; Alvarez et al., 2008). Similarly, in a rabbit model of myocardial infarction, CBD treatment of 100 μg/kg modestly reduced the size of myocardial ischaemic injury and increased myocardial BF (Feng et al., 2015). Together, this suggests that further investigation on the effects of CBD on regional BF, particularly in pathological situations, is warranted.

There was no relationship found between the dose of CBD and the effect size. However, in conscious monkeys, toxicology studies showed that very large doses of CBD (150–300 mg/kg) caused bradycardia, and CBD doses >200 mg/kg caused heart failure and death (Rosenkrantz et al., 1981). However, it is worth noting that this would be equivalent to a dose of 14,000 mg in a 70 kg human. Intracisternal administration of CBD had no effect on the increase in BP or HR induced by acute restraint stress in rats while systemic administration did (Granjeiro et al., 2011). Similarly, no effect was seen in BP after intracerebral

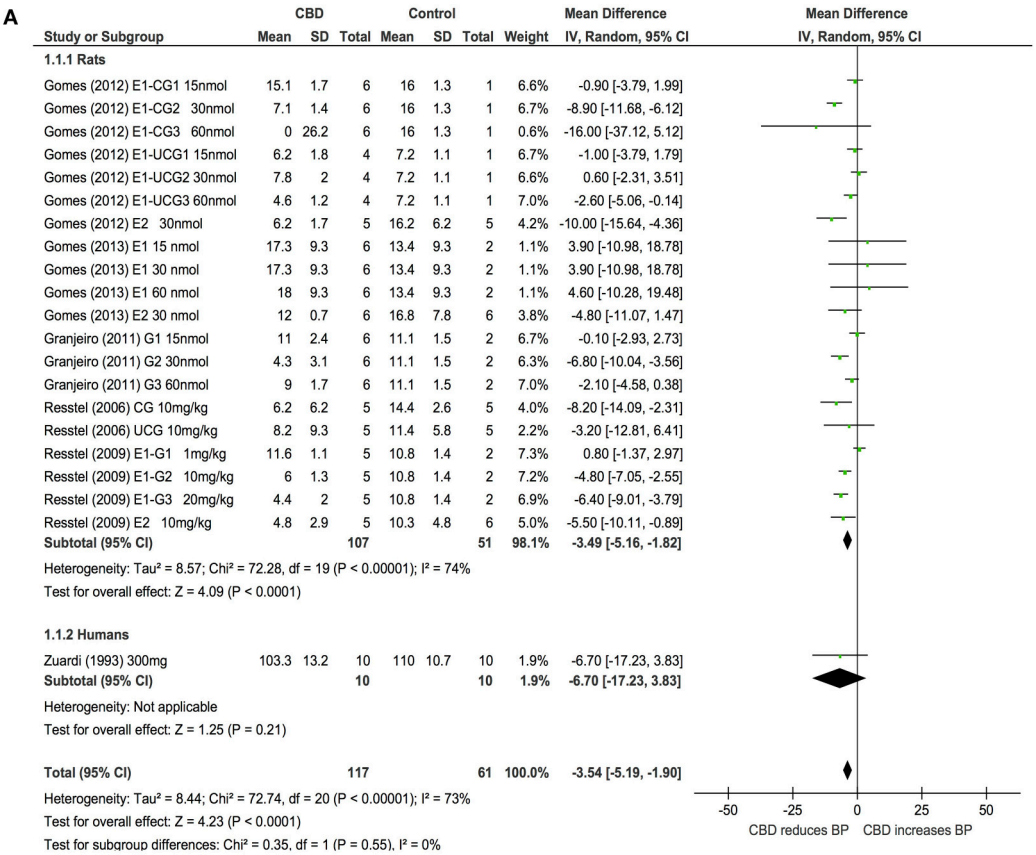

FIGURE 4 | Changes in BP (A) and HR **(B)** induced by acute CBD dosing under stressful conditions.

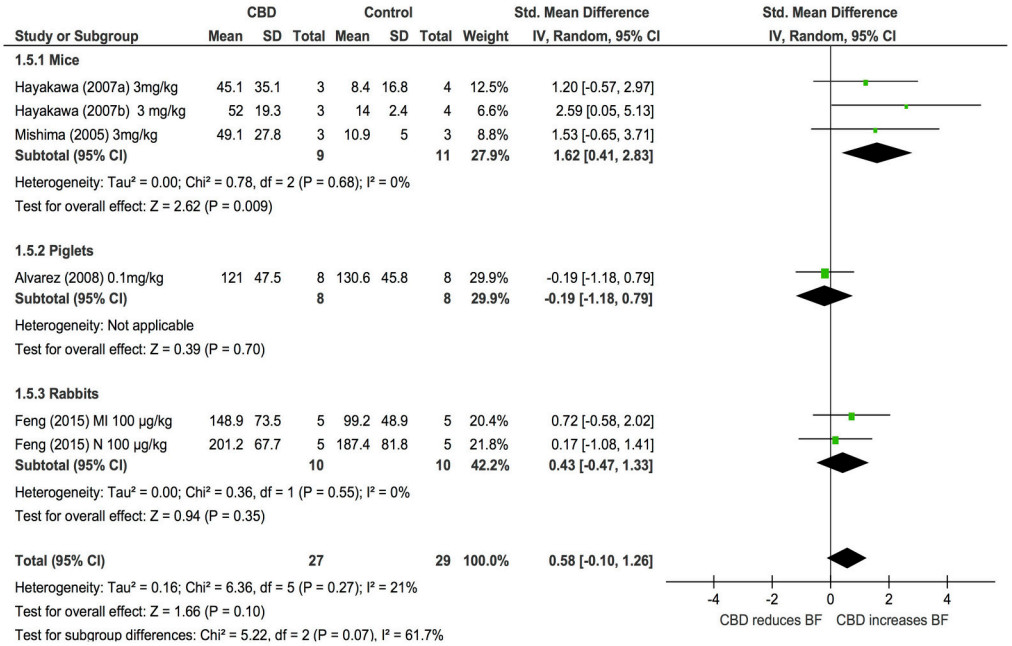

FIGURE 5 | Changes in regional blood flow induced by acute CBD dosing.

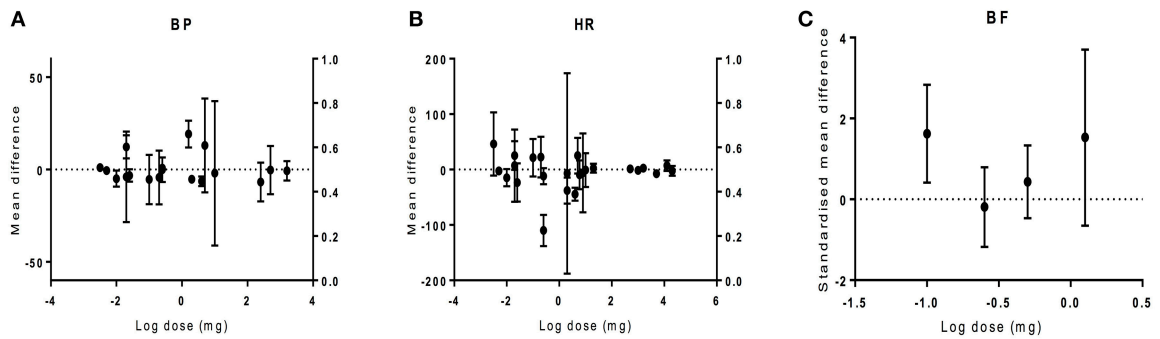

FIGURE 6 | The effect of CBD dose on haemodynamic responses *in vivo*. The mean difference (MD) in blood pressure (BP, **A**) or heart rate (HR, **B**), and standardized mean difference (SMD) in blood flow (BF, **C**) is plotted against the log dose (mg) for each study. Error bars represent 95% confidence intervals (CI).

CBD injection in rats (Gomes et al., 2013). This suggests that systemic administration of CBD is required to observe changes in haemodynamics.

Although CBD did not affect haemodynamics under control conditions, our analysis did reveal effects of CBD in pathological situations. For example, in piglet models of hypoxic injury, intravenous administration of CBD (0.1 mg/kg) maintained a stable BP after hypoxic injury compared to control animals where a reduction in BP was observed (Alvarez et al., 2008). Also, in rats conditioned to stress (i.e., restraint or fear), CBD reduced the increase in HR and MBP (Resstel et al., 2006, 2009; Gomes et al., 2012). However, in mouse models of stroke there was no significant change in MBP or HR post-CBD (Mishima et al., 2005; Hayakawa et al., 2007a, 2008). This suggests that CBD may regulate the haemodynamics when they are altered at times of stress or acute illness.

Most of the identified relevant studies were pre-clinical, and data concerning the effects of CBD on haemodynamics in humans is limited ($n = 36$ for BP, $n = 87$ for HR). A single oral dose of CBD (320 µg/kg, 1 mg/kg, 100, 300, 600 or 1200 mg) had no effect on BP or HR in healthy volunteers under control or stressful situations (Belgrave et al., 1979; Zuardi et al., 1982, 1993; Gong et al., 1984; Borgwardt et al., 2008; Bhattacharyya et al., 2009, 2010; Fusar-Poli et al., 2009; Bergamaschi et al., 2011; Hallak et al., 2011; Winton-Brown et al., 2011; Martin-Santos et al., 2012). Likewise, after repeated CBD dosing of 1200 mg or 3 mg/kg for 20 or 30 days, respectively there were no apparent effects on HR, BP, or ECG compared with other treatment groups (Cunha

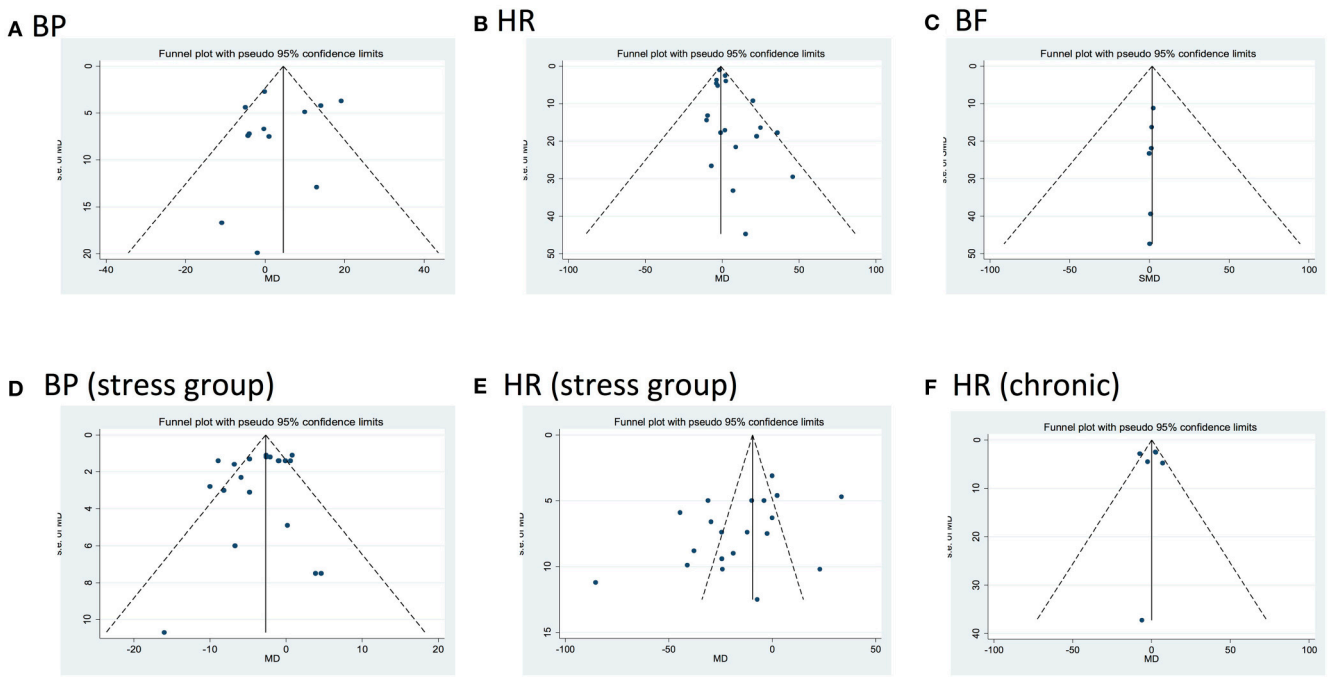

FIGURE 7 | Funnel plots for each outcome evaluating publication bias. Standard error (SE) of the mean difference (MD) in blood pressure (BP) and heart rate (HR), or of the standardized mean difference in blood flow (BF, MD, or SMD, y axes) for each study is plotted against its effect size (horizontal axes). There was significant bias in HR after acute dosing (B, HR control $p = 0.001$; E, HR stress $p = 0.049$).

et al., 1980; Gong et al., 1984). In clinical trials, chronic administration of CBD 300 mg, 10 mg/kg or 800 mg for 4½ months, 6 or 4 weeks, respectively, incurred no changes on the ECG, BP, or HR in patients of epilepsy, Huntington or schizophrenia disorders (Cunha et al., 1980; Consroe et al., 1991; Leweke et al., 2012). However, repeated oral dosing of CBD increasing from 100 to 600 mg/day over 6 weeks induced a reduction in standing BP by 10–20 mmHg in patients with dystonic movement disorders (Consroe et al., 1986). Conversely, a single dose of CBD (40 mg) given to patients with intraocular pressure increased systolic BP at 60 and 90 min post-sublingual administration (Tomida et al., 2006). Two studies of healthy volunteers and patients with social anxiety disorders showed CBD (400 mg) increased cerebral BF on the left parahippocampal and right posterior cingulate gyrus, respectively, but not in other brain regions when compared to control (Crippa et al., 2004, 2011). Bhattacharyya et al. (2010) suggested that the CBD effects on regional brain function during multi-tasking may be related to its effects on CBF (Bhattacharyya et al., 2010). Conversely, Borgwardt et al. (2008) suggested that the neural effects observed after CBD administration are unlikely to be a consequence of vascular effects, including CBF (Borgwardt et al., 2008). Together, this data would suggest that there are limited haemodynamic effects of CBD in humans, although further studies where this is the primary endpoint are warranted based on pre-clinical data reviewed in the present study.

Limitations

There are several factors that limit the interpretation of the results of these studies and the understanding of the CBD effects on haemodynamics. In general, the primary aim of the studies reviewed was not to assess the haemodynamic effects of CBD. Some studies did not include an impartial measurement of BP or HR which may lead to bias in their outcome, or did not state the method of measurement. Due to the presence of heterogeneity in publications, outcomes after acute and chronic dosing should be interpreted with caution. After acute dosing, changes in haemodynamics at 2 h or the closest time point available to 2 h post-drug were used for analysis, however, depending on the route of administration, the peak changes in plasma CBD and therefore associated cardiovascular changes, will be different. In chronic studies, the length of drug administration also varied. For the analysis of the relationship between drug dose and effect size, the total dose up to the time point in which the haemodynamic was measured was used in the analysis, this also may affect review conclusions. Only 9 out of 24 publications used randomisation and 6 reported blinding assessment of outcome, parameters that should impact on study quality. However, we found no relationship between quality and effect size. Finally, in some publications involving comparison of several doses, the number of animals per control group was divided into the number of comparison groups to avoid re-counting the same animal more than once, thus resulting in smaller sample sizes and broader estimates of the variance.

CONCLUSION

This meta-analysis and systematic review has highlighted the haemodynamic effects of CBD administration *in vivo*. The positive effects induced by CBD include maintaining the fall in BP after global hypoxia, reducing the increase in MBP and HR post-stress, and increasing BF in ischaemia-reperfusion models. It is possible that beneficial effects of CBD on haemodynamics occurs when the cardiovascular system is abnormally altered, suggesting that CBD may be used as a treatment for various cardiovascular disorders, such as hypertension, myocardial infarction and stroke. However, the findings from the reviewed studies were predominately preclinical and significant effects were only observed in animals. Data from human studies investigating the effects of CBD on haemodynamics is still very limited and we suggest that further research in humans under pathological conditions is required.

AUTHOR CONTRIBUTIONS

SO and TE: Substantial contributions to the conception or design of the work. All authors: The analysis and interpretation of data for the work; Drafting the work or revising it critically for important intellectual content; Final approval of the version to be published; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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11. General references

These references correspond to those contained in the introduction (chapter 2), general methods and principles (chapter 3), and general discussion (chapter 9), while each other chapter contains its own references therein.

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