PLASMA VOLUME IN NORMAL AND SICKLE CELL PREGNANCY

By

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ABSTRACT

Plasma volume (PV) rises by up to 50% in normal pregnancy, a phenomenon associated with a favourable pregnancy outcome. A previous study of pregnant women with sickle cell (haemoglobin SS) disorder found that PV paradoxically contracts in late pregnancy.

A cross-sectional study was performed to determine PV (Evans blue method) and volume regulatory hormones and electrolytes in pregnant women with haemoglobin (Hb) SS and in non-pregnant and Hb AA controls.

PV rose in pregnant HbAA and was significantly correlated with plasma angiotensinogen. Non-pregnant Hb SS women had supranormal PV measurements and reduced glomerular filtration rate (GFR). Their PV did not rise in pregnancy and was not correlated with angiotensinogen. Their plasma renin concentration also failed to rise significantly by 36 weeks gestation and was significantly less than in Hb AA pregnancy although aldosterone concentration was raised as expected.

A general vasoconstriction in pregnancy can cause inactivation of the renin-angiotensin system and could explain this, with aldosterone being elevated by non Angiotensin II dependent stimulation such as plasma potassium, which was significantly higher in the pregnant Hb SS women. Further studies demonstrating a deficiency of vasodilator substances in pregnant Hb SS women will strengthen this hypothesis.

ABSTRACTS OF CONFERENCE PRESENTATIONS

AFOLABI BB, OLADIPO OO, ABUDU OO, AKANMU AS, SOFOLA OA, BROUGHTON PIPKIN F. Disordered regulation of plasma volume in pregnant women with sickle cell disorder. Presented at the Society for Gynaecological Investigation International Conference in Glasgow, March, 2009.

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LIST OF ABBREVIATIONS USED IN THE THESIS

^{0}C – degrees centigrade

AII – angiotensin II

ACTH – adrenocorticotropic hormone

ADH – arginine vasopressin or antidiuretic hormone

AMP – adenosine monophosphate

ANP – atrial natriuretic peptide

Aogen – plasma angiotensinogen concentration

ATP – adenosine triphosphate

BNP – brain natriuretic peptide

Bo – maximum binding

BMI – body mass index

BP – blood pressure

BPD – diastolic blood pressure

BPS – systolic blood pressure

BSA – body surface area

cAMP – cyclic adenosine monophosphate

CG – Cockcroft-Gault

cGMP – cyclic guanosine 3'5' –monophosphate

CoV – coefficient of variation

DBP – diastolic blood pressure

dt – delay time

ECF – extracellular fluid

EDTA – ethylenediamine tetra-acetic acid

EIA – enzyme immunoassay

ELISA – enzyme-linked immunosorbent assay

fl – femtolitres

g – gram

GFR – glomerular filtration rate

Hb – haemoglobin

HbS – sickle haemoglobin

Hepsal – heparinised saline

HRP – horseradish-peroxidase

ICF – intracellular fluid

IQR – interquartile range

IU – international units

IUGR – intrauterine growth restriction

kg – kilogram

L or l – litre

L-NAME – N^{G-}nitro-L-arginine methyl ester

LUTH – Lagos University Teaching Hospital

m – metre

MDRD – ”Modification of Diet in Renal Disease”

mg – milligram

ml – millilitre

mmol – milli mole(s)

mosm – milli osmole(s)

Na^+ K^+ ATPase – sodium potassium adenosine triphosphatase

ng – nanogram

NO – nitric oxide

NSB – non-specific binding

PABA – p-aminobenzoic acid

PCV – packed cell volume

PDHT – pre-diastolic hypertension

pg – picogram

pNpp – p-NitroPhenyl Phosphate

Posmo – plasma osmolality

PP – pulse pressure

PRA – plasma renin activity

PRC – plasma renin concentration

PSHT – pre-systolic hypertension

PV – plasma volume

RAAS – renin angiotensin aldosterone system

RIA – radioimmunoassay

RISA – radio-iodine labelled serum albumin

RPF – renal plasma flow

Rpm – revolutions per minute

SBP – systolic blood pressure

SCD – sickle cell disorder

SD – standard deviation

SPSS – Statistical Package for the Social Sciences

SS – sickle cell

SST – serum separator tube

TMB – tetramethyl benzidine

TNF-α - tumour necrosis factor alpha

tt – transit time

Uosmo – urinary osmolality

Uvol – urinary volume

VLA4 – very late activation antigen 4

1. INTRODUCTION

The Cardiovascular system undergoes significant changes in pregnancy. Plasma volume is known to increase during the course of normal pregnancy and is associated with a positive pregnancy outcome (Hytten & Paintin, 1963; Pirani *et al*., 1973; Hays *et al*., 1985). Sickle cell disorder (SCD) is a haemoglobinopathy that is relatively common in Nigeria; in fact the country probably has the highest population of individuals living with the disorder worldwide (WHO, 2006a) and many afflicted women are surviving till pregnancy and beyond. In this thesis I explored plasma volume changes in pregnant Nigerian women with sickle cell disorder as well as hormones and electrolytes that are known to regulate body fluids. In doing so, I compared them with non-pregnant women with SCD and pregnant and non-pregnant women with normal haemoglobin AA. This Introduction begins by describing the context in which the study is carried out then goes on to comment on the physiology of sickle cell disorder in pregnant and non-pregnant women, starting with general then cardiovascular physiology in the non-pregnant before going further to discuss what is known to happen in pregnancy. It then explores the control of plasma volume in the non-pregnant and pregnant states.

The second chapter of my thesis is the Methods chapter where I specify and discuss the subject selection, specimen collection and processing, as well as the laboratory methods, data handling and analysis.

The third chapter details the results in non-pregnant women, starting with the demographic characteristics to the plasma volume measurements and ending with

the various hormone and electrolyte measurements and their interactions. The fourth chapter continues with the results of the pregnant sickle cell women in the same vein and also compares all the parameters of the pregnant with the non-pregnant women. I then discuss the findings of the two chapters in the fifth chapter and end the thesis with a final chapter that summarises my conclusions.

1.1. OVERVIEW AND GENERAL PHYSIOLOGY OF SICKLE CELL DISORDER

Sickle cell disorder (SCD) is an abnormality of haemoglobin that was first recognized in people of West African ancestry (Serjeant & Serjeant, 2001). Approximately 230,000 babies are born in Africa yearly with SCD (Modell & Darlison, 2008) and about 150,000 of these are born in Nigeria (WHO, 2006a). More than 50% of the children born with severe forms of the disease such as sickle cell anaemia i.e. those with haemoglobin (Hb) SS, die before the age of 5 years (WHO, 2006b) from bacterial and malarial infections. However, in recent times there has been increased survival due to increased access to hospital services particularly in Nigeria (Akinyanju *et al.*, 2005) and more girls are surviving till pregnancy and beyond.

I carried this study out in the Lagos University Teaching Hospital, in Lagos, Nigeria, a 761-bed hospital, 89 of which are obstetric and 60 gynaecological. It is one of two tertiary hospitals in Lagos state, the population of which is just under 10 million people. The ethnicity of the patients is homogenous; despite the fact that there is a mixture of tribes mostly from the South of Nigeria, where Lagos is situated, the women are all black African. Majority of the women have tertiary education although their earning power is not commensurate to their educational status as more than 60% of Nigerians live below $1 a day (Klugman, 2010) Despite the fact that the study hospital is a public one, patients still have to pay for all their services, albeit at a subsidised rate. There is a relatively new National Health Insurance Scheme in the country but it does not as yet cover deliveries or other secondary care.

Nigeria is an oil rich company but plagued with problems of infrastructure such as irregular electricity and water supply, poor roads and maintenance culture in general. The electricity supply to the hospital is also affected and is epileptic at best thus power generating sets which consume huge amounts of diesel have to be provided as back up. There is a blood bank within the hospital that prepares and provides blood for transfusion but rare blood types often have to be sourced from outside the hospital.

Another issue that requires a brief mention at this stage is that of malaria, a disease that is endemic to Nigeria. Malaria is caused by infection of the red blood cells by the protozoan genus Plasmodium, which is spread by the bite of an infected anopheles mosquito. This causes a flulike illness characterised by fever, chills, muscular aches and headache, which can be fatal especially in non-immune individuals if not promptly treated (Greenwood *et al*., 2005). In pregnancy, the acquired immunity to repeated episodes of malaria is transiently lost and it can become very severe, causing organ failure and death. Even in moderate cases, it can cause anaemia and miscarriage in the mother and low birth weight, prematurity and congenital malaria in the fetus (Duffy & Fried, 2005; Menendez *et al*., 2000). In order to prevent these complications, pregnant women are given prophylactic antimalarial therapy (Falade *et al*., 2007). Women with SCD who are more likely to suffer serious complications from malaria are given a daily antimalarial prophylactic drug – Proguanil, which is very effective (Garner & Gulmezoglu, 2006). Non-pregnant women with SCD are also asked to take Proguanil daily, as they are prone to severe morbidity and mortality from malaria as well (Ibidapo & Akinyanju, 2000). Pregnant women without SCD are

given sulphadoxine/pyrimethamine as intermittent preventive therapy, twice in the pregnancy after the first trimester at least one month apart, according to national guidelines (Health, 2010).

The genesis of SCD is as follows: A single point mutation in the 6th codon of the β globin haemoglobin subunit leads to substitution of glutamic acid for valine, resulting in the formation of 'sickle haemoglobin', or HbS. Upon deoxygenation, HbS forms hydrophobic interactions with adjacent S globins, ultimately resulting in the polymerisation of HbS (Bunn, 1997). This polymerization of HbS is the pathophysiological hallmark of SCD, leading to its various clinical manifestations (Bunn, 1997). The homozygous disease in which two S globin genes are inherited is known as sickle cell anaemia (HbSS) (Schnog *et al.*, 2004). Other genotypes that cause SCD include double heterozygous types such as HbSC and HbSβ-thalassaemia, with HbSC being the more common of the two. People who carry just one S globin gene are said to have the sickle cell trait (HbAS) and are generally asymptomatic (Serjeant & Serjeant, 2001).

Various triggers can induce this polymerization. Decrements in pH, increase in temperature (Schnog *et al.*, 2004), a high concentration of HbS in the erythrocytes and the presence of other haemoglobins are all determinants of polymerization (Schnog *et al.*, 2004). The presence of HbF and HbA2 limits the polymerization to a certain extent. HbSS individuals have about 85% of HbS but those with relatively high levels of HbF tend to have less severe disease manifestation (Bunn, 1997; Schnog *et al.*, 2004).

For this polymerisation to occur, the time taken for the red cells to traverse the circulation – transit time (tt) has to be longer than that taken for the red cells to form polymers – delay time (dt) (Bunn, 1997). Adherence of sickle cells to vascular endothelium results in intimal hyperplasia in larger vessels and causes increased tt (Hebbel *et al.*, 1980). The exact cause of this adherence is unknown but several red blood cell surface molecules, factors and proteins have been implicated (Setty *et al.*, 2002). These include red blood cell surface receptors and molecules such as the very late activation antigen 4 (VLA4) and the thrombospondin receptor CD36, and proteins such as fibrinogen and fibronectin (Joneckis *et al.*, 1996). However, because the transit time in the smaller vessels (arterioles and capillaries) is much shorter, polymers do not form in most of the cells during blood flow in the microcirculation (Mozzarelli *et al.*, 1987). Adherence to leucocytes and platelets as well as the vascular endothelial cells is now thought to contribute to the increased transit time (Frenette, 2002). Also the high white blood cell count found in most patients with sickle cell anaemia results in the production of injurious cytokines such as tumour necrosis factor – alpha (TNF-α). This together with the endothelial adherence mentioned above and a hypercoagulable state contribute to sickle cell vaso-occlusion (Hebbel *et al.*, 1980; Frenette, 2002; Ataga & Key, 2007).

The polymerisation of HbS causes the change of the affected red blood cells into stiff sickle shaped cells which then obstruct the capillaries resulting in early destruction i.e. haemolysis, and ischaemia from the resulting occlusion of the vessels. These lead to the various clinical manifestations which include clinical

jaundice and gallstones due to haemolysis, anaemia, increased bilirubin excretion and subsequent formation of pigment stones, splenic manifestations (splenic sequestration, hypersplenism), susceptibility to infections, stroke, complications in pregnancy, bone pain crises and acute chest syndrome (Serjeant & Serjeant, 2001; Schnog *et al.*, 2004). The pregnancy complications include anaemia, severe crises (the term used for the various clinical manifestations of the disease especially the vaso-occlusive type), pulmonary disease and infections and death (Rajab *et al.*, 2006; Villers *et al.*, 2008; Afolabi *et al.*, 2009). Perinatal mortality rates are higher than those for their haemoglobin AA counterparts worldwide often caused by fetal growth restriction, prematurity and intra-uterine fetal death (Villers *et al.*, 2008; Al Jama *et al.*, 2009; Barfield *et al.*, 2010).

Life expectancy and disease manifestation also vary with environmental and socioeconomic factors. There is a high early mortality in sub-saharan Africa due to malaria, malnutrition and infections and the average survival is less than 5 years (Serjeant & Serjeant, 2001). However, with improving medical conditions and early diagnosis, many affected individuals are surviving to the age of 40 and beyond. In areas with more developed public health and services, average survival is better. The Cooperative study of sickle cell disease, which was carried out in the USA, showed a median survival for SS disease at 42 years for males and 48 years for females (Platt *et al.*, 1994). A study carried out in Jamaica reporting on 3301 people attending a dedicated sickle cell clinic between 1987 and 1996 found a median survival of 53 years for men and 58.5 years for women (Wierenga *et al.*, 2001).

An additional reason for differences in survival between individuals with SCD is the variability in severity of clinical and haematological factors of the disease in the different genotypes, with a minority having few complications, particularly those with haemoglobin SC, and the rest with intermediate to severe complications, the latter being more common with haemoglobin SS individuals (Kato *et al.*, 2007).

The most common cause of death varies between different environments. In a report of 241 Jamaican SS individuals, acute chest syndrome (a syndrome consisting of chest pain, fever, cough and dyspnoea with abnormal radiological chest signs), acute splenic sequestration (a condition in which the spleen enlarges significantly and traps a large amount of red blood cells causing a profound anaemia), renal failure and meningitis were the most common causes of death (Thomas *et al.*, 1982). In sub-Saharan Africa, other causes such as malaria, bacterial infections, malnutrition and sudden death during pregnancy have been reported (Athale & Chintu, 1994; Serjeant & Serjeant, 2001; Van-Dunem *et al.*, 2007).

1.1.1. Cardiovascular physiology

One of the consequences of haemolytic anaemia in HbSS individuals is a hyperdynamic circulation and an expanded plasma volume as a result of an attempt to compensate for the reduced oxygen carrying capacity (Schnog *et al.*, 2004). Cardiac output was reported to be about 50% above normal in steady state HbSS individuals with a haemoglobin concentration of 6 – 8 g/dl (Lindsay *et al.*, 1974). Heart rate has been found to be higher at rest in sickle cell anaemia patients (Balfour *et al.*, 1984) but the contribution of heart rate to the increase in

cardiac output is minimal; the main cause is an increase in stroke volume (Covitz *et al.*, 1995). It is thus expected that their heart chambers are dilated and this has been found in several studies (Gerry *et al.*, 1976; Rees *et al.*, 1978; Balfour *et al.*, 1984). In a prospective, multicentre study of 191 sickle cell subjects with blinded echocardiography readings, all the heart chambers were found enlarged and all except the right ventricle had dimensions that were inversely proportional to haemoglobin concentration (Covitz *et al.*, 1995). However, contrary to other findings (Rees *et al.*, 1978; Denenberg *et al.*, 1983), left ventricular contractility was found to be normal in most patients suggesting that the individual with SCD has a dilated heart but normal left ventricular function (Covitz *et al.*, 1995). This difference could be because the numbers studied were fewer, 44 in one study (Rees *et al.*, 1978) and 11 in the other (Denenberg *et al.*, 1983) respectively than in Covitz *et al*, and patient selection was unbalanced with more severely affected patients being included in the studies which found abnormal left ventricular function.

1.1.1.1. Plasma volume

As mentioned above, plasma volume is supranormal in individuals with sickle cell disorder in the steady state (Barreras *et al.*, 1966; Steinberg *et al.*, 1977; Hatch *et al.*, 1989). Several reports have found a decrease during crises (Jenkins *et al.*, 1956; Erlandson *et al.*, 1960; Barreras *et al.*, 1966) although one Jamaican study (Wilson & Alleyne, 1976) found no significant change in plasma volume during the painful crisis. An explanation for this may be the differences in severity or duration of the crises at the time of plasma volume estimation (Serjeant & Serjeant, 2001).

The mechanism of the plasma volume expansion is not known. Despite the fact that most anaemias are associated with some degree of expansion of plasma volume (Serjeant & Serjeant, 2001), that in SCD exceeds that found in other haemolytic anaemias with similar haematocrits (Erlandson *et al.*, 1960; Steinberg *et al.*, 1977). It is thought that the extra volume expansion could be an adaptation in order to reduce the blood viscosity found in them (Serjeant & Serjeant, 2001). The regulation of plasma volume depends on the balance of fluids between the extracellular and the intracellular compartments, which in turn is dependent on osmotic forces exerted by plasma proteins and electrolytes and the hydrostatic forces (Jordan & Marshall, 1995).

Plasma volume is particularly dependent on the quantity of sodium in the body (Ganong, 2005) thus dietary intake and excretion of sodium is important. All the hormones that affect sodium regulation (e.g. renin-angiotensin-aldosterone system, progesterone and prolactin) also control plasma volume (Ganong, 2005) and will be discussed in detail in the section on the control of plasma volume below. In addition, although a lot of fluid may be lost from the skin in hot climates, most of the fluid lost from the body is by excretion through the kidneys (Jordan & Marshall, 1995) thus renal function greatly affects plasma volume regulation. Finally, ingestion of water inspired by thirst is another factor that can affect plasma volume and this brings osmolality and the hormones that control it such as vasopressin into play.

1.1.2. Renal physiology

1.1.2.1. Haemodynamics

The kidneys function to filter a plasma-like fluid through the glomerular capillaries into the renal tubules (Ganong, 2005) in a process known as glomerular filtration. In a normal adult the glomerular filtration rate (GFR) is about 120-130 ml of fluid per minute. In children with SCD, renal hemodynamic parameters are generally supranormal (Addae, 1975; Ataga & Orringer, 2000). The renal plasma flow (RPF), blood flow and GFR have been found to be increased in young SCD patients (Etteldorf *et al.*, 1952; Allon *et al.*, 1988) but fall with increasing age. However, although these parameters appear to be influenced by age, the age at which the decline sets in is not certain. Previous studies found RPF and GFR declined to normal levels towards adolescence (Etteldorf *et al.*, 1952; Etteldorf *et al.*, 1955; Hatch *et al.*, 1970) but a more recent study found that GFR, RPF and blood flow were still supranormal at a median age of 20.8. (Thompson *et al.*, 2007) and other authors found that although GFR (measured by creatinine clearance) tended to decline below normal over the age of 40, there was significant variability and two patients over the age of 60 had creatinine clearance of 120 ml/min/1.73m^2 or more (Morgan & Serjeant, 1981). It is difficult to determine the timing of the decline in GFR as it may be influenced by the frequency and severity of crises (Addae, 1975). GFR has been reported as reduced during crises (Addae, 1975).

The cause of the supranormal GFR in children and young adults is not known. The GFR in many other types of anaemia is either normal or reduced (Addae, 1975). Also correction of the anaemia has not been shown to alter the GFR in

short-term studies (Keitel *et al.*, 1956; Statius van Eps *et al.*, 1967). It has been postulated that the increased renal haemodynamic parameters are as a result of increased prostaglandin synthesis in the kidneys of SCD individuals (de Jong *et al.*, 1980). The use of indomethacin, a prostaglandin inhibitor, resulted in significant falls in GFR and RPF in them compared with normal controls, despite having high or normal values at the beginning of the experiment (de Jong *et al.*, 1980; Allon *et al.*, 1988). It is thought that the high prostaglandin synthesis in them could be as a result of renal medullary ischaemia due to sickling (Serjeant & Serjeant, 2001). The low oxygen tension, acidic pH and hypertonicity found in the renal medulla are conducive to sickling, resulting in vaso-occlusion within the vasa recta (Statius van Eps *et al.*, 1970a). This hypothesis has been extended to explain the decline in renal function with age in SCD individuals. The authors of a review of the pathophysiology of sickle cell nephropathy suggest that the prostaglandin mediated glomerular hyperfiltration may eventually promote the occurrence of glomerulosclerosis, leading to a decline in renal function and ultimately renal failure (de Jong & Statius van Eps, 1985). The proteinuria that is frequently seen in these individuals may be as a result of this glomerulosclerosis.

Nitric oxide may also play a role in the increased GFR as increased levels of nitric oxide synthases have been found in transgenic mice with high levels of haemoglobin S, compared with normal control mice. The transgenic HbS mice also demonstrated an increased GFR compared to the control mice (Bank *et al.*, 1996).

1.1.2.2. Hyposthenuria

Another major renal manifestation of SCD is hyposthenuria. Hyposthenuria, an inability to concentrate urine maximally, is the most widely known renal abnormality in sickle cell disease patients (Addae, 1975; Ataga & Orringer, 2000). The urine is both profuse in volume and dilute, resulting in a significantly lower osmolality than in normal controls after an 8-10 hour water deprivation (Allon *et al.*, 1988). The administration of vasopressin does not improve urine concentration in them thus it is not thought to be due to a deficiency of this hormone (Statius van Eps *et al.*, 1970b; De Jong *et al.*, 1982), but could be due to down-regulation of the V2 receptors as has been shown in nephrectomised rats (Teitelbaum & McGuinness, 1995). Although blood transfusion has been shown to reverse the concentration defect in younger SCD individuals, the effect is less after the age of 15 (Statius van Eps *et al.*, 1970b). Another reason why anaemia alone is not the likely cause of the hyposthenuria is that it does not occur in other chronic anaemias and it still occurs with normal haemoglobin levels in both HbSC and HbAS individuals (Serjeant & Serjeant, 2001). As hyposthenuria has been noted to develop in a normal donor kidney following its transplantation into a patient with SCD (Spector *et al.*, 1978), it can be surmised that the defect is likely due to a sickling related pathology in the kidneys. The current thinking is that the destruction of the vasa rectae system in SCD individuals leads to a loss of the deep juxtamedullary nephrons that are necessary for maximal urine concentration (Ataga & Orringer, 2000; Serjeant & Serjeant, 2001).

1.1.2.3. Water balance

As the outer medulla is relatively spared, individuals with SCD are capable of concentrating their urine to the required extent in the steady state (Statius van Eps *et al.*, 1970a). In the steady state, they also excrete large volumes of urine and consequently drink large amounts of fluid (Saxena *et al.*, 1966; Addae & Konotey-Ahulu, 1971). However, during crises and water deprivation states, they are known to often be in negative water balance (Addae, 1975). The urine volume is reduced from their usual polyuric state but it remains dilute (Hatch & Diggs, 1965). It is thought that the relative oliguria seen in crisis states may be due to the dehydration that occurs before crises (Hatch & Diggs, 1965).

Sodium excretion in steady state sickle cell patients has been found to be similar to (Hatch *et al.*, 1989; Olowu *et al.*, 1995) or less than (Addae & Konotey-Ahulu, 1971) their haemoglobin AA counterparts. This is likely to be an important defence against their tendency to being dehydrated as sodium conservation is linked with the maintenance of an adequate, in their case often supranormal intravascular volume (Addae, 1975). A study that found high urinary sodium losses and hyponatraemia in a group of sickle cell patients, actually studied non-steady state sickle cell disease children (Radel *et al.*, 1976). Urinary potassium excretion was however found to be reduced in 6 individuals with SCD when compared with 5 age-matched healthy controls after receiving intravenous potassium chloride infusion (DeFronzo *et al.*, 1979). This was despite normal plasma renin activity (PRA) and aldosterone levels and it has been corroborated in other studies of individuals with SCD (Batlle *et al.*, 1982; Yoshino *et al.*, 1982). It is thought to be due to the ischemia caused by vasa

recta occlusion from the sickling process, which affects the distal tubules more despite overall renal function still being adequate (DeFronzo *et al.*, 1979).

There are varying reports on PRA in SCD individuals. It appears that PRA and aldosterone concentration are generally normal in the steady state (DeFronzo *et al.*, 1979; de Jong *et al.*, 1980) although high levels have also been reported (Matustik, 1979). The study reported by Matustik (Matustik, 1979) was in a much younger group (ages 6-20) whilst that reported by de Jong (de Jong, 1980) had an older age range (14-63, mean 28 years), and that by DeFronzo (DeFronzo *et al.*, 1979) also had older subjects (19-37, mean 25). As there was no clinical evidence of hyper-activity of the RAAS in the subjects studied by Matustik (Matustik, 1979), a possible explanation of this finding is that the high levels found may be a compensatory mechanism in order to conserve sodium in these individuals.

1.1.3. Physiology in pregnancy

There is limited data available on the physiology, especially cardiovascular, of SCD in pregnancy perhaps due to the infrequency of this condition in affected individuals in the past, as well as the high incidence of maternal mortality in them (Serjeant *et al.*, 2004; Rajab *et al.*, 2006). In a study comparing 15 women with SCD in their third trimester of pregnancy with 40 AA women, cardiac output was found to be significantly higher in the SCD women and their left ventricular systolic function was found to be normal (Veille & Hanson, 1994), similar to findings in the large study of non pregnant subjects mentioned in section 1.1.1 above (Covitz *et al.*, 1995). As expected, the ventricular heart chambers were also found to be enlarged in the SCD women (Veille & Hanson, 1994) as they were in the non-pregnant subjects.

It would be interesting to compare pregnant SCD with non-pregnant to discover whether the heart chambers are more enlarged in pregnancy and if the enlargement is a reversible one i.e. if they revert to previous sizes post-partum. The clinical significance of this could also be examined by observing if pregnancy increases the risk of SCD women for future cardiovascular disease when compared to those who were never pregnant.

Another study comparing plasma volume in pregnant and non-pregnant SCD and HbAA women found a decrease in plasma volume at 36 weeks gestation in the SCD women compared with their 16 week values, and a 17% increase over non-pregnant HbSS females as opposed to a 68% increase at 36 weeks gestation in the HbAA women over values from non-pregnant HbAA women (Abudu & Sofola, 1988). Low aldosterone levels were found in three pregnant haemoglobin SS patients in their third trimester but the significance was not certain, as dietary and sodium excretion parameters were not evaluated and the numbers were small (Lindheimer *et al.*, 1987). If this finding is consistent, it could mean that the activity of the renin angiotensin aldosterone system (RAAS) is reduced in HbSS pregnant women and could explain the contracted plasma volume reported. The examination of the other hormones and electrolytes discussed in section 1.2 below, as well as renal water handling would also be useful.

1.2. CONTROL OF PLASMA VOLUME IN PREGNANCY

Plasma is the fluid component of blood and suspended in it are the formed elements – the red cells (erythrocytes), white cells (leukocytes) and platelets (thrombocytes). On centrifuging a blood sample, the formed elements fall to the bottom of the tube and the plasma is seen above as a clear yellow liquid above them (Pocock, 2004). Before I discuss the plasma volume (PV) in pregnancy, I must first of all mention the components of plasma and the control of its volume in the non-pregnant state.

The body has three fluid compartments – the intracellular fluid (ICF) within the cells, and the extracellular fluid (ECF) outside the cells, which are separated by the barrier of the cell membrane. The ECF is further divided into interstitial fluid and plasma. The distribution of water amongst these compartments is that the ICF contains about 67% of the body's water while the ECF contains 33%. This 33% is further split between the interstitial fluid (75% of ECF water) and plasma (25%), so that the plasma volume is <10% of the total (Silverthorn, 2007). Despite accounting for a small proportion of total body water, plasma is a very important compartment as it contains a large number of components and is responsible for many essential bodily functions. The functions of plasma include the transport of nutrients, waste products, hormones and gases, maintenance of acid-base balance and body temperature, defence due to its carriage of antibodies and antitoxins, haemostasis and maintenance of blood pressure (Pallister, 1994; Frenkel, 2006; Silverthorn, 2007; Stanfield, 2008).

As only highly permeable capillary membranes separate plasma and interstitial fluid, their ionic composition is similar, the main difference being the higher concentration

of proteins in plasma. This is because the capillary membranes have a low permeability to the plasma proteins (Guyton, 2006; Stanfield, 2008).

1.2.1. Components of plasma

The plasma volume in normal adults is about 35 – 45 ml per kilogram body weight. The PV when corrected for height and body surface area is significantly higher in males than females (Boer, 1984) and it accounts for about 4% of body weight. In general, water constitutes about 50% of total body weight in females of age 17 to 39, and about 60% in males of the same age group (Silverthorn, 2007). Apart from water, plasma is made of mineral and non-mineral ions such as sodium, potassium, calcium, chloride, bicarbonate, inorganic phosphate, magnesium and hydrogen. Small organic molecules such as amino acids, fatty acids and glucose, cholesterol and plasma proteins such as albumin, α-, β-, and γ- globulins, fibrinogen, prothrombin and transferrin are also plasma components (Pocock, 2004). Albumins provide most (~70%) of the colloid osmotic pressure (oncotic pressure) that regulates the passage of water and solutes through the capillaries and are thus important in body fluid balance and help to regulate plasma volume. This is because the capillary membranes are impermeable to albumin, which exerts a force of about 25mm Hg and pulls water into the blood (Pocock, 2004).

The chief inorganic cation of plasma is sodium with a concentration of 135-145 mmol/L. Other cations of much lower concentration are potassium (3.5 -5.0 mmol/l), calcium (2.1 – 2.6 mmol/l), magnesium, iron and trace metals. The latter are important in enzyme activity. Chloride is the principal anion (100 – 106 mmol/L) and plasma is neutralized by the presence of other anions such as

bicarbonate (24 – 30 mmol/l), phosphate, sulphate, protein and organic anions. The ions maintain the osmolality (280 – 300 mOsm/kg water) and pH of plasma (7.35 – 7.45), particularly bicarbonate and phosphate, within physiological limits (Pocock, 2004). Sodium is the most important determinant of plasma volume because of its effect on plasma osmolarity and will be discussed further below. Osmolarity is a measure of the concentration of biological solutions and is the number of particles per litre of solution i.e. osmoles per litre. Osmolality, on the other hand, is concentration expressed in osmoles per kilogram of water. As biological solutions are dilute, e.g. plasma, and very little of their weight comes from solute, both terms are used interchangeably (Silverthorn, 2007). Osmolality is used more in clinical situations as it is more accurately measured.

1.2.2. Factors affecting plasma volume regulation

Plasma volume is a dynamic quantity that can vary under physiological conditions. Its regulation depends on the balance of fluids between the extracellular and the intracellular compartments, which in turn is dependent on osmotic forces exerted by plasma proteins and electrolytes and hydrostatic forces (Jordan, 1995).

1.2.2.1. Sodium

The amount of sodium (Na^+) in the ECF is the most important determinant of ECF volume because sodium and chloride are the most abundant osmotically active solutes in the ECF and changes in chloride are mostly secondary to changes in sodium (Ganong, 2005). The equilibrium between the ECF and plasma volume is determined by Starling forces (i.e. the forces governing the movement of fluid across capillary walls which include capillary and oncotic pressure) so any change in total body sodium will affect both the ECF and the plasma volume (Pocock, 2004).

In healthy individuals, the effective circulatory volume is constant and sodium and water loss are balanced by dietary intake. There is an appetite for salt that is mainly regulated according to need. However, tastes differ and some people ingest salt in excess of what they need. The stimulus for water intake is thirst, which may be defined as an appetite for water (Pocock, 2004).

Sodium excretion is controlled by several mechanisms. The glomerular filtration rate (GFR) and tubular reabsorption are thought to be the major determinants of sodium and water regulation in the body (Schrier & Niederberger, 1994). About 96% of filtered sodium is reabsorbed into the ECF (Ganong, 2005). In the late distal tubules and the cortical collecting tubules, sodium is re-absorbed according to the needs of the body (Pocock, 2004). This is in exchange for potassium or hydrogen that are secreted into the tubular lumen and occurs in specialized cells known as the principal cells of these tubules (Silverthorn, 2007;Guyton, 2006) through the activity of the sodium potassium adenosine triphosphatase (Na^+ K^+ ATPase) pump. The early part of the distal tubule reabsorbs sodium and chloride ions via a symporter,

which is a membrane protein that transports 2 or more substances simultaneously (Pocock, 2004). When ECF decreases, the blood pressure falls leading to a reduction in glomerular capillary pressure and consequently, GFR. This reduces the amount of sodium filtered by the kidneys (Ganong, 2005).

The factors affecting sodium reabsorption include the circulating level of aldosterone and other adrenocortical hormones, atrial natriuretic peptide (ANP) and other natriuretic hormones and the rate of tubular secretion of H^+ and K^+ (Ganong, 2005). Plasma volume contraction can also occur via salt and water shift into the interstitial space and the lymphatic system, and a diuresis. ANP and endothelin play a central role in this transcapillary efflux (Zimmerman *et al.*, 1990; Zimmerman *et al.*, 1992).

1.2.2.2. Effects of adrenocortical steroids on sodium regulation

Aldosterone is a mineralocorticoid i.e. a steroid hormone with predominant effects on sodium and potassium excretion (Ganong, 2005). It increases the tubular reabsorption of sodium in the late distal and cortical collecting tubules as mentioned above, together with chloride and in association with the secretion of potassium ions, or hydrogen ions when total body potassium concentration is low.

As well as primary hyperaldosteronism, conditions that increase aldosterone secretion include reduction of dietary intake of salt, a decrease in the ECF, increase in dietary potassium, standing, and secondary hyperaldosteronism e.g. some cases of congestive cardiac failure and cirrhosis. Others include surgery, anxiety, physical trauma and haemorrhage (Ganong, 2005). The actual regulatory factors involved

include adrenocorticotropic hormone (ACTH) from the pituitary, renin from the kidney via Angiotensin II (AII) and a direct stimulatory effect of a rise in potassium ions on the adrenal cortex.

Desoxycorticosterone is also a mineralocorticoid but has only 3% of the mineralocorticoid activity of aldosterone (Ganong, 2005). Its effects on sodium excretion are therefore usually negligible (Ganong, 2005). Cortisol is a glucocorticoid i.e. a steroid hormone with predominant effects on glucose and protein metabolism. It possesses weak mineralocorticoid activity but plasma levels are much higher than those of aldosterone (Ganong, 2005) and it is thus potentially important. However, in the kidney, its mineralocorticoid actions are regulated by the action of the enzyme 11-β-hydroxysteroid dehydrogenase, which inactivates circulating cortisol by conversion to cortisone (Ganong, 2005).

1.2.2.3. Effects of Angiotensin II (AII) and renin

AII is formed in the body from angiotensin I (AI) through the action of renin on circulating angiotensinogen. Renin is secreted from the juxtaglomerular cells that surround the renal afferent arterioles as they enter the glomeruli and aldosterone secretion is regulated via the increase in AII in a feedback fashion (Ganong, 2005). A very recent paper reports that AII influences expression of a group of genes which themselves modulate the transcription of the two end-point genes affecting aldosterone synthesis, 11β-hydroxysteroid dehydrogenase and aldosterone synthase (Romero *et al*., 2007).

A drop in extracellular fluid volume leads to a reflex decrease in renal nerve discharge and a fall in renal arterial pressure. Both these changes increase the secretion of renin, which in turn normally increases AII and the rate of secretion of aldosterone. Aldosterone then causes sodium and water retention, expanding ECF volume and shutting off the initial stimulus that caused the increased renin secretion (Ganong, 2005).

AII also acts directly on the proximal nephron to increase the reabsorption of sodium (Navar, 1999). AII receptors are located on both the proximal and the distal nephron segments but it is those of the proximal segment that have been studied most extensively (Navar, 1999). These dual effects of AII, i.e. increase in aldosterone and direct effect on proximal tubules, act synergistically to enhance sodium reabsorption in the renal tubules (Navar, 1999).

Dietary sodium restriction also increases aldosterone secretion via the RAAS probably due to a reflex increase in the activity of the renal nerves. This is thought to occur because aldosterone and renin release are increased when there is reduced dietary sodium intake before any consistent fall in blood pressure takes place (Ganong, 2005).

1.2.2.4. Effect of ACTH

ACTH stimulates the output of aldosterone as well as that of glucocorticoids and sex hormones, when first administered. ACTH appears to prime the glomerulosa cells for the action of AII. It increases aldosterone secretion by binding to the glomerulosa cell-surface melanocortin-2 receptor, by activating adenylate cyclase and increasing intracellular cyclic adenosine monophosphate (cAMP) (Arai, 2007). However, the effect is transient and even if ACTH levels remain high, aldosterone output declines in 1 – 2 days (Ganong, 2005).

1.2.2.5. Effects of electrolytes

An acute decline of plasma sodium of about 20 mmol/L stimulates aldosterone secretion although such large changes are rare. However, increases in plasma potassium of just 1 mmol/L (a 20 – 25% rise) are enough to stimulate aldosterone secretion and a meal rich in potassium e.g. bananas, tomatoes and spinach can cause this. Potassium acts on the steroid biosynthetic pathway to increase the production of aldosterone. Low dietary potassium also decreases the sensitivity of the AII producing areas of the kidney to a low sodium diet (Ganong, 2005).

1.2.2.6. Arginine Vasopressin (AVP)

Apart from the sodium control of extracellular volume, there is also volume control of water excretion (Ganong, 2005). A rise in ECF volume inhibits AVP secretion while a decline in ECF increases AVP secretion. AVP is also increased by an increase in osmotic pressure but volume stimuli override the osmotic stimulation of AVP secretion (Ganong, 2005).

AVP increases the permeability of the collecting ducts of the kidney so that water enters the interstitium of the renal pyramids. The urine thus becomes concentrated and its volume decreases thus it is also called the antidiuretic hormone. Water is thus retained when there is an increase in the secretion of AVP. Other factors that increase AVP include pain, emotion, surgical stress and exercise, nausea and vomiting, standing, clofibrate and carbamazepine, and angiotensin II (Ganong, 2005).

1.2.2.7. Osmoreceptors

The state of water balance is monitored by the osmoreceptors of the anterior hypothalamus, which regulate the amount of AVP secreted by the posterior pituitary, increasing it during dehydration and decreasing it during a water load. When osmolarity rises by about 4 mOsm/l the desire to drink is stimulated (Pocock, 2004). Thus the osmoreceptors influence water balance in two ways – via AVP and by inducing thirst. This mechanism is triggered by the osmolality of the body fluids (Pocock, 2004).

Angiotensin II is a direct dipsogen i.e. it induces thirst and promotes the ingestion of fluids. It does this centrally by acting on the subfornical organ, a specialized receptor area in the diencephalons, to stimulate the neural areas concerned with thirst (Ganong, 2005). It is thought that it may also act on the organum vasculosum of the lamina terminalis (Ganong, 2005). However, angiotensin-blocking drugs do not completely block the thirst response to hypovolaemia. Thus baroreceptors in the heart and blood vessels may also be involved in this. On the other hand, fluid

volume per se is regulated by adjustment of sodium intake and excretion. Thus osmolality and fluid volume are regulated independently of each other.

1.2.2.8. Prolactin and dopamine

Prolactin has also been reported to be implicated in osmoregulation by a possible effect on renal retention of water, sodium and potassium (Campbell, 1982;Cowie, 1973). This has apparently been documented in sheep and not humans. However, water retention and release of vasopressin and oxytocin have been documented in hyperprolactinaemic women (Laczi, 1998). The water retention may be due to increased renal retention of sodium and chloride as seen in other mammals (Horrobin, 1980). Alternatively, the secretion of vasopressin and oxytocin may also be implicated (Laczi, 1998). More recent studies in rats found prolactin to be a natriuretic and diuretic hormone that acts by inhibiting proximal convoluted tubule sodium potassium adenosine triphosphatase (Na^+ K^+ ATPase) activity, achieving its full effect by interacting with the intrarenal dopamine system (Ibarra *et al.*, 2005). Dopamine is also involved in osmoregulation and is thought to act as an intrarenal natriuretic agent, which acts by inhibiting the action of Na^+ K^+ ATPase (Hansell & Fasching, 1991; Eklof *et al.*, 1997).

1.2.2.9. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP)

These two hormones are peptides that are secreted by the heart. C-type natriuretic peptide is also a member of this family but very little of it is present in the heart and circulation and it appears to be primarily a paracrine mediator. They are released when there is a stretch on the atrial myocytes caused by expansion of ECF.

ANP and BNP act on the kidneys to increase sodium excretion by apparently dilating afferent arterioles and relaxing mesangial cells, thus increasing glomerular filtration. They also act on the proximal collecting tubules and cortical collecting ducts of the kidney to inhibit sodium reabsorption. Other actions include an increase in capillary permeability, relaxation of smooth muscle in arterioles and venules and inhibition of renin secretion. When the plasma volume is expanded due to an increase in total body sodium, the action of renin is inhibited by ANP (Pocock, 2004). ANP also decreases the responsiveness of the zona glomerulosa to AII thus reducing aldosterone secretion (Ganong, 2005) and consequently inhibiting sodium chloride uptake from the distal tubules and collecting ducts of the kidneys (Pocock, 2004). All this ultimately reduces the ECF and blood pressure (Sabrane *et al.*, 2005). The overall action of these peptides is to counter increases in blood volume caused by the RAAS.

1.2.2.10. Nitric oxide (NO) pathways

NO pathways have been found to be involved in the regulation of plasma volume, with chronic inhibition causing a decrease in plasma volume in rats injected with N^{G-}nitro-L-arginine methyl ester (L-NAME) (Filep & Foldes-Filep, 1993; Balaszczuk *et al.*, 2002). NO is locally generated and has a very short half-life so it plays a role in the moment-to-moment homeostatic control of renal sodium excretion and extracellular fluid volume.

1.2.3. Pregnancy

The control of plasma volume in pregnancy is a fascinating area, involving the interplay of most of the substances mentioned above. Plasma volume rises by up to 50% in pregnancy and this rise has been shown to begin as early as 6 weeks after the last menstrual period (Whittaker & Lind, 1993; Bernstein *et al.*, 2001). One of the earliest cardiovascular changes in pregnancy is peripheral arterial vasodilation, which is thought to trigger haemodynamic and hormonal responses leading to sodium and water retention and subsequently, plasma and extracellular fluid volume expansion (Schrier & Niederberger, 1994).

The initiating factor for plasma volume expansion in pregnancy is now thought to be the early peripheral arterial vasodilation (Schrier & Niederberger, 1994). Arguments for this include the fact that systolic and diastolic blood pressures fall during the first trimester of pregnancy, despite an increase in blood volume (Davison, 1987; Duvekot *et al*., 1993; Chapman e*t al*., 1998). Also activity of the RAAS increases in pregnancy (Weinberger *et al.*, 1976; Wilson *et al.*, 1980; August *et al.*, 1990) and this is noticed from as early as the 6^{th} week of gestation (Chapman *et al.*, 1998). This supports an arterial vasodilation stimulus as opposed to a primary volume expansion one as the RAAS usually kicks in when there is a reduction in peripheral vascular resistance (Ganong, 2005).

Since similar cardiovascular changes to those in pregnancy, i.e. reduction in arterial pressure and peripheral resistance and activation of the RAAS, occur in the luteal phase of the menstrual cycle, it is likely that the hormonal stimulus responsible for the overall decrease in vascular tone is also present in the luteal phase of the

menstrual cycle (Chapman *et al.*, 1997). Likely candidates are oestradiol, progesterone, prostacyclin and nitric oxide. Indeed it may be due to the interaction of some of these substances. Plasma oestradiol, which is increased in both the luteal phase of the menstrual cycle and in pregnancy, has been shown to increase local prostacyclin production and to increase nitric oxide synthase production (Weiner *et al.*, 1994) which cause vasodilation. Supporting this is the finding that oestradiol levels appear to be reduced in pre-eclamptic pregnancies, a condition in which plasma volume is reduced and the activities of the RAAS are also reduced (August *et al.*, 1990; Weiner *et al.*, 1994; Salas *et al.*, 2006). Why interplay of factors is likely is that individual factors do not appear sufficient to cause the large differences seen. Prostaglandins have been postulated to contribute (Everett *et al.*, 1978; Pedersen *et al.*, 1983) but do not appear to be fully responsible for the degree of vasodilation found (Venuto & Donker, 1982; Conrad & Colpoys, 1986). A prospective study of urinary prostacyclin and thromboxane metabolites during the first three months of pregnancy also showed that these did not rise significantly until rather later (Al Kadi *et al.*, 2005). Furthermore, in studies done with chronically instrumented conscious pregnant rats, the administration of indomethacin did not result in a reversal of the AII mediated blood pressure attenuation (Conrad & Colpoys, 1986). However, there are some subtle differences in cardiovascular change in pregnancy between rats and humans and the above evidence may not be sufficient to disregard the prostaglandin hypothesis. Maternal plasma volume, for example, expands to a maximum before the end of pregnancy and is maintained till term in humans, whilst it continues to expand progressively till term in rats (Baylis, 1984).

Nitric oxide (NO) has also been postulated to be important because increased levels of NO and those of its secondary messenger, cyclic guanosine 3'5' –monophosphate (cGMP) have been found in animal pregnancy (Conrad *et al*., 1993; Conrad & Vernier, 1989; Weiner *et al*., 1994). Hormones known to increase in pregnancy, such as oestrogen and progesterone, have been shown to induce nitric oxide synthase in in-vitro experiments (O'Connor & Moncada, 1991; Hayashi *et al.*, 1992). However, reduced plasma levels of NO and increased urinary levels of its secondary messenger, cGMP, have been found in human pregnancy (Chapman *et al*., 1998), which is another difference between human and rat pregnancy. This latter negative finding may also be accounted for by the problems in assessing nitric oxide levels in humans (Benjamin, 2001). Nitric oxide is rapidly oxidized to inorganic nitrate, which has a very complex metabolism including active reabsorption in the kidney by an anion pump that appears saturable (Benjamin, 2001). As such the measurement of plasma nitrate is a poor indicator of nitrate synthesis. Urinary excretion of nitrate is also unreliable because of the fact that its reliability depends on a low nitrate diet and Western diets and even water are rich in nitrate (Benjamin, 2001).

Placental oestrogen may also play a role in volume expansion through hepatic stimulation of angiotensinogen synthesis. However, oestradiol concentration has been found to be no different from normal controls in women with pre-eclampsia and idiopathic fetal growth restriction who had inadequate plasma volume expansion at the time when they were studied (Salas *et al.*, 2006).

The osmolar threshold for the release of AVP is reduced in pregnancy, thus as plasma volume increases and osmolality falls, AVP still continues to be released (Davison *et al*., 1984). Despite a significantly lower plasma osmolality, AVP was found to be similar in pregnant Sprague-Dawley rats compared to virgin ones (Durr *et al.*, 1981). This allows the expanded plasma volume of pregnancy to be viewed as normal (Barron *et al.*, 1984). A study that examined eight pregnant women also found a reduced osmotic threshold for the release of AVP and for thirst (Davison *et al.*, 1988).

The role of ANP in maintaining plasma volume in pregnancy has been more controversial. Many studies found conflicting results including an increase in ANP (Milsom *et al.*, 1988; McCance *et al.*, 1990; Steegers *et al.*, 1991) and no change in ANP (Lowe *et al*., 1992). However, none of the studies that found an increase controlled sodium intake. In a longitudinal study where sodium intake was controlled by restricting the women to 100mmol of sodium for 4 days before the study, there was no significant change in plasma ANP levels from the first to the third trimester (Lowe *et al*., 1992). This was thought to be because the increased plasma volume in pregnancy may be "perceived" as normal. A meta-analysis showed a 41% increase in ANP levels in the third trimester, compared with non-pregnant and it also suggested that atrial stretch receptors sense the expanded blood volume in pregnancy as normal or moderately raised (Castro *et al.*, 1994). However, as noted, a majority of these studies did not control for sodium intake.

1.2.3.1. Maintenance of plasma volume expansion in pregnancy

Plasma volume increases steadily from early pregnancy to at least 30 weeks gestation on average, by approximately 1250 ml, after which it remains fairly steady till term (Pirani *et al.*, 1973; Whittaker & Lind, 1993). Salas et al found that it reached a maximum between 34 and 36 weeks gestation (Salas *et al*., 2006), while the first 2 studies suggested a maximum at 30 – 34 weeks, and at 28 – 36 weeks respectively. The findings in these 3 studies are very similar and the slight differences in maximum plasma volume expansion may be due to the different intervals of gestational age at which the women were studied.

Posture appears to affect plasma volume in late pregnancy. Studies performed with the women in the supine position cause an apparent decrease in plasma volume due to an incomplete mixing of the Evans blue dye because of the weight of the pregnant uterus on the inferior vena cava (Chesley & Duffus, 1971; Letsky, 1991). When lying on their sides, apparent plasma volume is greater and mixing of the dye complete within 10 minutes (Chesley & Duffus, 1971; Pirani *et al*., 1973).

The prime determinant of ECF in pregnancy as well as in the non-pregnant, is renal sodium handling (Brown & Gallery, 1994). Approximately 950 mmol of sodium accumulates in the body during pregnancy (Baylis & Davison, 1998). As greater than 30,000 mmol/day of sodium is filtered by the glomeruli, an increase from non-pregnant levels of about 10,000 mmol/day, several changes occur to effect the reabsorption required to maintain maternal and fetal stores (Baylis & Davison, 1998). Antinatriuretic hormones such as aldosterone and desoxycorticosterone increase substantially during pregnancy, as there is increased activity of the RAAS

(Baylis & Duffus, 1998; Chapman *et al*., 1998). Specifically, it appears that maternal adrenal secretion maintains the normal increase in plasma volume in pregnancy. A study that gave aldosterone or cortisol to adrenalectomised pregnant ewes, found that reduction of aldosterone or cortisol resulted in a decrease in plasma volume (Jensen *et al*., 2002). The concentration of natriuretic hormones also increases in pregnancy however (Baylis & Davison, 1998). Perhaps ECF volume is therefore maintained by the response of the antinatriuretic hormones to the perceived hypovolaemia created by the natriuretic hormones, as well as the peripheral vasodilation of pregnancy. More likely, plasma volume regulation in pregnancy is multifactorial and not due to a simple rise in the level of one or more antinatriuretic hormones (Khraibi *et al.*, 2002).

With this likelihood and the fact that a previous study in my centre had reported a contraction in plasma volume in SCD women later in pregnancy (see 1.1.3 above), I decided to perform a study to examine several different hormones and electrolytes that could affect plasma volume. I felt this was pertinent because SCD pregnancies are also often complicated with intrauterine growth restriction and perinatal mortality (section 1.1). If the reported plasma volume contraction was an accurate representation, it would be similar to what occurs in pre-eclampsia, a condition in which there is also a contraction in plasma volume associated with low birth weight and increased perinatal mortality, and a disruption of the renin- angiotensin system (Brown *et al*., 1997; Al Kadi *et al*., 2005).

This thesis was thus designed to test the following hypotheses:

Hypothesis 1. The Evans blue PV distribution in pregnant SCD women is lower than their haemoglobin AA counterparts.

Hypothesis 2. The activity of components of the RAAS is reduced in pregnant SCD women compared to their haemoglobin AA counterparts.

Hypothesis 3. Plasma vasopressin concentration and urinary osmolality are lower, and plasma osmolality is higher in pregnant SCD women than their haemoglobin AA counterparts.

Hypothesis 4. Plasma sodium concentration is reduced and fractional excretion of sodium is increased in pregnant SCD women as compared with their haemoglobin AA counterparts.

The primary outcome measures were thus plasma volume in millilitres and in millilitre per metre squared, plasma renin concentration in ng/ml/hour, plasma angiotensinogen concentration in g/ml and serum aldosterone concentration in ng/ml. The secondary outcome measures were plasma vasopressin concentration in pg/ml, plasma and urinary osmolality in mosmo/kg and plasma sodium concentration in mmol/l.

2. METHODS

This study was conducted at the Lagos University Teaching Hospital (LUTH), Lagos, Nigeria, which is a 761 bed hospital, 89 of which are obstetric and 60 gynaecological. It is one of two tertiary hospitals in Lagos state, the population of which is just under 10 million people.

The study protocol was approved by the Research and Ethics Committee of the Lagos University Teaching Hospital, Reference number ADM/DCST/221/Vol.9, dated 11th February 2004. All women gave written, informed consent to participation. They were allowed to take the 'volunteer information leaflet' (Appendix 1) home to show their partners and were included after they had understood the information and signed the consent forms (Appendix 1).

2.1. SUBJECT SELECTION AND INCLUSION CRITERIA

There were 4 groups of subjects:

1. Pregnant haemoglobin SS genotype (Hb SS) women.
2. Non-pregnant haemoglobin SS genotype women.
3. Pregnant Haemoglobin AA genotype (Hb AA) women.
4. Non-pregnant haemoglobin AA genotype women.

All the women were healthy at recruitment and in a steady clinical state. The pregnant women were recruited from the antenatal clinic of the consultant out-patient department at booking, where they were fully counselled about the study. The non-pregnant haemoglobin AA women were recruited from members of staff and students while the non-pregnant haemoglobin SS women were

recruited from the sickle cell clinic, which is a clinic run by the internal medicine department of the hospital. All women were between ages 20 and 40 and all had normal regular menstrual periods. The pregnant women all had known last menstrual periods, all had their gestational ages checked by early ultrasound scans and were non-smokers. The pregnant AA women had oral ferrous gluconate 300 mg three times daily, folic acid 5 mg daily and intermittent preventive treatment for malaria with sulphadoxine/pyrimethamine (500mg/25 mg) twice, 8 – 10 weeks apart during the pregnancy, avoiding the first trimester and the last 4 weeks of pregnancy, as is routine for antenatal patients in this hospital (see section 1.1). All the SS women (pregnant and non-pregnant) had routine folic acid 5mg twice daily and proguanil 200mg daily, the latter for malaria prophylaxis (see section 1.1). They are not routinely given iron supplements in my hospital as they have been reported to usually have sufficient iron stores presumably due to recurrent haemolysis (Akinyanju *et al.*, 1987; Abudu *et al.*, 1990; Aken'Ova *et al.*, 1997). Anaemia in pregnancy is common in Nigeria (Anorlu *et al.*, 2006; Dim & Onah, 2007; Kagu *et al.*, 2007; Ukaga *et al.*, 2007) thus all pregnant women without contraindications to iron supplements are given supplements in the form of ferrous sulphate and folic acid.

2.2. EXCLUSION CRITERIA

1. Women who had received or donated blood in the 6 months prior to the study.
2. Women with a history of sickling crises in the 4 weeks prior to the study.
3. Women with known cardiovascular disease (including chronic hypertension), thyroid, renal or metabolic disease, and diabetes mellitus.
4. Women on the oral contraceptive pill or other hormonal preparation.

2.3. SAMPLE SIZE

It was calculated that 15 pregnant SCD women and 15 age and parity matched pregnant haemoglobin AA controls would give an 80% power of reproducing the difference in plasma volume (PV) between both groups of about 1 litre, seen at 36 weeks gestation in a previous study (Abudu & Sofola, 1988) at the 5% significance level. An attempt was made to recruit at least 20 women each to allow for dropouts.

2.4. STUDY DESIGN

Measurement of plasma volume using the Evans blue dye dilution method (el-Sayed *et al.*, 1995), and of concentrations of osmoregulatory hormones, including vasopressin, aldosterone, progesterone and prolactin, plasma renin concentration, plasma angiotensinogen concentration, urinary and plasma electrolytes – sodium and potassium, urinary creatinine, full blood count including haemoglobin concentration and white cell count, and genotype estimation were carried out on all the women. All measurements were done in

the follicular phase of the menstrual cycle for the non-pregnant women.

For the pregnant women, all the measurements were done at 16 and 36 weeks gestation. It was originally intended to study all the pregnant women prospectively i.e. a longitudinal study but due to the invasive nature of the study and also to the fact that the women with SCD often had to have a blood transfusion during pregnancy or developed severe crisis or other illness, they could not be studied again at 36 weeks. Thus other women had to be recruited to take their place and it became a cross-sectional study.

2.5. INTERVENTION

The general outline of the study is presented in this section, which is then followed by the detailed methodology. The women were admitted for 24 hours from the night before the PV measurements. They were weighed and had their heights measured and it was these measurements and the calculated body mass index (BMI) figures that were recorded in the study data. All urine passed by them during that time was collected and the volume carefully measured. About half of the women, evenly spread amongst the groups, did not want to be admitted and rather than lose them for that reason, they were instructed on collecting their urine at home. They were all verbally instructed on 24-hr urine collection (see below: “24h urine collection”), given a form with the instructions written on it (See Appendix 2) and asked at the end of the collection about the completeness of their collection. Urinary osmolality and fractional excretion of sodium, potassium and creatinine were estimated for each sample. Free water clearance was then calculated.

During the estimation of plasma volume, blood was taken for the measurement of plasma sodium, potassium and creatinine, plasma angiotensinogen concentration (Aogen), plasma renin concentration (PRC), progesterone, prolactin, aldosterone and vasopressin levels in the steady state (see Appendix 3). All but Aogen and PRC were measured by Dr O.O. Oladipo, a senior lecturer and consultant chemical pathologist in the Lagos University Teaching Hospital. Dr Peter Marsters measured the Aogen and PRC in Nottingham.

The blood collection was done between 0900 and 1100 hours in the morning, at the midpoint of the urine collection. The women were fasted overnight and rested for 30 minutes before the blood was taken. They were however allowed to drink clear fluids liberally, particularly because dehydration is a trigger factor for Hb SS crises and they are usually encouraged to drink a lot of water.

Plasma and urinary osmolality, electrolyte and creatinine concentrations were measured using standard clinical chemistry techniques (Appendix 3) also by Dr Oladipo. I then calculated the fractional excretions of sodium and potassium as well as the free water clearance.

Haemoglobin concentration (Hb) and packed cell volume (PCV) were estimated in all the patients at the time of study. Haemoglobin genotype was also confirmed in all the patients using cellulose paper electrophoresis at an alkaline pH of 8.4. The babies were carefully weighed at birth and data collected on their mothers' gestational age at delivery, their birth weight and their gender.

2.6. PLASMA VOLUME MEASUREMENT AND SPECIMEN COLLECTION

2.6.1. 24-hour urine collection

The 24-hour urine collection was done as follows: The women were given a 5-litre plastic container (jerry-can) and a 1-litre plastic jug. They were instructed to note the time and date of starting the urine collection and to write these on a label on the 5-litre container which also bore their names and study code. They were to pass urine into the toilet at the start time and to subsequently collect all other urine passed into the jerry can. They were instructed to pass urine and put in the jerry can before starting to take a shower or bath, or before going to move their bowels, as these were times when urine could be inadvertently lost from collection. They were to stop the collection at exactly 24 hours after they started, with the difference being that they were to also put the last bit of urine into the jerry can. This was gone over several times with each patient and they were asked to repeat it as understood, emphasizing that the first urine at the start time was to go into the toilet, the last urine, into the container, and all others in-between into the container. The women were also given the instruction sheet (Appendix 2). At the end of the collection, the women were questioned about their method of collection and they all reported proper collection.

2.6.2. Blood collection

The materials required for the plasma volume and blood collection experiment are listed in Appendix 4. All clinical disposables were supplied by the hospital community pharmacy unless otherwise stated.

The Evans blue was prepared in 3-monthly batches for the duration of the study. It was supplied in powder form (Sigma-Aldrich, Missouri, U.S.A.) and prepared as follows: One gram of Evans blue dye was weighed each time and dissolved in distilled water. The solution was then made up to 1L with distilled water and dispensed into 40 ml glass tubes, sealed and autoclaved. Two random samples from each batch were cultured after each preparation and found to be negative for organisms. The remaining tubes were then stored in the refrigerator till they were used.

Prior to each visit, all necessary specimen tubes were labelled with the subject's study code and one of the two ethylenediamine tetra-acetic acid (EDTA) tubes was put in ice.

Two syringes were filled with heparinised saline 10IU/ml – one 10ml and one 5ml. Fifteen millilitres of Evans blue dye solution was withdrawn into a 20ml syringe. Unfortunately they could not be weighed as I did not get sufficient funding to buy a sensitive weight balance and there was no functional one available for me to use on a regular basis. The Evans blue was thus carefully drawn out by me 80% of the time and by another person – Dr Olaleye, a senior registrar – (trained by me) about 20% of the time. I later managed to borrow a

weight balance and I measured the before and after weights of 10 syringes into which I drew Evans blue and calculated a coefficient of variation (CoV) for these. The between sample CoV for the syringes with Evans blue was 0.4%, the CoV for the syringes emptied of Evans blue was 1% and the CoV of the difference between both weights was 0.7%. The mean weights were 24.89, 9.85 and 15.05 grams respectively.

As each woman arrived, she was welcomed into the research room and her details taken down into a data form (Appendix 4). After she had been lying down for 30 minutes, a 20-gauge (G) cannula was inserted into a large vein (usually one of the veins in the antecubital fossa). Twenty-two millilitres of blood was withdrawn each time and handed to an assistant who gently released it into the specimen tubes in the following volumes:

5ml each (15ml) into the plain serum separator tube (SST) gel tubes
3ml into the lithium heparin tube
2ml each (4ml) into the cold and room temperature EDTA tubes

The 3-way tap was attached and the cannula stuck down with plaster. The cannula was flushed with a small volume of heparinised saline and the 3-way tap was closed. The stopwatch was then set to 10 minutes.

Fifteen ml of Evans blue dye was injected into the cannula within 1 minute. As the injection was started, we also started the stopwatch. The cannula was flushed with heparinised saline through the pink covered outlet and through the 3-way

tap, till all traces of dye were gone. After this, another 1ml of heparinised saline was pushed in and the 3-way tap was closed.

At 10 seconds, 5ml of blood was slowly withdrawn and the stopwatch was reset to 10 minutes. The 5 ml syringe filled with blood was gently emptied into the plain tube labelled "10 minutes". The syringe containing the mixture of blood and heparinised saline was then reattached and 5ml of this was pushed back into the vein in order to keep it patent.

The cycle was repeated after 10 minutes and then after another 10 minutes, putting the blood into the "20" and "30 minute" plain bottles, respectively. All the blood/heparinised saline mixture was re-injected after the 30-minute sample had been taken and the cannula was taken out of the vein. This method of using the same venous line has been validated by a previous study (el-Sayed *et al.*, 1995).

2.6.3. Specimen processing

The plain SST tubes were left to stand for one hour so the serum could separate. All the tubes were centrifuged at 3000 revolutions per minute (rpm) for 10 minutes. The EDTA tube on ice was centrifuged in a cold centrifuge at -4^{o} degrees centigrade I, at the same speed. The serum from the plain bottles was separated and 2ml was stored in a plain tube at -20^{o} centigrade I, for the analyses of aldosterone, progesterone and prolactin (see Appendix 5). The remaining approximately 5 – 7 ml was used for the standard curve, the preparation of which will be discussed below.

The plasma from the cold EDTA bottles was separated and split into two parts – one part was for the analysis of plasma angiotensinogen concentration and plasma renin concentration, while the other was for the analysis of vasopressin.

The plasma from the lithium heparin and the EDTA bottles was also separated and put in plain tubes. All the tubes containing serum and plasma were stored at -20 degrees centigrade until they were ready to be analysed.

2.7. LABORATORY METHODS

2.7.1. Plasma volume measurements

The dye-dilution method of PV measurement works on the principle that when a substance is added to a compartment and allowed to mix freely and completely without being lost, its concentration at a point in time can be used to calculate the volume of the compartment (Jacob *et al.*, 2007). This is by calculating the volume of distribution of the substance.

The volume of the compartment is calculated thus: Amount of substance added to the compartment divided by the concentration of substance after complete mixing within the compartment. The concentration at the point of injection of the substance is unknown but can be obtained by taking blood samples at timed intervals after complete mixture, measuring the concentration of the substance using colorimetry, and extrapolating back to time zero by plotting a time/concentration graph (el-Sayed *et al.*, 1995).

Measurement of plasma volume requires a substance or tracer which is mostly limited to the plasma compartment, is evenly distributed and non-toxic. This is achieved by using a tracer that binds to albumin. The tracers commonly used are the azo dye known as Evan's blue (or T1824), which binds avidly to albumin, or radio-iodine labelled serum albumin (RISA) or indocyanine green (Jacob *et al.*, 2007). The strengths and weaknesses of these various types of tracer are discussed in section 2.9.4.

2.7.2. Plasma volume measurements in this thesis

The plasma volume measurements were done using the Evans blue dye-dilution technique as previously described (el-Sayed *et al.*, 1995; Bernstein *et al.*, 1998).

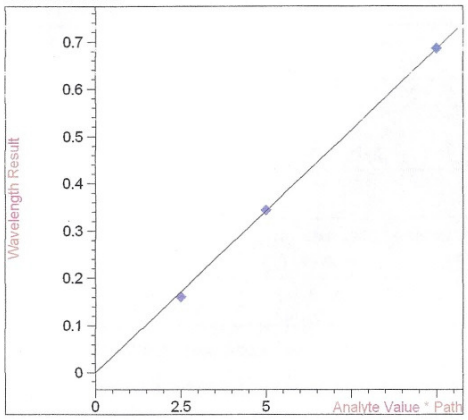
Known concentrations of Evans blue were prepared using the stock 1mg/ml solution as described above. In this case, the concentrations used were 5, 10 and 20 g/ml respectively. Equal volumes of each of these known concentrations were then diluted with equal volumes of the serum for the standard curve. In this case we used 1ml of known Evans blue to 1 ml of serum, resulting in concentrations of 2.5, 5 and 10 g/ml of Evans blue respectively. Two millilitres of plain serum was used as a blank.

An ultraviolet spectrophotometer with a visible range (Agilent 8453; Supplier) was used to measure the absorbances at a wavelength of 610 nanometres. The blank and the three Evans blue/plasma serum mixtures were measured each time with the absorbances plotted against the concentrations in order to draw a

standard curve (Figure 2.1).

The concentrations of the timed samples were then obtained from the curve and plotted against time and extrapolated to zero (Figure 2.2). The time zero concentration was used to calculate the plasma volume using the calculation mentioned above.

Calibration Curve



Calibration Table

#	Standard Name	Evans blue(ug/ml)	Abs<610nm>	%Error
1	Evans blue	2.50000	0.16140	6.10
2	Evans blue	5.00000	0.34469	-0.64
3	Evans blue	10.00000	0.68614	-0.17

Calibration Result Summary

Analyte Name	Evans blue	Std.Dev. of k1	0.13365 ug/ml
Number of Standards	3	*Std.Dev. of Calibrat*	*0.10487 ug/ml*
Calibration Curve	C = k1 * A	Correl. Coeff. (R^2)	0.99983
Coefficient k1	14.60000 ug/ml		

Figure 2.1. An example of a standard curve

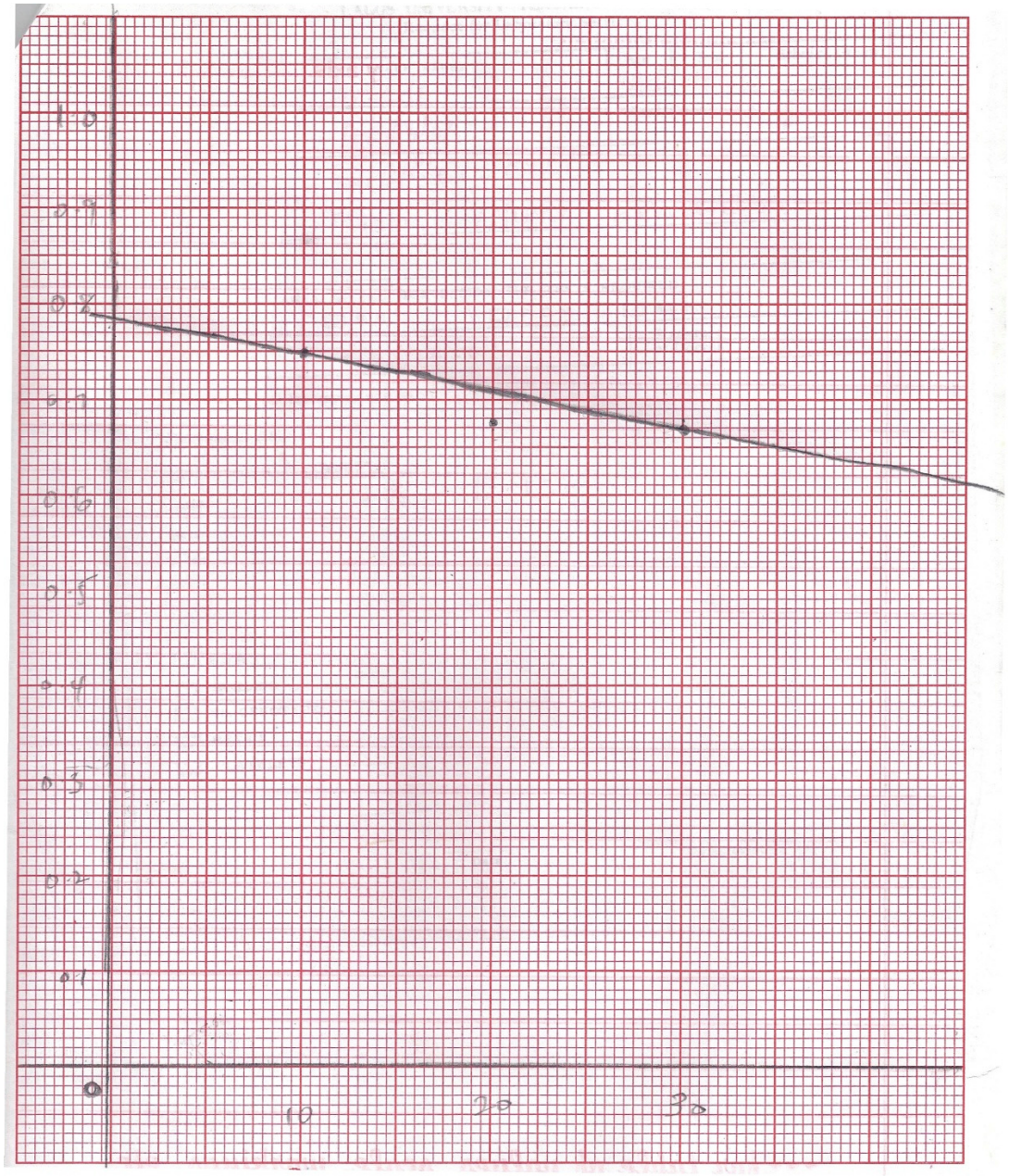


Figure 2.2. An example of a concentration/time plot

2.7.3. Radioimmunoassay and enzyme immunoassay

All the hormones were measured by enzyme immunoassay (EIA) or radioimmunoassay (RIA). First, I will outline the general principles of RIA and EIA then go on to discuss the specific methods used in this thesis.

Radioimmunoassay (Skelley *et al.*, 1973) is a very sensitive method for measuring small amounts of substances in blood using the antigen-antibody binding principle. Unknown amounts of substances, hormones in this case, can be determined by their competitive binding for the binding sites of antibodies bound to known concentrations of radiolabelled hormones, thus displacing the radiolabelled hormone. Small amounts of radiolabelled samples (known antigen) of the substance to be measured are mixed with antibody and then incubated either with known concentrations of standard antigen or with a plasma sample containing an unknown concentration of the hormone to be measured. The unknown antigen in the plasma sample then displaces the labelled known antigen proportionally to its concentration and binds with the antibody. As more unknown antigen is added, more known is displaced and becomes free known antigen. Higher concentrations displace more, reducing the ratio of bound to free known antigen. The bound antigens are then physically separated from the free or unbound ones and the radioactivity of the free fraction is measured. This is used to determine the concentration of unknown antigen (hormone). Berson and Yalow developed RIA in 1959 (Yalow & Berson, 1960) and Rosalyn Yalow was awarded the Nobel Prize in Physiology or Medicine in 1977 for her work on the development of the RIA for insulin (Kahn & Roth, 2004).

In EIA, an enzyme, as opposed to radioactivity, acts as the measurable label. An antigen is labelled with an enzyme and then reacted with an antibody thus inhibiting the enzymatic reaction. An unknown amount of antigen or substance to be measured is then added and mixed with the antigen-enzyme-antibody complex. If the unknown substance is the same as the known antigen, it competes with it for the limited amount of antibody thus reducing the amount of antibody available to inhibit the enzyme. Enzyme substrate is then added and the quantity of the unknown antigen can be measured (Voller, 1978). In 'traditional' or homogenous EIA, the measurement is performed without any physical separation during the analysis. In heterogeneous EIA which is often used synonymously with ELISA, the bound antigen is separated from the free material by washing the microtiter plate before enzyme activity is estimated by addition of substrate (Voller, 1978; Lequin, 2005).

Plasma samples were air freighted on dry ice to Nottingham for assay of plasma renin and angiotensinogen concentrations (PRC; Aogen), using established radioimmunoassay (Tetlow & Broughton Pipkin, 1983; Broughton Pipkin *et al.*, 1984). Samples were assayed in duplicate, and all samples from individual patients were run in the same assay. Dr Peter Marsters assayed all the samples in the City Hospital, Nottingham. The reagents used and techniques are as in Appendix 3. The inter- and within assay coefficient of variation (CoV) was 14.8% and 5.6% for PRC respectively and 13% and 5.1% for Aogen respectively.

2.7.4. Aldosterone

Serum aldosterone was assayed with an aldosterone ELISA kit Catalogue number 1875, manufactured by Alpha Diagnostic International, Texas, USA. All the assays were done by one person- Dr O. O. Oladipo as mentioned above. The technique is as shown in Appendix 3. A formal validation in pregnancy was not done, as there is very low cross-reactivity with other steroids. The between assay CoV was 6.2% (n=5) and the intra-assay CoV was 3.4% (n=20). The minimal detectable concentration was 15pg/ml (n=10).

2.7.5. Arginine-Vasopressin (AVP)

Arginine-vasopressin was assayed with an EIA kit Catalogue number 900-017, manufactured by Assay Designs, Michigan, USA. The details of the technique and its rationale are as shown in Appendix 3. A formal validation in pregnancy was not done, as here again the cross-reactivity to other related compounds such as oxytocin is extremely low. The between assay and intra-assay CoV were 7.8% (n=5) and 6.6% (n=10) respectively.

2.7.6. Progesterone

Progesterone was assayed with an EIA kit Catalogue number PrOG-96 manufactured by Teco Diagnostics, California, USA; the details of the technique and rationale are as in Appendix 3. The intra and inter-assay CoVs were 3.8% (n=10) and 4.7% (n=6) respectively.

2.7.7. Prolactin

Prolactin was assayed with an EIA kit Catalogue number PROL-96 manufactured by Teco Diagnostics. The intra and inter-assay CoVs were 4.5% (n=10) and 8.7% (n=10). The details of the technique and rationale are as in Appendix 3.

2.7.8. Osmolality measurements

The Wescor Vapor Pressure Osmometer (Wescor Incorporated, Utah, USA) was used for all the osmolality measurements (Appendix 3). Although it is an indirect method, it has a significant advantage over the previous methods of indirect measurements i.e. that of freezing point depression or boiling point elevation. This is because it can be performed without the necessity for a change in the physical state of the specimen and is thus free from measurement artefacts that can occur due to physical alteration (Tornheim, 1980; Knepper, 1982). The intra- and inter- assay CoVs were 1.4 % (n=10) and 3.5% (n=10) respectively.

2.7.9. Creatinine

The principle used was the colorimetric reaction – Jaffe reaction (Hervey, 1953), of creatinine with alkaline picrate measured kinetically at 490nm without any pre-treatment step (Appendix 3). The kit was provided by Biolabo Reagents (Maizy, France) and measured using a VWR spectrophotometer by Dr Oladipo. The intra- and inter- assay CoVs were 3.6% (n=10) and 5.4% (n=10) respectively.

2.7.10. Electrolytes (Sodium and Potassium)

Flame photometry was used to analyse plasma sodium and potassium, the principle of which is as follows:

Atoms on ground state get excited and unstable by the absorption of energy (heat). The atoms immediately return to ground state and release energy in the process in the form of light. The light emission is at wavelengths specific for each element and can be quantified.

Plasma separated from lithium heparin tubes was used. The intra- and inter-assay CoVs for sodium were 2.3% (n=10) and 4.2% (n=10) respectively. Those for potassium were 3.9% (n=10) and 4.8% (n=10) respectively. The Jenway Flame Photometer (Jenway, Essex, England) was used for the assays and the details are in Appendix 3.

2.8. DATA HANDLING

The baseline characteristics of each patient were entered from the data form mentioned above (Appendix 5) into the Statistical Package for the Social Sciences (SPSS) version 15 for Windows (SPSS Inc, Chicago, IL, USA) statistical software programme, using a file structure which I developed. As the collected blood and urine samples were analysed, the results were entered into the same file. All the data in which the samples were noted to have haemolysed before analysis were omitted. Any measurements that fell outside the range compatible with life were also omitted from analysis, as it was assumed that the results generated were erroneous. The data were initially entered into SPSS by a research assistant and subsequently checked over completely by me.

Missing data and outliers were checked in the original proformas and corrected if transcription errors were found. Checking the patient's case files or calling the patient to request for them e.g. birth weights and sex of their babies retrieved other data.

2.8.1. Statistical analysis

Data were tested for normality of distribution using the frequency distribution analysis. If they were not normally distributed, they were normalised by logarithmic transformation or non-parametric tests were used as necessary. Normally distributed data were expressed summarised and presented as means ± standard deviation (sd), with subsequent analysis by Student t-test or ANOVA as appropriate. Pearson's correlation coefficient was used for measurement of association between variables. The Mann-Whitney U test and Kruskal Wallis ANOVA were used for non-parametric data, which were presented as median and interquartile ranges.

2.9. DISCUSSION

2.9.1. Subject selection and inclusion criteria

As mentioned earlier, iron supplements are not given to individuals with HbSS in our centre as they are known to haemolyse frequently without external bleeding, due to the fragility of their red blood cells, which have a short life span. Most studies measuring the red cell survival rate give an average of less than 20 days (McCurdy, 1969; Solanki *et al.*, 1988; Serjeant *et al.*, 1996). They therefore conserve body iron and for this reason, iron deficiency is not thought to be a cause of their anaemia (Akinyanju *et al.*, 1987; Abudu *et al.*, 1990). In order to avoid the risk of iron overload in them, supplementary iron is only given if they have proven iron deficiency anaemia. Although there are reports, which have found pregnant Hb SS women to be iron deficient (Anderson, 1972; Oluboyede, 1980), the weight of evidence for iron sufficiency in pregnant women with Hb SS (Fleming, 1969; Akinyanju *et al.*, 1987; Abudu *et al.*, 1990) is more than that for its deficiency.

The consequences of malaria in pregnancy include miscarriage, severe malaria (which can cause coma, renal failure and death), preterm labour and perinatal mortality from prematurity and intrauterine growth restriction (Sholapurkar *et al.*, 1988; Menendez *et al.*, 2000; Duffy & Fried, 2005). As malaria is endemic in Nigeria, with pregnant women being particularly susceptible despite being semi-immune when not pregnant, they are given antimalarial prophylaxis during pregnancy. Intermittent sulphadoxine/pyrimethamine is used for non-HbSS women and has been found to be very effective in preventing malaria and its consequences (Rogerson *et al.*, 2000; Garner & Gulmezoglu, 2006; Falade *et al.*, 2007). Proguanil

is known to be very effective (Mutabingwa *et al.*, 1993; Garner & Gulmezoglu, 2006) but relatively more expensive and has to be taken daily. As pregnancy is fraught with complications in pregnant HbSS women, they are given proguanil and regularly screened for malaria so as to ensure they do not get the disease. None of the volunteers for this study contracted malaria during the period of the study.

One of the exclusion criteria was anyone who was transfused in the 6 months prior to the study. This was to ensure the study participants, especially those with SCD, were truly in a steady clinical state. Thus they were not representative of the average person with SCD. It was important to select them in this manner, as my primary objective was to examine the physiological determinants of their plasma volume differences.

2.9.2. Sample preservation

Most frustratingly, I lost a lot of data during the course of this study for various reasons, the most significant being the poor power supply in my country generally, a situation that also prevails in universities and hospitals. The freezers were thus often thawing out and I could only analyse samples where I was sure of their authenticity i.e. those that I could salvage before a major power failure. The freezer in which the urine samples were kept was different from that of the plasma and serum samples; the creatinine assays were done last and only a few of the samples could be salvaged for analysis.

2.9.3. Twenty-four hour urine collection

Twenty-four hour urine collection is notoriously inaccurate, thus despite instructing all the subjects in a study carefully on the proper method of collection and the importance of accuracy, it is still important to check the completion of sample collection objectively. The ideal way to do this is by getting the studied women to ingest p-aminobenzoic acid (PABA), which can then be measured in urine in which it is completely excreted (Bingham & Cummings, 1983). Thus if at least 85% of the total dose of PABA is recovered, the collection can be deemed complete. However, as it may be a bit cumbersome and costly to add this to a study, an alternative method based on the examination of the 24-hour creatinine output in relation to body weight is usually used. In a study comparing five different urinary creatinine based methods with the PABA method, the Knuiman method which uses a formula based on 24-hour urinary creatinine excretion (Knuiman *et al.*, 1986), was found to be the most useful as it had a moderate sensitivity and excellent specificity (Murakami *et al.*, 2008). I could not use it in this study however as I did not get a lot of creatinine results (see above).

2.9.4. Plasma volume measurement

There are various methods of plasma volume measurement and most of them are based on dilution principles that measure the apparent volume of distribution of the particular substance used (Wilson & Mills, 1970). The radio-labelled albumin method is thought to be the most reliable and reproducible method (1980) but is usually not used in pregnancy as it involves the administration of radioactive substances and human blood products to the individual.

The dye-dilution method includes the use of substances such as Evans blue (also known as T-1824), Coomassie blue and Indocyanine green. Other substances such as dextran (labelled and unlabelled) and iron-dextran (Semple *et al.*, 1958; Wilson & Mills, 1970; van Kreel *et al.*, 1998) have also been used. Evans blue is one of the most commonly used dye-dilution methods, particularly in pregnancy. It and the other dyes all bind to albumin and thus measure the albumin distribution which reflects the true plasma volume only if the capillaries remain impermeable to albumin and the dye stays within the intravascular space. As this is not often the case e.g. with traumatic injuries, renal impairment and with pre-eclampsia where albumin escapes from leaky capillaries, they tend to overestimate the plasma volume (Valeri *et al.*, 1973; Campbell & Campbell, 1983). Another problem with Evans blue is the variability of its absorbance in turbid plasma (Brown *et al.*, 1992a). This can be overcome by the use of methods such as extraction procedures which were popular in the 1950s and 60s (Allen, 1951; Hobsley & Dew, 1958; Discombe, 1961; Hobsley & Thurn, 1963), a two wavelength method at 610 and 740 nm ((Nielsen & Nielsen, 1962; Brown *et al.*, 1992a) and the polyethylene glycol method (Brown *et al.*, 1992a).

In the 'Recommended Methods for the Measurement of Red Cell and Plasma Volume' by the International Committee for Standardisation in Haematology published in 1980 (1980), it was noted that there was evidence for the use of a protein with molecular weight larger than albumin which would give a more accurate indication of the true plasma volume, but that this was not yet in routine practice. Substances such as gamma globulin (Andersen, 1962), fibrinogen (Baker & Wycoff, 1961) and alkaline phosphatase (Posen *et al.*, 1965), have been used. The

problem with the use of dextran is that its chemical assay is tedious and using iron (^{59}Fe) labelled dextran which is less laborious, introduces the use of radioactivity. Dextran 70 has been used successfully to measure plasma volume and found to be comparable to iodine-labelled albumin with a simpler laboratory method required for its quantification. This involves the estimation of glucose, which is a product of its hydrolysis (van Kreel *et al.*, 1998). The CoV of this method was found to be 5% compared to 3% using ^{125}I-labeled albumin that is taken as the gold standard (van Kreel *et al.*, 1998). However, I could not use it in this study because I did not have access to some of the equipment required to carry out the complete experiment in my laboratory in Lagos.

2.9.5. Renin-angiotensin assays

I could not set up the renin and angiotensinogen assays in Lagos, as we do not have a gamma counter or safety procedures for handling ^{125}I. The assay was developed and standardized in Nottingham and all the expertise is available there (Tetlow & Broughton Pipkin, 1983; Broughton Pipkin *et al.*, 1984). I learnt to do the method myself whilst in Nottingham but did not do the actual assays of my samples as I could only spend a limited time in Nottingham at each visit. There was a very experienced technologist in the laboratory that did all the assays. I however did all the data analysis.

2.9.6. Study design and recruitment.

Longitudinal studies provide a stronger methodology to study the natural history or aetiology of disease (Vandenbroucke, 2008; Kirkwood & Sterne, 2003) unlike cross-

sectional ones which are carried out at one point in time and are best suited to measuring prevalence of disease (Kirkwood & Sterne, 2003).

The study was originally meant to be longitudinal but the plan failed due to a number of reasons. The greatest problem was the fact that pregnant women with sickle cell disease frequently suffer various crises (during which their red blood cells get haemolysed or sequestrated in their spleens or livers), and infections that cause them to require blood transfusion. As the study was to measure plasma volume, one of the exclusion criteria had to be a recent blood transfusion. Unfortunately more women were transfused than expected, possibly because of the worsening socio-economic condition of average Nigerians, leading to poor nutritional habits and increased susceptibility to infections and crises. The endemicity of malaria in Nigeria and the increased susceptibility of people with sickle cell disorder and pregnant women in particular, even despite the use of prophylactic medication in pregnancy, contribute to the increased risk of haemolysis, severe anaemia and need for blood transfusion in them.

I also encountered some difficulty in persuading a few of the Hb AA women to return for either their 36 weeks' session. This was due to a general suspicion about medical research particularly that necessitating the provision of bodily fluids, the fact that these women were not actually the study group; rather they were healthy with uncomplicated pregnancies and because of the inconvenience of coming to the hospital when they have no clinical complaints.

2.9.7. Study data

2.9.7.1. Calculation of body surface area

The body surface area (BSA) was calculated using the Mosteller formula as it has been found to be more applicable regardless of body weight and age, and also easier to calculate than most other formulae (Verbraecken *et al.*, 2006; Ahn & Garruto, 2008). The Dubois and Dubois formula that was popularly used was derived from just 9 subjects (DuBois & DuBois, 1916).

2.9.7.2. Calculation of glomerular filtration rate (GFR)

Historically GFR used to be calculated by using a 24-hour urine collection. However, 24-hour collections have been found to be inaccurate both in non-pregnant and pregnant populations (Cote *et al.*, 2008) usually because they are time consuming and inconvenient. Also as age, gender, weight and race are known to affect the relationship of serum creatinine and GFR, it is now generally recommended that creatinine based equations be used to estimate GFR rather than creatinine clearance measurements from 24 hour urine collections (Lamb *et al.*, 2005).

The "Modification of Diet in Renal Disease" (MDRD) equation and the Cockcroft-Gault (CG) are two of the most frequently used ones. Although the MDRD equation (Levey *et al.*, 1999) is now preferred for use outside pregnancy, its use has been heavily criticised in pregnancy as it was found to underestimate the GFR substantially in women studied serially from early through late pregnancy and post-partum (Smith *et al.*, 2008). Both the CG and the MDRD were reported to respectively overestimate and underestimate GFR in pre-eclamptics in a

retrospective study (Alper *et al.*, 2007) and to correlate poorly with creatinine clearance in third trimester pregnant women in a brief communication (Delemarre & Schoenmakers, 2008). However, as it is easier to calculate and has not been discredited in early pregnancy, as has the MDRD, I decided to use the CG in this study. Furthermore, none of the women I studied developed pre-eclampsia.

3. NON-PREGNANT SICKLE CELL STUDY RESULTS

I have split the sickle cell results section into two as it covers a lot of material. I will therefore be commenting on and displaying the results from all the non-pregnant women in this chapter and that from all the pregnant women in the 4th chapter. I will then discuss both sets of results in the 5th chapter.

3.1. STUDY DESIGN AND RECRUITMENT

The study was originally designed to be longitudinal, with samples taken at 16 and 36 weeks of gestation (see section 2.4). Additional, opportunistic, cross-sectional data were to be added as they became available, and non-pregnant women would be studied as controls. However, logistic problems (see section 2.9.6) meant that longitudinal data were only collected from 7 women, all haemoglobin AA (Hb AA). Thus, for the final analysis it was decided to use a cross-sectional design.

One hundred and fifty-four women were approached in total, 56 non-pregnant and 98 pregnant. A total of 140 women agreed to participate, comprising 51 non-pregnant women and 89 pregnant. The five non-pregnant women who declined all had haemoglobin AA and were either afraid of needles (3) or gave no reason (2). Of the 9 pregnant women who declined, 6 were haemoglobin AA while 3 were SS. The reasons given by the AA women were 'husband disapproved' (2) and 'afraid the blue dye might harm baby' (3). The sixth woman gave no reason. The three SS women were afraid it might make them unwell.

Out of the 140 women, measurements of plasma volume were made in 45 non-pregnant women, 37 women at ~16 weeks' gestation, 29 at ~36 weeks and 11 at ~8 weeks post-partum, a total of 122 women. Plasma volume measurements were technically unsuccessful in the remaining 18 women. Eighty-eight women were eventually studied in the final cross-sectional analysis as 34 women were excluded for statistical reasons (see 3.1.2). Below are the reasons for elimination of the 52 women not studied.

3.1.1. Technical reasons (18 women)

Non-pregnant women (6): One woman initially identified as SS was subsequently found to be SC; erythrocytes in samples from 4 Hb SS women haemolysed due to difficulty with collection. One AA woman's plasma volume result was lost.

Pregnant women (12): Erythrocytes in samples from four SS women haemolysed due to difficulty with collection; a further six SS women became ineligible since they were found to have been transfused before the plasma volume was measured. One AA woman had clotted samples and one became ineligible after being diagnosed as diabetic.

3.1.2. Methodological reasons (34 results)

In a true cross-sectional study, only one measurement from one volunteer can be used otherwise there will be autocorrelation as data from each individual tends to behave in a similar way even though the stages of gestation are different. The data were therefore balanced by systematically removing the first repeated measure of each pregnant woman. By doing this, data from 34 women became ineligible.

3.2. CHARACTERISTICS OF STUDIED WOMEN

Most of the women were nulliparous and in their third decade of life (Table 3.1).

Table 3.1. Characteristics of all studied women, irrespective of pregnancy status.

	Age (y) (mean±SD)	Parity (median & IQR)	Weight (kg) (mean±SD)	Height (m) (mean±SD)	Body mass index (kg/m2) (mean±SD)
N	88	88	87	88	87
Mean/median	26	0	61.8	1.62	23.4
SD or IQR	5	0 (0,0)	11.9	0.06	3.9

N = Number in each group, SD = standard deviation and IQR = Interquartile range.

3.2.1. Characteristics of studied women within groups (Table 3.2)

Haemoglobin AA women. The non-pregnant Hb AA women were significantly younger and more likely to be nulliparous than the pregnant AA women and, as would be expected, weighed less and thus had lower BMI.

Haemoglobin SS women. In this case, the non-pregnant were younger and had a lower BMI than the pregnant women.

Non-pregnant Hb SS were significantly younger and had a significantly lower weight and BMI than the Hb AA.

At **16 weeks of pregnancy**, Hb SS women had fewer children and were shorter, lighter and as such had a lower BMI than Hb AA women as expected.

At **36 weeks of pregnancy**, Hb SS women were lighter and had a lower BMI than their Hb AA counterparts.

Table 3.2 Comparison of studied women within groups.

	AA (N=39)		
	Non-pregnant (N=19)	**16 weeks pregnant (N=10)**	**36 weeks pregnant (N=10)**
Age (y)	25.1±4.3	28.8±4.7 *	30.4±4.9 **
Parity	0 (0,0)	1(0,2) **	0 (0,1) *
Weight (kg)	60.5±9.0	74.0±10.3 ***	78.6±10.6 ****
Height (m)	1.63±0.06	1.66±0.05	1.64±0.04
BMI (kg/m^2)	22.7±3.3	27.0±3.3 ***	29.3±3.4 ****
	SS (N=49)		
	Non-pregnant (N=25)	16 weeks pregnant (N=12)	36 weeks pregnant (N=12)
Age (y)	22.3±3.3 #	30.1±3.6 ****	28.4±4.6 ****
Parity	0 (0,0)	0 (0,1) #	0 (0,0)
Weight (kg)	53.3±6.9 ###	57.2±7.9 ####	62.3±6.6 *** ####
Height (m)	1.61±0.07	1.59±0.05 ##	1.63±0.06
BMI (kg/m^2)	20.6±1.9 #	22.6±2.9 * ###	23.4±1.8 *** ####

N = number studied in each group. Age, body weight, height and BMI are reported as mean ± standard deviation. Parity is reported as median and interquartile range in parentheses. * refers to comparisons of pregnant groups with non-pregnant, # refers to comparison of SS women with AA. *P< 0.05, **P<0.01, ***P<0.005, ****P<0.001; #P<0.05, ##P<0.01, ###P<0.005, ####P<0.001.

3.3. NON-PREGNANT WOMEN

3.3.1. Haematological variables amongst non-pregnant women

As expected from the two genotypes, there were very marked differences in haematological variables between the HbAA and HbSS women. Summary data are shown in Table 3.3. The much greater degree of scatter in the samples from HbSS women was particularly marked with respect to the white blood cell, neutrophil and lymphocyte counts, for each of which the F (also known as Levene) test was also statistically highly significant ($P<0.005$ for all).

3.3.2. Plasma volume amongst non-pregnant women

The mean plasma volume in the 44 non-pregnant women studied was 2477±825ml. Women of genotype HbSS had significantly higher plasma volumes than women of HbAA (2714±949ml compared with 2165±417ml; $P = 0.018$). The range of values was considerably higher in HbSS women, and an F test showed this to be statistically significant ($F = 5.092$, $P =0.029$).

Table 3.3. Comparison of haematological values between the non-pregnant women.

	HbAA	HbSS
Hb concentration (g/L)	115±11 (N=19)	79±11 (N=24) ****
Haematocrit (%)	0.37±0.02 (N=14)	0.24±0.04 (N=23) ****
Mean cell volume (fl)	89.6±6.7 (N=14)	87.3±8.3 (N=22)
Mean cell haemoglobin (pg)	28.6±2.0 (N=14)	29.0±3.4 (N=23)
Mean cell Hb concentration (g/l)	319.4±9.8 (N=14)	329.4±19.0 (N=23) *
RBC count x 10^9 (cells/L)	4.1±0.4 (N=14)	2.8±0.5 (N=23) ****
WBC count x 10^9 (cells/L)	4.3±0.8 (N=14)	8.4±2.2 (N=21) ****
Lymphocyte count x 10^9 (cells/L)	2.1±0.3 (N=13)	3.5±1.0 (N=18) ****
Neutrophil count x 10^9 (cells/L)	1.7±0.6 (N=11)	3.9±1.6 (N=13) ****
Platelet count x 10^9 (cells/L)	183±54 (N=14)	367±141 (N=23) ****

Hb – haemoglobin concentration, fl – femtolitres, pg – picograms, WBC – white blood cell. (N) refers to number of women studied in each group. Data are reported as mean ± standard deviation. *P<0.05, ****P<0.001.

Plasma volume is often quoted per unit weight (Lund & Donovan, 1967; Hutchins, 1980), or per unit BSA (Viart, 1976; Boer, 1984). Table 3.2 shows that women with Hb SS were significantly lighter than those with HbAA and had significantly lower BMIs. There are a number of equations for calculating the BSA, usually based on those of DuBois & DuBois (DuBois & DuBois, 1916). That of Mosteller (Mosteller, 1987) is now widely-used, and was used to calculate BSA for the women of this thesis.

$$\mathbf{BSA} = \sqrt{\frac{weight * height}{3600}} \qquad \text{Equation 3.1}$$

where weight is measured in kg and height in cm.

The mean BSA for HbSS women was $1.54 \pm 0.13 m^2$ while that for HbAA women was $1.65 \pm 0.14 m^2$ ($P = 0.007$). Table 3.4 summarises the plasma volume in the two groups expressed in terms of body weight, BMI and BSA; significantly higher values are noted in the Hb SS women whichever correction factor is used. There is a statistically significant higher degree of scatter in the Hb SS women when plasma volume is expressed in terms of BMI and BSA respectively ($P<0.05$ in both cases).

Table 3.4. Comparison of plasma volume measurements between the non-pregnant women

	HbAA (N=19)	HbSS (N=25)
PV/bodyweight (ml/kg)	36.1±8.3	51.1±16.8***
PV/BMI (ml/kg/m^2)	97.1±24.4	131.4±42.8***
PV/BSA (ml/m^2)	1308±281	1762±593***

PV – plasma volume, BMI – body mass index, BSA – body surface area. Data are reported as mean ± standard deviation. ***P<0.005.

3.3.3. Plasma and urinary osmolality and electrolytes

It was expected that HbSS women would have higher urine outputs and lower plasma and urinary osmolality and that this would be reflected in the plasma and urinary concentrations of the major electrolytes. The results are summarised in Table 3.5.

However, although both plasma sodium and potassium are slightly higher in SS than AA, this difference is not statistically significant. Both the urinary sodium concentration and urinary Na:K ratio are significantly lower in Hb SS women, though urinary potassium is not. That being so, I went on to calculate the urinary outputs for sodium and potassium, and their fractional excretions, together with the osmolar and free water clearances (Table 3.6). It should be noted that, unfortunately, not all laboratory measurements were available for all samples (see section 2.9.2).

Table 3.5. Urine volume, plasma and urinary electrolyte concentrations in non-pregnant HbAA and HbSS women

	HbAA	HbSS
Urine volume (L)	1.1 (0.8,1.3) N=18	1.9 (1.4,2.4)**** N=23
Posmo (mosmo/kg)	277 (269,283) N=18	273 (268,280) N=25
Uosmo (mosmo/kg)	377 (231,486) N=18	306 (158,386) N=24
Plasma Na (mmol/L)	142(138,145) N=15	143 (138,146) N=22
Urinary Na (mmol/L)	86 (59,118) N=17	58 (46,77)* N=23
Plasma K (mmol/L)	3.8 (3.6,3.9) N=16	3.9 (3.7,4.3) N=25
Urinary K (mmol/L)	8.7 (6.8,22.8) N=17	13.1 (7.5,22.4) N=22
Urinary Na:K ratio	8.1 (4.4,10.3) N=17	5.7 (3.0,7.2)* N=22

Posmo – plasma osmolality, Uosmo – urinary osmolality, Na – sodium, K – potassium. Data are reported as median (interquartile range). N – number of women studied. *P<0.05, ****P<0.001.

Table 3.6. Comparison of osmolar indices between the non-pregnant women

	HbAA	HbSS
Total urinary Na (mmol/day)	78.9 (64.9,128.1) N=17	100.7 (84.2,189.6)* N=23
Total urinary K (mmol/day)	9.4 (7.4,24.0) N=17	22.6 (14.5,38.6)* N=22
FE Na	0.77 (0.46,1.22) N=12	0.76 (0.45,1.44) N=12
FE K	6.14 (3.04,8.05) N=13	6.04 (4.35,14.02) N=13
Osmolar clearance (L/day)	1.20 (0.90,2.03) N=18	1.71 (1.32,2.50)* N=23
Free H20 clearance (L/day)	-0.35 (-0.69,0.18) N=18	-0.10 (-0.68,0.67) N=23

FE Na – fractional excretion of sodium, FE K – fractional excretion of potassium, Free H20 clearance – free water clearance. Data are reported as median (interquartile range). N – number of women studied. *P<0.05.

It can be seen from the above that total urinary sodium and potassium outputs, as well as osmolar clearance, are higher in the HbSS women. It was felt that these differences might reflect alterations in the GFR and this was thus calculated. Although the gold standard for measuring GFR is from 24-hour urine collections, a number of formulae are now available for approximating the GFR from single urine measurements, mostly based on the CG equation (Cockcroft & Gault, 1976). Although the MDRD equation (Levey *et al*., 1999) is now preferred for use outside pregnancy, its use has been heavily criticised in pregnancy (Smith *et al.*, 2008) and I have accordingly used the original CG equation in this thesis.

$$\mathbf{GFR} = \frac{(140 - age) * weight * 1.04}{plasma[creatinine]} \qquad \text{Equation 3.2}$$

where weight is measured in kg and plasma [creatinine] in mmol/L.
The GFR for Hb AA women (N=16) was higher than that of Hb SS (N=14) – 134 (92,156) vs 92 (68,118) ml/min respectively but this stopped short of being statistically significant, p = 0.051.

3.3.4. Hormones

The increased plasma volume in association with HbSS was identified over 30 years ago (Barreras *et al.*, 1966; Steinberg *et al.*, 1977) but the cause is unknown. Endocrine factors that might influence renal tubular sodium handling have not previously been systematically studied. Table 3.7 summarises the median and interquartile range (IQR) values for plasma renin, angiotensinogen and arginine vasopressin concentrations, and serum aldosterone, progesterone and prolactin

concentrations by genotype. No statistically significant differences were observed between non-pregnant HbSS and HbAA women except in prolactin concentration, which was higher in HbAA women.

Table 3.7. Comparison of hormone concentrations in the non-pregnant women

	HbAA	**HbSS**
PRC (ng/ml/hr)	12.6 (6.6,20.8) N=16	11.8 (7.2,18.2) N=23
Aogen (µg/ml)	0.95 (0.45,1.20) N=15	0.98 (0.46,1.48) N=24
ADH (pg/ml)	4.00 (3.40,4.58) N=16	3.80 (3.40,4.73) N=22
Aldosterone (ng/ml)	80.1 (55.8,96.7) N=19	62.7 (42.6,104.0) N=24
Progesterone (ng/ml)	0.8 (0.1,2.1) N=18	0.6 (0.3,1.0) N=23
Prolactin (ng/ml)	80.1 (31.6,138.0) N=19	35.0 (16.3,104.3)* N=25

PRC – Plasma renin concentration, Aogen – angiotensinogen, ADH – arginine vasopressin. Data are reported as median (interquartile range).*P<0.05.

3.3.5. Association between plasma volume and measured indices

With the differences noted above, I went ahead to see if there were any correlations with plasma volume. I also examined other parameters that were likely to be associated. These are shown in figures 3.1 to 3.7 below.

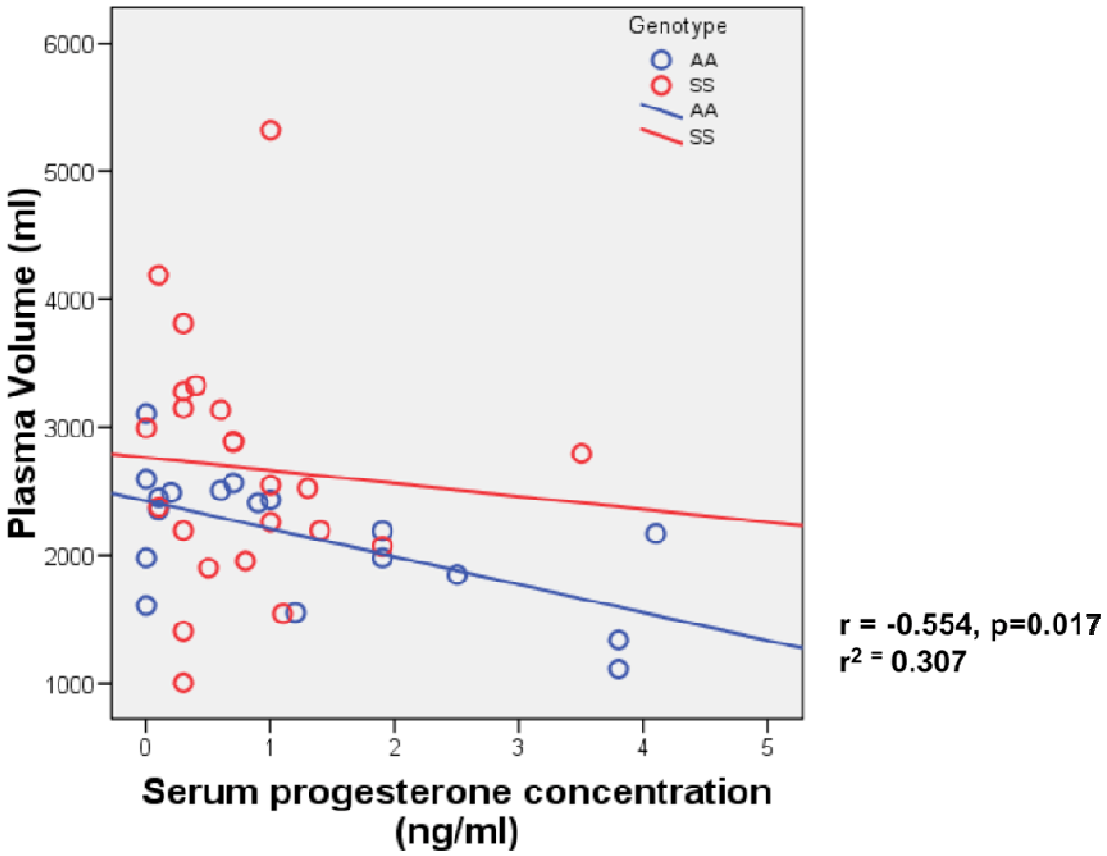


Figure 3.1. A scatter plot showing the relationship between serum progesterone concentration and plasma volume, in non-pregnant Hb AA (N=18) and Hb SS (N=23) women. The computed best line of fit for both groups of women, derived from a linear equation, is displayed. The equation is y = mx + c, where y = plasma volume, x = serum progesterone concentration, c = constant and m = slope.

There is a significant negative correlation between serum progesterone concentration and plasma volume both overall ($r = -0.335$; $P = 0.033$) and in Hb AA women as shown in Figure 3.1. There was a substantial scatter of plasma volumes in the Hb SS group that was not reflected in the serum progesterone concentrations; in these women, the association between the two variables was largely dependent on data from a single woman with high progesterone concentration.

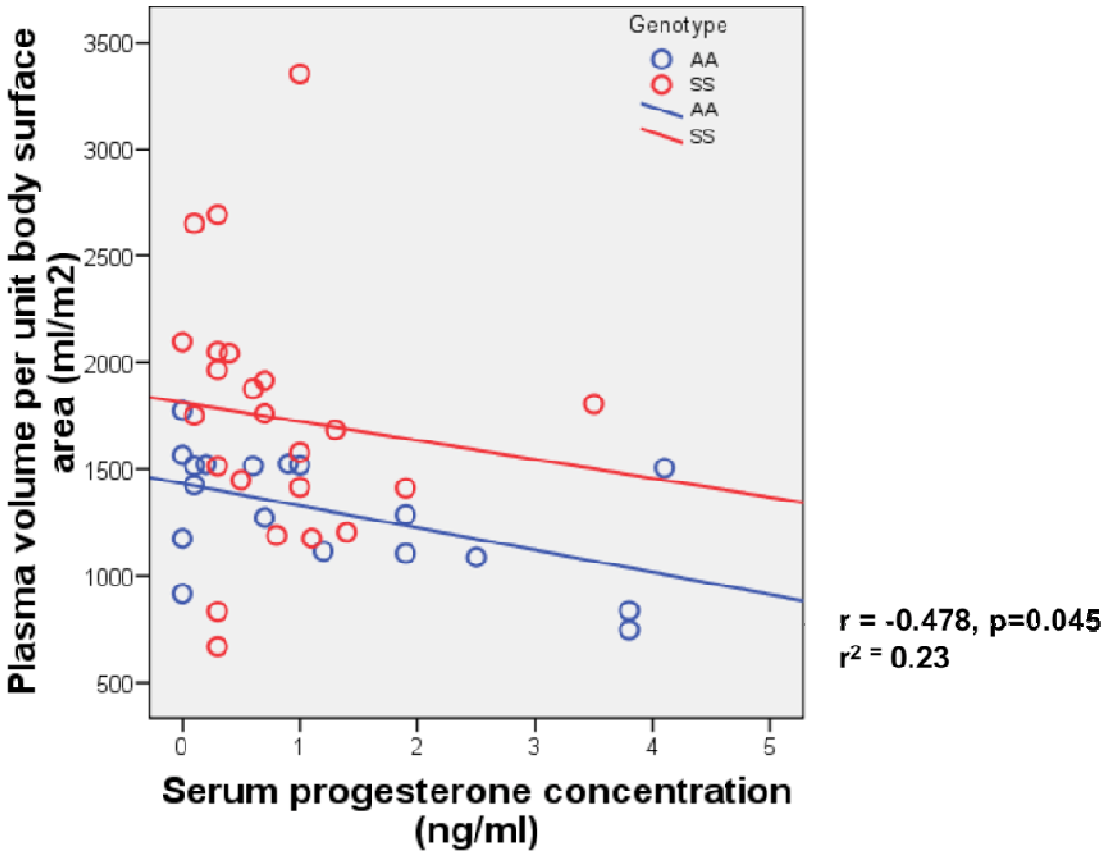


Figure 3.2. A scatter plot showing the relationship between serum progesterone concentration and plasma volume per unit body surface area in non-pregnant Hb AA women (N=18), and Hb SS (N=23) women. The computed best line of fit for both groups of women is displayed as in Fig 3.1. There is a significant negative correlation in Hb AA women as shown.

Here also (Figure 3.2), there was a significant negative correlation between serum progesterone concentration and plasma volume both overall ($r = -0.316$; $P = 0.044$) and in Hb AA women as shown. There was also a greater scatter in the Hb SS women's plasma volume per unit BSA measurements than in the Hb AA women.

There was a positive correlation between serum prolactin concentration and plasma volume in Hb AA women ($r = 0.442$; $P = 0.058$), which stopped short of being statistically significant. However, as shown in Figure 3.3, when plasma volume was expressed per unit BSA, the correlation was statistically significant.

The trend of much greater variability in Hb SS women continues with the positive correlation of plasma volume (Figure 3.4) and plasma volume per unit body surface area (Figure 3.5), with plasma renin concentration in Hb AA women. The greater scatter in Hb SS women's measurements was seen in both scatter plots as well.

With plasma ADH concentration, there is a significant negative correlation with both plasma volume and plasma volume per unit BSA (Figures 3.6 and 3.7 respectively) in Hb SS women, albeit with a larger scatter than that seen in the Hb AA measurements.

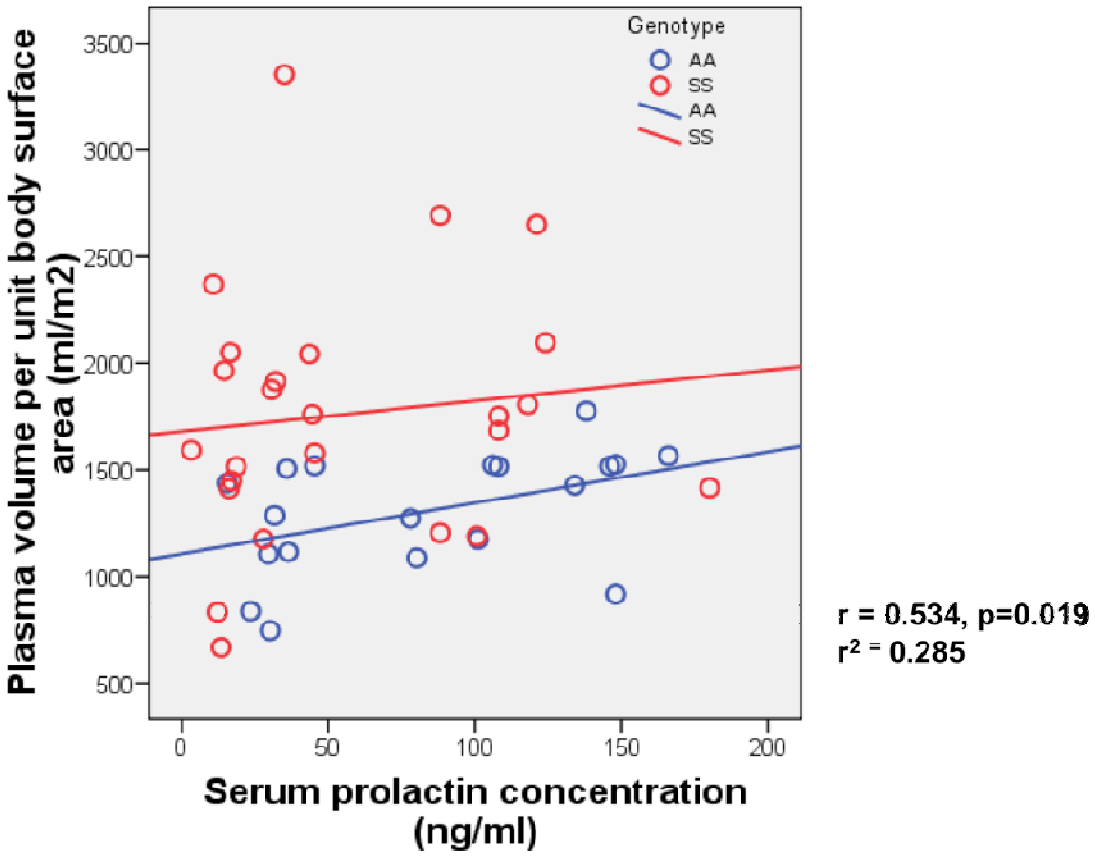


Figure 3.3. A scatter plot showing the relationship between serum prolactin concentration and plasma volume per unit body surface area in non-pregnant Hb AA (N=19) and Hb SS (N=25) women. The computed best line of fit for both groups of women is displayed as in Fig 3.1. There is a significant positive correlation in Hb AA women as shown.

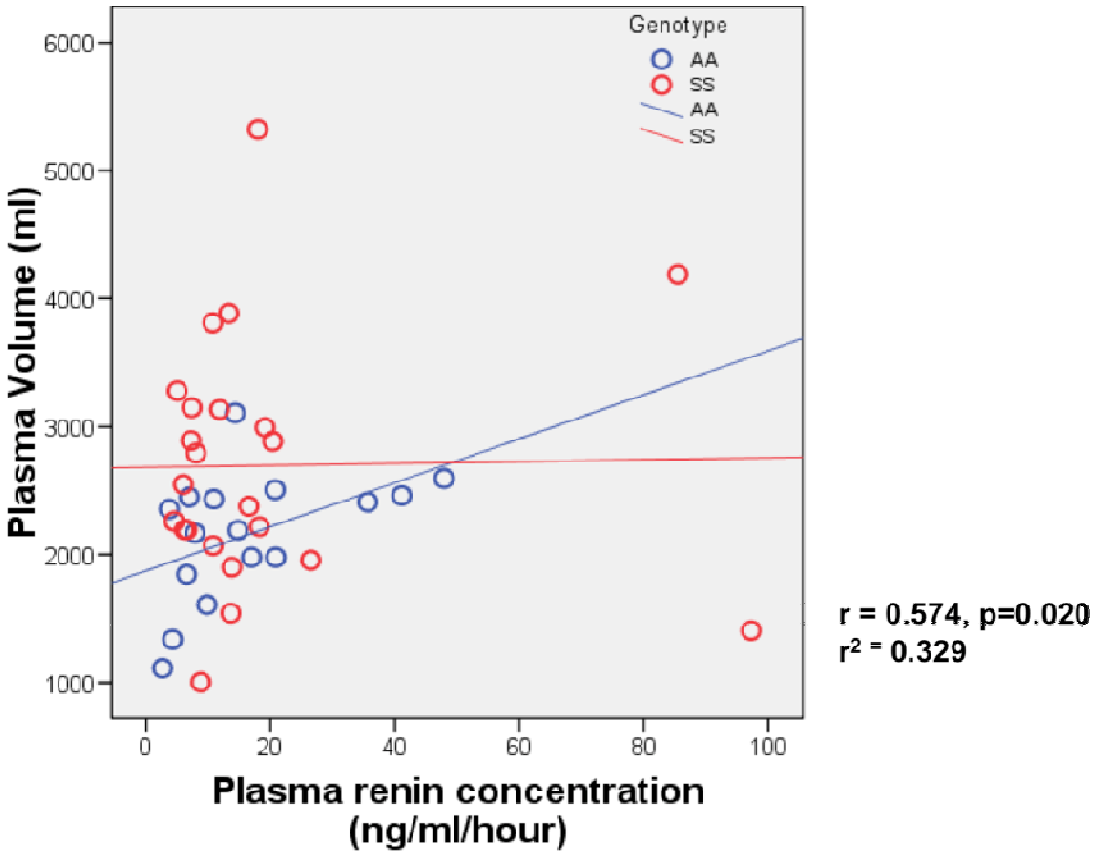


Figure 3.4. A scatter plot showing the relationship between plasma renin concentration and plasma volume in non-pregnant Hb AA (N=16) and Hb SS (N=23) women. The computed best line of fit for both groups of women is displayed as in Fig 3.1. There is a significant positive correlation in Hb AA women as shown.

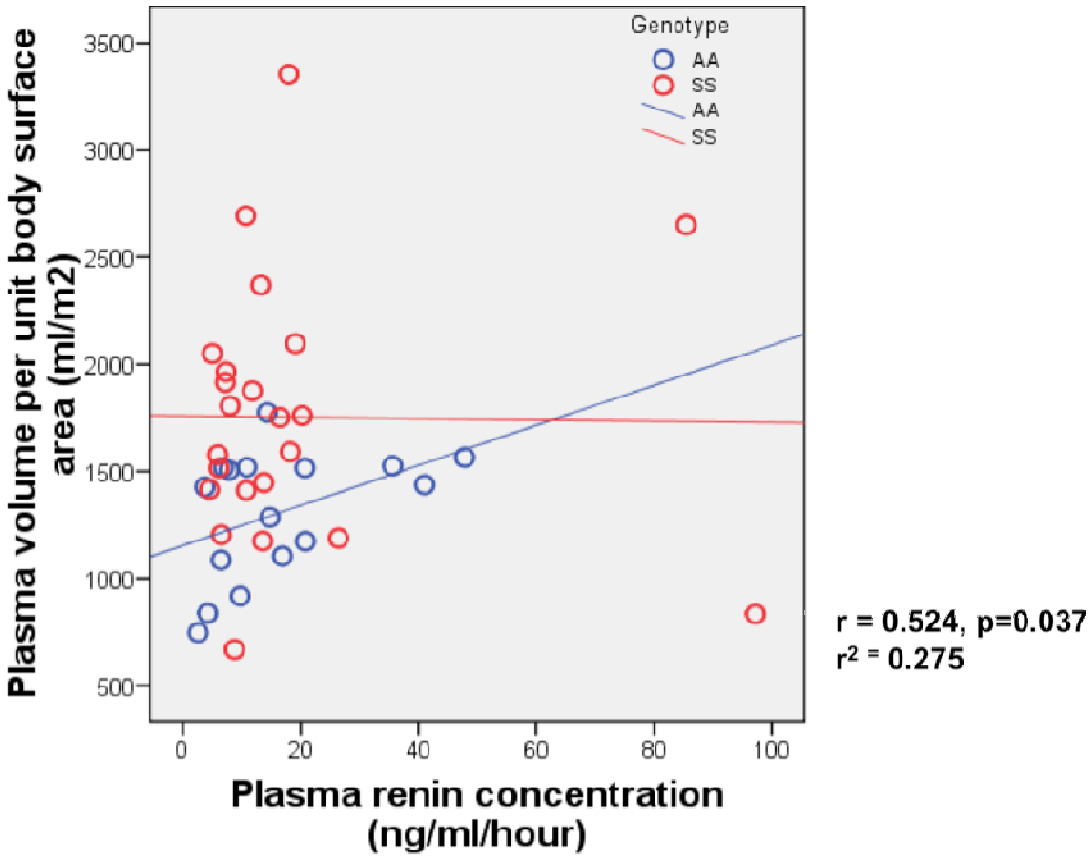


Figure 3.5. A scatter plot showing the relationship between plasma renin concentration and plasma volume per unit body surface area in non-pregnant Hb AA (N=16) and Hb SS (N=23) women. The computed best line of fit for both groups of women is displayed as in Fig 3.1. There is a significant positive correlation in Hb AA women as shown.

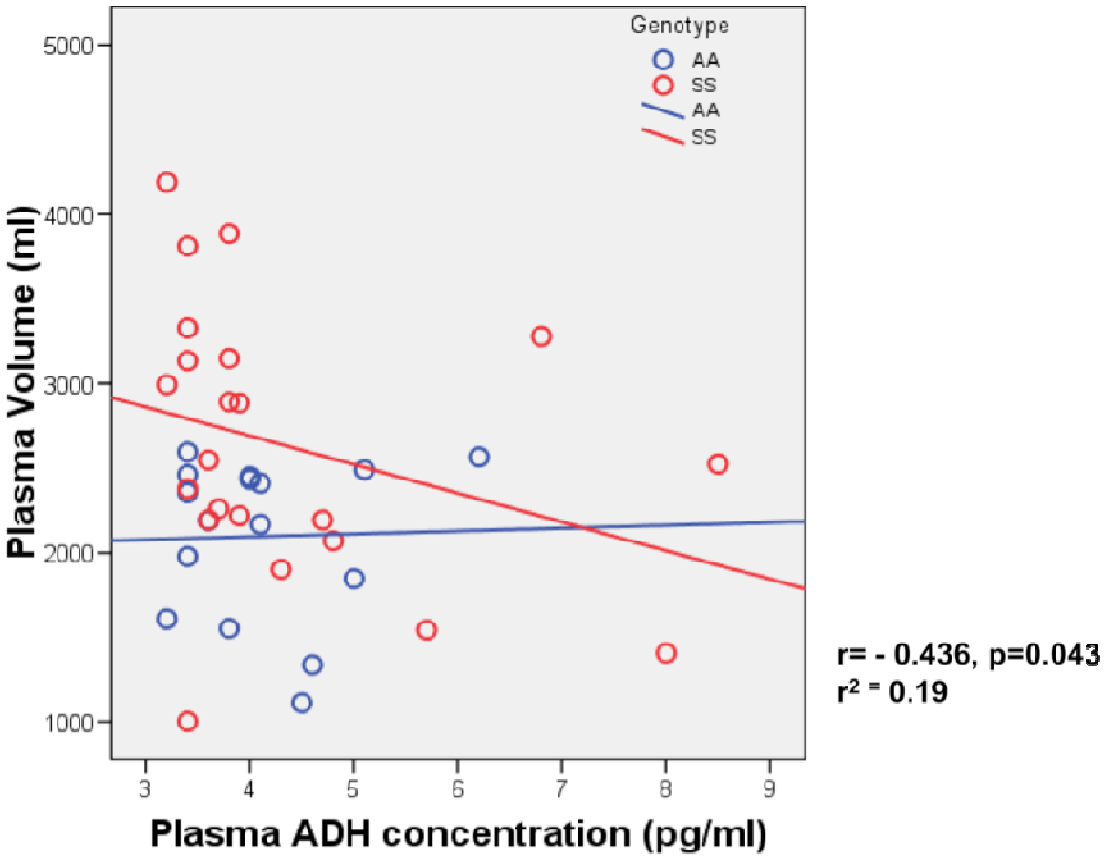


Figure 3.6. A scatter plot showing the relationship between plasma Arginine Vasopressin (ADH) concentration and plasma volume in non-pregnant Hb AA (N=16) and Hb SS women (N=22). The computed best line of fit for both groups of women is displayed as in Fig 3.1. There is a significant negative correlation in Hb SS women as shown.

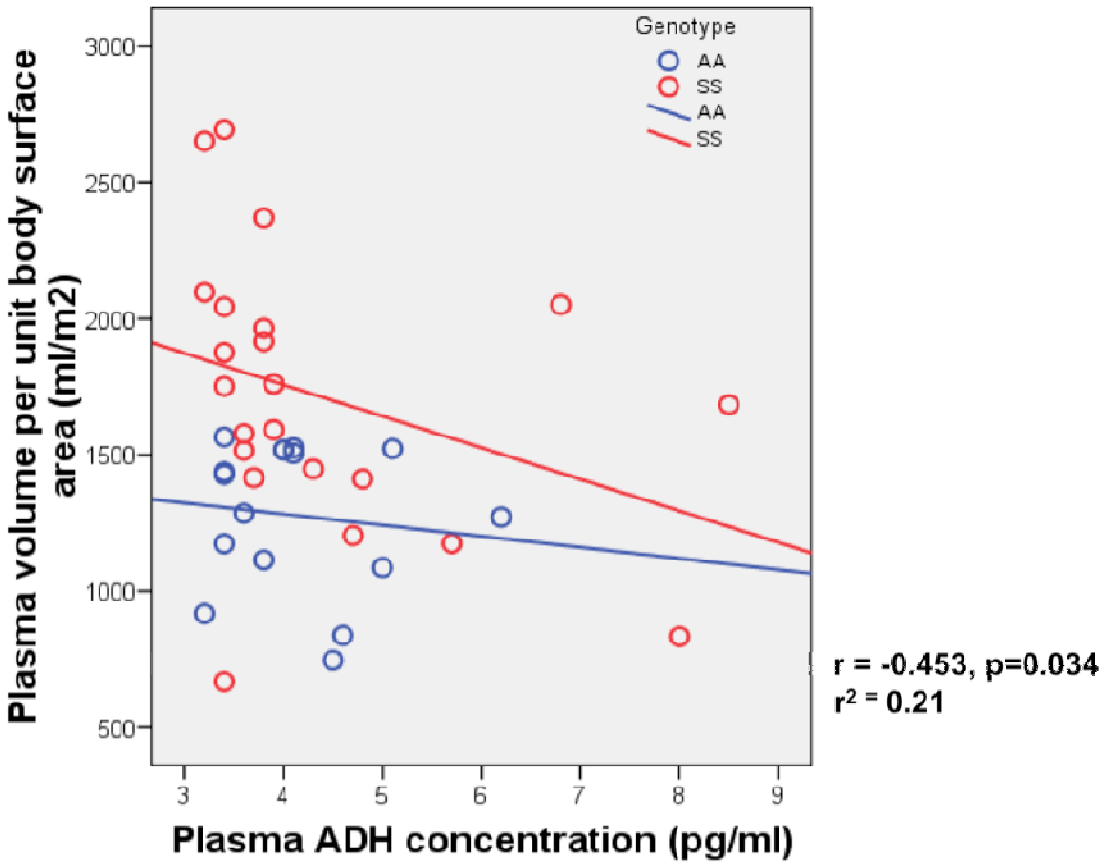


Figure 3.7. A scatter plot showing the relationship between plasma Arginine Vasopressin (ADH) concentration and plasma volume per unit body surface area in non-pregnant Hb AA (N=16) and Hb SS women (N=22). The computed best line of fit for both groups of women is displayed as in Fig 3.1. There is a significant negative correlation in Hb SS women as shown.

4. PREGNANT SICKLE CELL STUDY RESULTS

In this chapter, I will report results from all the pregnant women, compare them with the non-pregnant and also compare Hb SS pregnant with the Hb AA pregnant women.

4.1. ALL PREGNANT WOMEN

I decided to compare all the pregnant women together first, as done with the non-pregnant women, before splitting them into early and late pregnancy as appropriate.

4.1.1. Haematological variables amongst pregnant women

As in the non-pregnant women, there were also marked differences in most of the haematological parameters between HbAA and HbSS women (Table 4.1) but there was not much difference in scatter between the variables except with respect to platelet count ($F = 7.635$, $P = 0.009$).

Table 4.1. Comparison of haematological values between the pregnant women

	HbAA	HbSS
Hb concentration (g/L)	102±12 (N=19)	73±12 (N=23) ****
Haematocrit (%)	0.32±0.04 (N=17)	0.22±0.04 (N=23) ****
Mean cell volume (fl)	87.7±5.3 (N=16)	91.8±8.9 (N=23)
Mean cell haemoglobin (pg)	28.1±2.2 (N=16)	30.2±3.5 (N=23) *
Mean cell Hb concentration (g/L)	320.8±12.1 (N=16)	329.0±13.5 (N=23)
RBC count x 10^9 (cells/L)	3.7±0.4 (N=16)	2.4±0.4 (N=23) ****
WBC count x 10^9 (cells/L)	5.9±2.2 (N=16)	10.4±2.6 (N=22) ****
Lymphocyte count x 10^9 (cells/L)	1.5±0.5 (N=15)	3.0±1.3 (N=22) ****
Neutrophil count x 10^9 (cells/L)	4.2±1.8 (N=14)	6.5±1.8 (N=20) ****
Platelet count x 10^9 (cells/L)	192±60 (N=16)	429±129 (N=23) ****

Hb – haemoglobin concentration, fl – femtolitres, pg – picograms, WBC – white blood cell, RBC – red blood cell. (N) refers to number of women studied in each group. Data are reported as mean ± standard deviation. *P<0.05, ****P<0.001.

4.1.2. Plasma volume amongst pregnant women

Unlike the non-pregnant state, there was no significant difference in the various plasma volume measurements between all pregnant Hb AA and Hb SS women taken together (Table 4.2). There was also no significant difference when the two genotype groups were compared separately at 16 and 36 weeks gestation respectively. However, comparisons between pregnant and non-pregnant HbAA and HbSS women revealed some interesting differences (see Section 4.2 below).

Table 4.2. Comparison of plasma volume between all pregnant women

	Hb AA (N=20)	**Hb SS (N=22)**
Plasma volume (ml)	3000±1004	2881±1150
PV/bodyweight (ml/kg)	40.3±14.9	49.5±22.1
PV/BMI (ml/kg/m^2)	108.3±37.9	128.0±57.9
PV/BSA (ml/m^2)	1626±569	1785±744

PV – plasma volume, BMI – body mass index, BSA – body surface area. Data are reported as mean ± standard deviation.

4.1.3. Plasma and urinary osmolality and electrolytes

There was no significant difference in urine volume or plasma and urinary osmolality and electrolytes with the exception of plasma potassium, which was significantly higher in Hb SS women than Hb AA respectively (Table 4.3). Due to the limited urinary data available during pregnancy, fractional excretions, clearances and glomerular filtration rate were not subjected to statistical analysis.

Table 4.3. Urine volume, plasma and urinary electrolyte concentrations in pregnant Hb AA and Hb SS women

	Hb AA	Hb SS
Urine volume (L)	1.8 (1.2,2.4) N=17	2.4 (1.3,2.8) N=19
Posmo (mosmo/kg)	270 (264,275) N=15	268 (264,272) N=12
Uosmo (mosmo/kg)	323 (122,379) N=16	245 (222,287) N=16
Plasma Na (mmol/L)	142(138,147) N=18	138 (132,144) N=19
Urinary Na (mmol/L)	77 (59,113) N=17	68 (51,120) N=11
Plasma K (mmol/L)	3.4 (3.2,3.8) N=19	3.9 (3.6,4.6) N=21*
Urinary K (mmol/L)	8.8 (5.8,31.2) N=17	11.6 (7.6,14.7) N=11
Urinary Na:K ratio	7.0 (3.2,11.6) N=17	8.4 (4.9,10) N=11

Posmo – plasma osmolality, Uosmo – urinary osmolality, Na – sodium, K – potassium. Data are reported as median (interquartile range). N – number of women studied. *P<0.05.

4.1.4. Hormones (Table 4.4)

All the hormones studied apart from ADH usually rise in normal human pregnancy. When they were compared between genotypes there were no significant differences except in PRC and the scatter in this case was greater in Hb AA women than in Hb SS (Figure 4.1).

Table 4.4. Comparison of hormone concentrations in pregnant women

	HbAA	**HbSS**
PRC (ng/ml/hr)	49.85(20.53,66.88) N=17	21.97(11.15,33.93) N=19**
Aogen (µg/ml)	2.19(1.59,3.64) N=17	2.37(1.30,4.11) N=18
ADH (pg/ml)	4.00(3.80,8.20) N=19	3.65(3.40,4.28) N=10
Aldosterone (ng/ml)	149.4(87.1,200.6) N=18	126.4(81.4,151.3) N=16
Progesterone (ng/ml)	41.4(17.9,68.0) N=16	31.1(19.4,65.1) N=16
Prolactin (ng/ml)	156.1(133.0,312.2) N=19	162.1(83.9,291.1) N=17

PRC – Plasma renin concentration, Aogen – angiotensinogen, ADH – arginine vasopressin. Data are reported as median (interquartile range). **P<0.01.

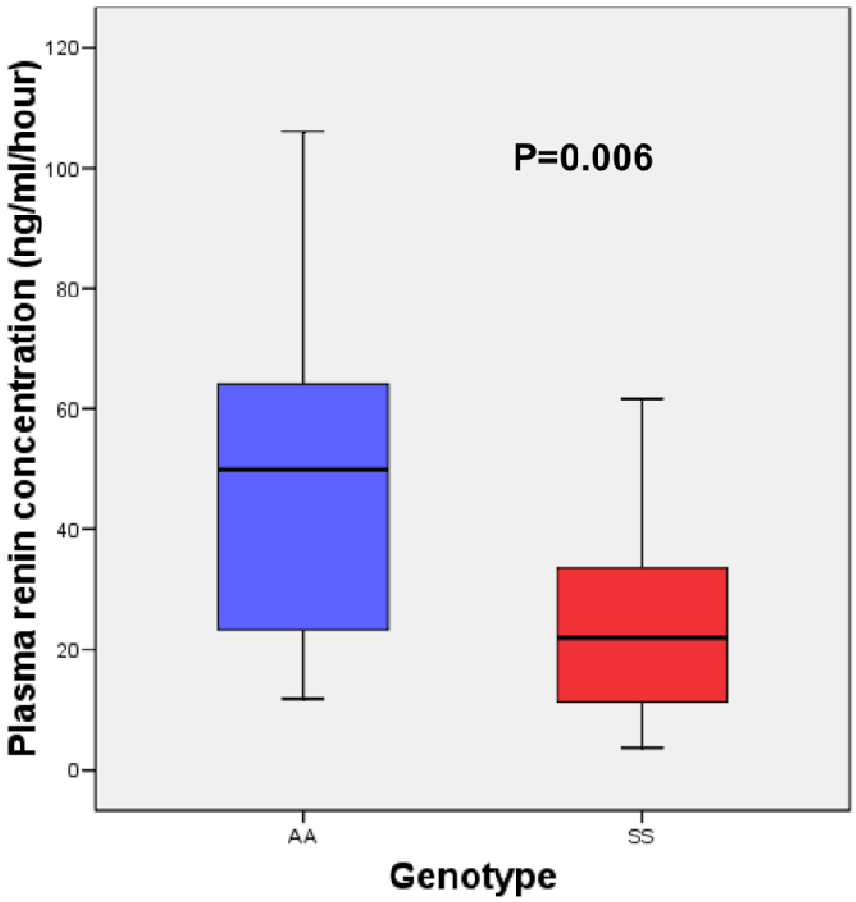


Figure 4.1. Box-plot of plasma renin concentration amongst pregnant women according to genotype. Data were available from 17 and 19 Hb AA and SS women respectively.

As there were no differences in plasma volume measurements when all the pregnant women were compared according to genotype (Table 4.2) although significant differences had been identified in the non-pregnant women, I decided to compare non-pregnant with pregnant women of the same genotype, first to see if there were any differences in plasma volume as previously found (Abudu & Sofola, 1988), and to examine the electrolytes and hormones in this context.

4.2. PREGNANT VERSUS NON-PREGNANT WOMEN

These comparisons enable us examine what happens in pregnancy in each individual genotype.

4.2.1. Pregnant versus non-pregnant Hb AA women

The comparisons were made between all pregnant and non-pregnant Hb AA women. In cases where the findings were felt to merit further consideration, comparisons were also made between non-pregnant and each of the gestational age groups.

4.2.1.1. Haematological variables in pregnant and non-pregnant AA women

Haematocrit, haemoglobin concentration and red cell count were lower in pregnancy, as expected whilst white cell count and neutrophil count were significantly higher (Table 4.5).

Table 4.5. Comparison of haematological values in non-pregnant and pregnant Hb AA women

	Non-pregnant	Pregnant
Hb concentration (g/L)	115±11 (N=19)	102±12 (N=19) ***
Haematocrit (%)	0.37±0.02 (N=14)	0.32±0.04 (N=17) ***
Mean cell volume (fl)	89.6±6.7 (N=14)	87.7±5.3 (N=16)
Mean cell haemoglobin (pg)	28.6±2.0 (N=14)	28.1±2.2 (N=16)
Mean cell Hb concentration (g/L)	319.4±9.8 (N=14)	320.8±12.1 (N=16)
RBC count x 10^9 (cells/L)	4.1±0.4 (N=14)	3.7±0.4 (N=16) *
WBC count x 10^9 (cells/L)	4.3±0.8 (N=14)	5.9±2.2 (N=16) *
Lymphocyte count x 10^9 (cells/L)	2.1±0.3 (N=13)	1.5±0.5 (N=15) ***
Neutrophil count x 10^9 (cells/L)	1.7±0.6 (N=11)	4.2±1.8 (N=14) ****
Platelet count x 10^9 (cells/L)	183±54 (N=14)	192±60 (N=16)

Hb – haemoglobin concentration, fl – femtolitres, pg – picograms, WBC – white blood cell, RBC – red blood cell. (N) refers to number of women studied in each group. Data are reported as mean ± standard deviation. *P<0.05, ***P < 0.005, ****P<0.001.

4.2.1.2. Plasma volume indices in pregnant and non-pregnant women

The expected rise in plasma volume in Hb AA pregnancy was seen and found to be significant both at 16 weeks and at 36 weeks pregnancy when compared to non-pregnant women (Table 4.6). The increase appeared to be almost complete by 16 weeks. There was also a rise in the other plasma volume indices but none of these reach statistical significance at either 16 or 36 weeks, although the rise in plasma volume per unit body surface area overall in pregnancy (Table 4.6) did achieve statistical significance.

Table 4.6. Comparison of PV in pregnant and non-pregnant women

	AA (N=39)			
	NP (N=19)	**16 weeks P (N=10)**	**36 weeks P (N=10)**	**All P (N=20)**
Plasma volume (ml)	2165±497	2911±1020*	3089±1035*	3000±1004***
PV/bodyweight (ml/kg)	36.1±8.3	40.1±14.4	40.6±16.1	40.3±14.9
PV/BMI ($ml/kg/m^2$)	97.1±24.4	108.9±37.8	107.8±40.0	108.3±37.9
PV/BSA (ml/m^2)	1308±281	1593±564	1659±603	1626±569*
	SS (N=47)			
	NP (N=25)	**16 weeks P (N=11)**	**36 weeks P (N=11)**	**All P (N=22)**
PV (ml)	2714±949	2758±913	3003±1382	2881±1150
PV/weight (ml/kg)	51.1±16.8	50.2±19.7	48.9±25.3	49.5±22.1
PV/BMI (ml/kg/m2)	131.4±42.8	126.5±51.9	129.5±66.0	128.0±57.9
PV/BSA (ml/m2)	1762±593	1768±623	1801±879	1785±744

N = number of women studied, P = pregnant, NP = non-pregnant. Data are reported as mean ± SD. *P<0.05, ***P<0.005. * refers to comparison with non-pregnant.

4.2.1.3. Plasma and urinary osmolality, electrolytes and renal indices

Only limited data could be obtained at 36 weeks' gestation; these are shown in Table 4.7 but were not subjected to separate statistical analysis other than trend analysis as indicated. Urine volume increased significantly in pregnancy as expected and plasma osmolality decreased also as expected. Trend analysis showed the fall in plasma osmolality and rise in urine volume to deepen progressively as gestation advanced in HbAA women ($P = 0.014$; $P<0.001$), but not in HbSS ($P = 0.084$; $P>0.5$). Overall, both plasma and urinary osmolality were inversely associated with urine volume ($P =0.027$; $P<0.001$). The only other significant changes were the reduction in plasma potassium concentration and the higher total urinary sodium output in pregnancy but there were no significant changes in plasma or urinary sodium concentration.

4.2.1.4. Hormone concentrations

As expected in normal Hb AA pregnancy, there was an increase in pregnancy of all the hormones measured except for arginine vasopressin (Table 4.8).

Table 4.7. Urine volume, plasma and urinary osmolality and electrolyte concentrations in Hb AA women

	Non-pregnant	16 weeks pregnant	36 weeks pregnant	**All pregnant**
Urine volume (L)	**1.1** (0.8,1.3) N=18	**1.5**(1.2,2.2) N=10*	**2.0**(1.2,3.1) N=7	**1.8**(1.2,2.4) N=17***
Posmo (mosmo/kg)	**277**(269,283) N=18	**270**(267,278) N=10	**264**(262,274) N=5	**270**(264,275) N=15*
Usomo (mosmo/kg)	**377**(231,486) N=18	**323**(171,421) N=10	**227**(87,417) N=6	**323**(122,379) N=16
Plasma Na (mmol/L)	**142**(138,145) N=15	**138**(135,146) N=9	**144**(140,149) N=9	**142**(138,147) N=18
Urinary Na (mmol/L)	**86** (59,118) N=17	**87**(71,123) N=10	**56**(43,79) N=7	**77** (59,113) N=17
Plasma K (mmol/L)	**3.8** (3.6,3.9) N=16	**3.4**(3.0,3.7) N=10**	**3.5**(3.4,4.1) N=9	**3.4** (3.2,3.8) N=19*
Urinary K (mmol/L)	**8.7** (6.8,22.8) N=17	**8.3**(6.3,25.9) N=10	**11.5**(4.8,41.9) N=7	**8.8** (5.8,31.2) N=17
Urinary Na:K ratio	**8.1** (4.4,10.3) N=17	**9.5**(4.7,15.2) N=10	**3.7**(2.4,7.9) N=7	**7.0** (3.2,11.6) N=17
Total urine Na (mmol/day)	**78.9**(64.9,128.1)N=17	**151.3**(83.8,216.5)N=10*	**99.4** (65.2,129.2) N=6	**124.2**(79.9,171.8)N=16*
GFR (ml/min)	**133.9** (92.2,156.3) N=16	**131.3** (94.8,196.1) N=10	**140.4** (N=3)	**132.6** (107.1,182.6) N=13

Posmo – plasma osmolality, Uosmo – urinary osmolality, Na – sodium, K – potassium. Data are reported as median (interquartile range). N – number of women studied. *P<0.05, **P<0.01, ***P<0.005. * refers to comparison with non-pregnant women.

Table 4.8. Hormone concentrations in Hb AA women

	Non-pregnant	16 weeks pregnant	36 weeks pregnant	**All pregnant**
PRC (ng/ml/hr)	12.59(6.58,20.79) N=16	32.04(19.15,58.64) N=8**	52.38(32.05,70.74) N=9***	49.85(20.53,66.88) N=17****
Aogen (µg/ml)	0.95 (0.45,1.20) N=15	1.78 (1.03,2.16) N=8*	3.58(2.39,4.28) N=9****	2.19(1.59,3.64) N=17****
ADH (pg/ml)	4.00 (3.40,4.58) N=16	3.90(3.55,6.90) N=10	4.20(3.90,8.50) N=9	4.00(3.80,8.20) N=19
Aldosterone (ng/ml)	80.1(55.8,96.7) N=19	103.1(74.5,158.3) N=9	197.1(149.4,205.6) N=9***	149.4(87.1,200.6) N=18***
Progesterone (ng/ml)	0.8 (0.1,2.1) N=18	17.9(9.3,28.1) N=8****	61.6(46.4,74.7) N=8****	41.4(17.9,68.0) N=16****
Prolactin (ng/ml)	80.1 (31.6,138.0) N=19	134.0(105.8,160.1) N=9*	295.2(148.5,419.5) N=10****	156.1(133.0,312.2) N=19****

PRC – Plasma renin concentration, Aogen – angiotensinogen, ADH – arginine vasopressin. Data are reported as median (interquartile range).*P<0.05, **P<0.01, ***P<0.005, ****P<0.001.

4.2.1.5. Association between hormones and plasma volume indices (Figures 4.2 to 4.6)

There was a significant positive correlation between plasma volume and PV per unit BSA respectively, and log10 angiotensinogen (P=0.01; P=0.018) in all pregnant AA women. At 16 weeks gestation, there was a significant positive correlation between PV and PV per unit BSA, and log 10 aldosterone (P=0.046; P=0.044) and at 36 weeks gestation, there was also a significant positive correlation between PV and log 10 angiotensinogen.

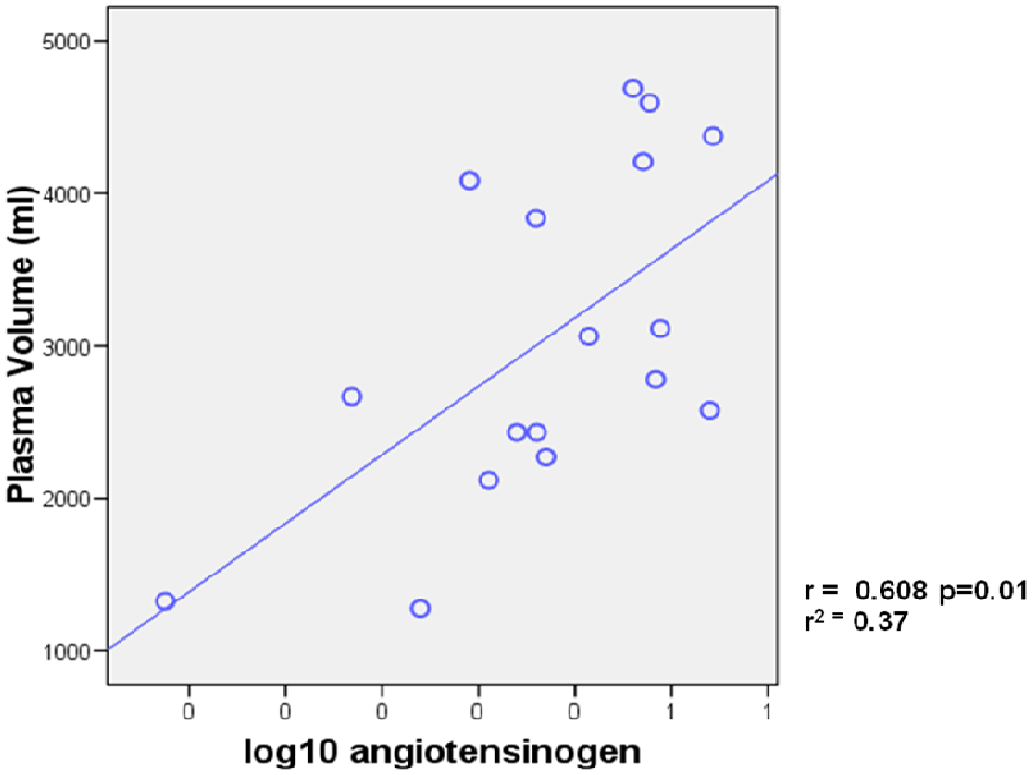


Figure 4.2. A scatter plot showing the relationship between angiotensinogen concentration and plasma volume in all pregnant Hb AA (N=17) women. The computed best line of fit derived from a linear equation, y = mx + c, is displayed, where y = PV, x = log 10 angiotensinogen, c = constant and m = slope.

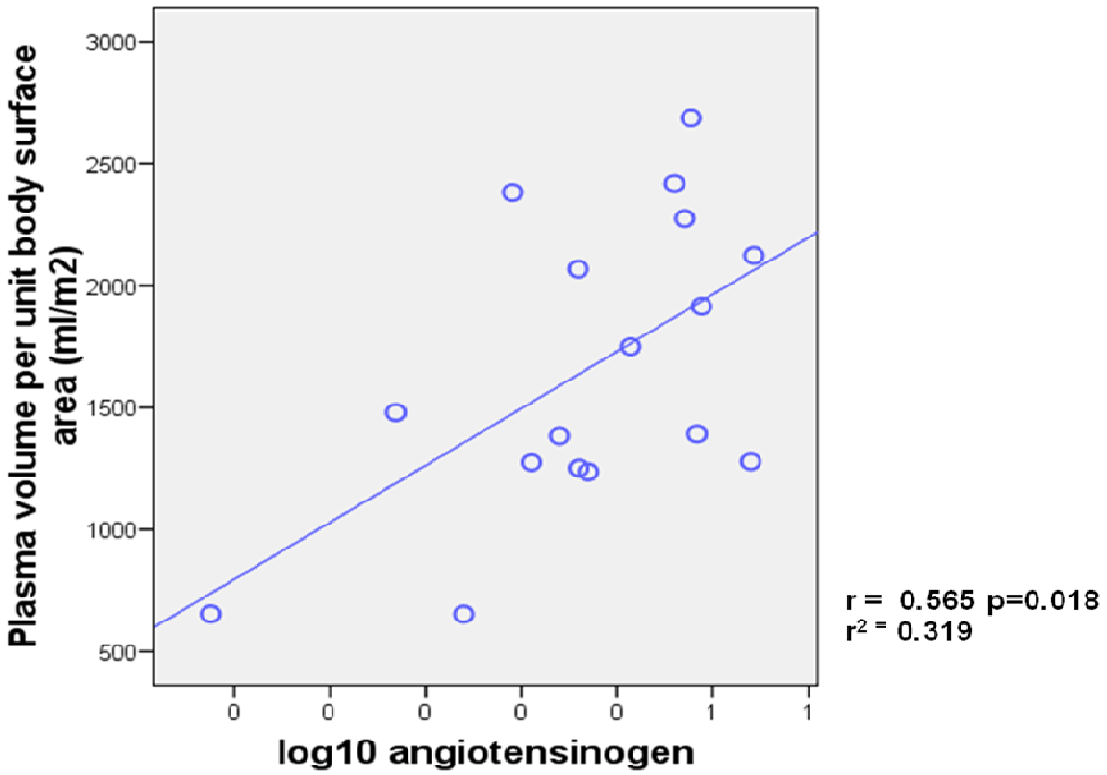


Figure 4.3. A scatter plot showing the relationship between angiotensinogen concentration and plasma volume per unit body surface area in all pregnant Hb AA (N=17) women. The computed best line of fit is displayed as in Fig 4.2 above.

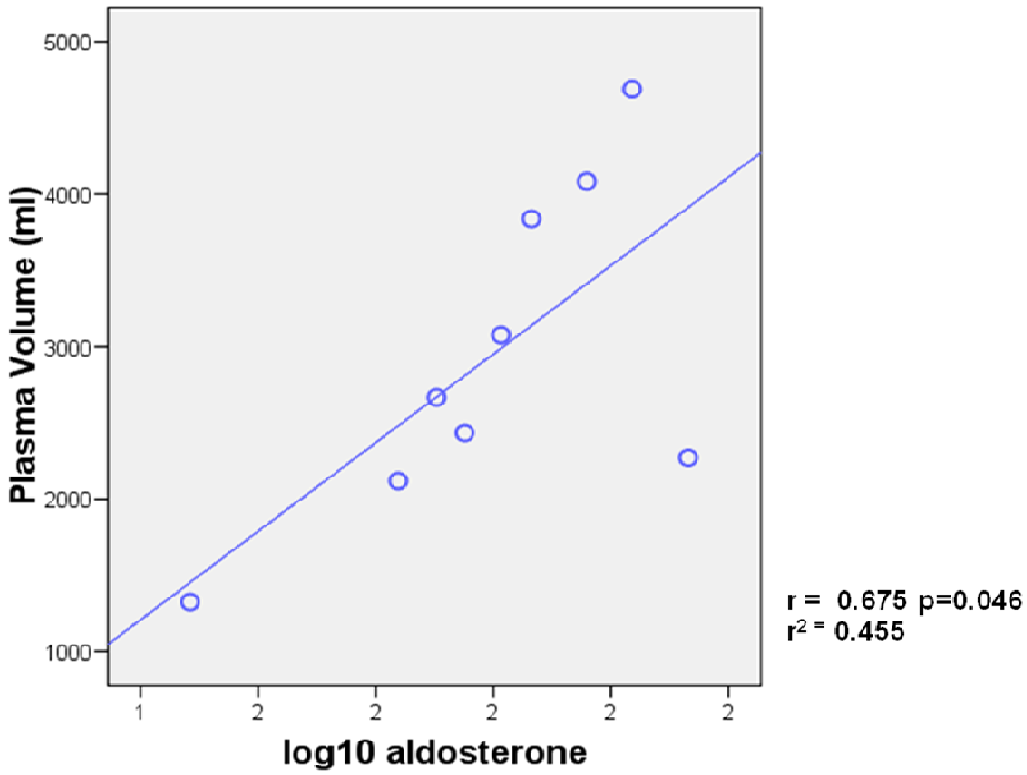


Figure 4.4. A scatter plot showing the relationship between aldosterone concentration and plasma volume in 16 weeks pregnant Hb AA (N=9) women. The computed best line of fit is displayed as in Fig 4.2 above.

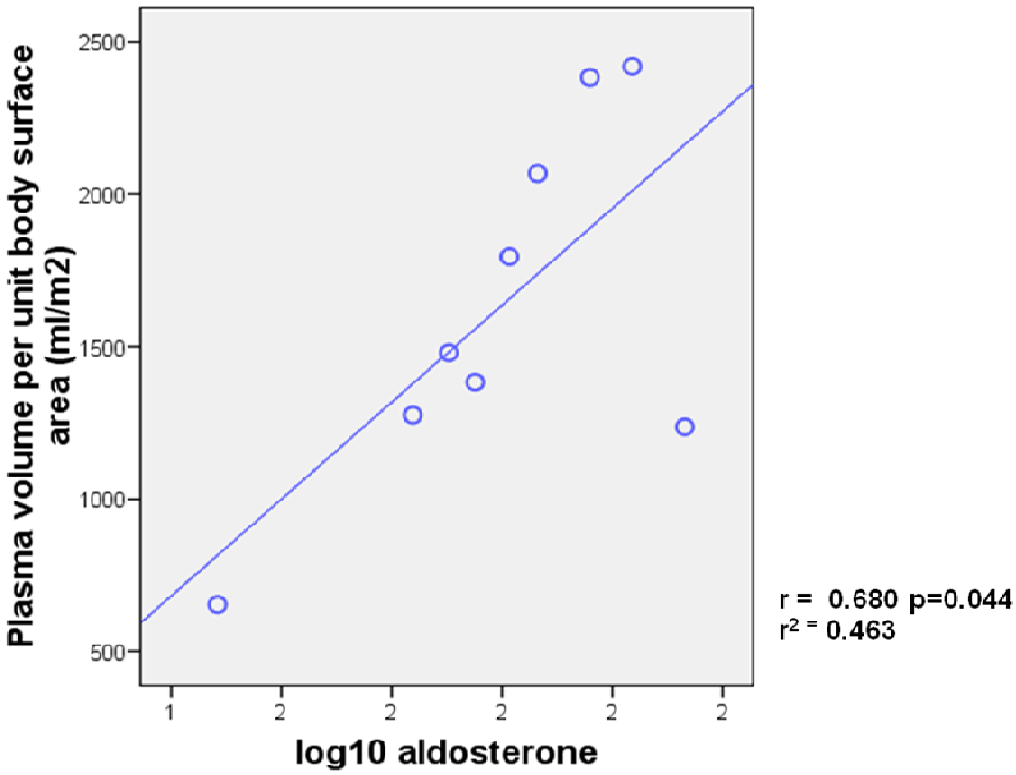


Figure 4.5. A scatter plot showing the relationship between aldosterone concentration and plasma volume per unit body surface area in 16 weeks pregnant Hb AA (N=9) women. The computed best line of fit is displayed as in Fig 4.2 above.

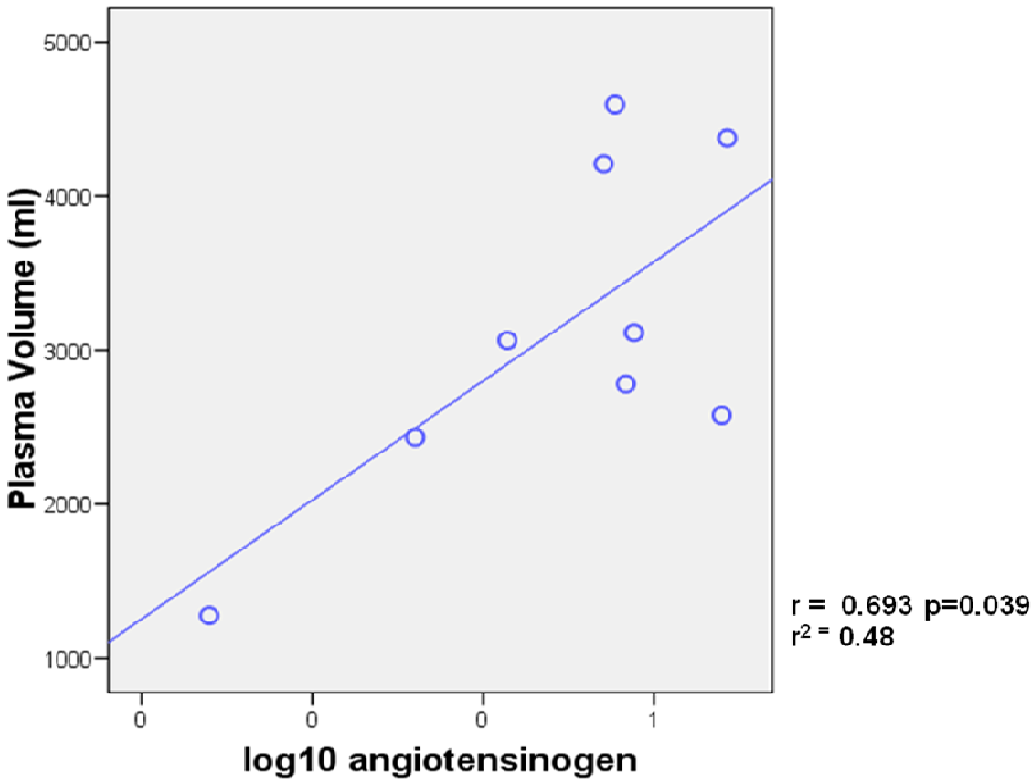


Figure 4.6. A scatter plot showing the relationship between angiotensinogen concentration and plasma volume in 36 weeks pregnant Hb AA (N=9) women. The computed best line of fit is displayed as in Fig 4.2 above.

4.2.2. Pregnant versus non-pregnant Hb SS women

The same comparisons done in Hb AA women were repeated in Hb SS women.

4.2.2.1. Haematological variables in pregnant and non-pregnant SS women

Unlike in Hb AA women, although both haematocrit and haemoglobin concentrations fell slightly in pregnant Hb SS women when compared with the non-pregnant, the difference was not significant. Similar to the AA women however, red cell count was significantly lower whilst white cell count and neutrophil count were significantly higher in Hb SS pregnancy (Table 4.9).

Table 4.9. Comparison of haematological values in Hb SS women

	Non-pregnant	Pregnant
Hb concentration (g/L)	79±11 (N=24)	73±12 (N=23)
Haematocrit (%)	0.24±0.04 (N=23)	0.22±0.04 (N=23)
Mean cell volume (fl)	87.3±8.3 (N=22)	91.8±8.9 (N=23)
Mean cell haemoglobin (pg)	29.0±3.4 (N=23)	30.2±3.5 (N=23)
Mean cell Hb concentration (g/L)	329.4±19.0 (N=23)	329.0±13.5 (N=23)
RBC count x 10^9 (cells/L)	2.8±0.5 (N=23)	2.4±0.4 (N=23)*
WBC count x 10^9 (cells/L)	8.4±2.2 (N=21)	10.4±2.6 (N=22)*
Lymphocyte count x 10^9 (cells/L)	3.5±1.0 (N=18)	3.0±1.3 (N=22)
Neutrophil count x 10^9 (cells/L)	3.9±1.6 (N=13)	6.5±1.8 (N=20)****
Platelet count x 10^9 (cells/L)	367±141 (N=23)	429±129 (N=23)

Hb – haemoglobin concentration, fl – femtolitres, pg – picograms, WBC – white blood cell, RBC – red blood cell. (N) refers to number of women studied in each group. Data are reported as mean ± standard deviation.

*P<0.05, ****P<0.001.

4.2.2.2. Plasma volume indices in pregnant and non-pregnant women (Table 4.6)

There were no statistically significant differences in plasma volume indices in pregnant Hb SS women, compared with non-pregnant. This is unlike Hb AA pregnancy where there is an increase in PV at 16 and 36 weeks and in PV/BSA overall.

4.2.2.3. Plasma volume at different gestational age groups

There were no statistically significant differences in any of the plasma volume measurements between Hb SS and Hb AA women, at 16 and at 36 weeks gestation (Table 4.6). Thus PV does not rise in Hb SS pregnancy and neither is it significantly different from the volumes in Hb AA pregnancy, regardless of the form of PV measurement.

4.2.2.4. Plasma and urinary osmolality and electrolytes

There were no significant differences in any of the renal indices that had sufficient data for a meaningful statistical analysis to be performed. All the available data are shown in Table 4.10 below.

4.2.2.5. Hormones

As in Hb AA pregnancy, there was an increase in most hormones in both early and late pregnancy and in pregnancy as a whole from the non-pregnant state (Table 4.11). The only exceptions to this rule were arginine vasopressin, which did not rise at all, and plasma renin concentration, which rose in early pregnancy and in pregnancy in general, but did not rise significantly in late pregnancy.

Table 4.10. Urinary volume, plasma and urinary electrolyte concentrations in Hb SS women

	Non-pregnant	16 weeks pregnant	36 weeks pregnant	All pregnant
Urine volume (L)	**1.9** (1.4,2.4)N=23	**2.4** (1.4,2.8)N=11	**1.6** (1.2,3.7) N=8	**2.4** (1.3,2.8) N=19
Posmo (mosmo/kg)	**273**(268,280)N=25	**267**(263,272)N=9	**268** N=3	**268** (264,272)N=12
Usomo (mosmo/kg)	**306**(158,386)N=24	**239**(229,282)N=11	**281**(136,386) N=5	**245** (222,287)N=16
Plasma Na(mmol/L)	**143**(138,146)N=22	**144**(137,148)N=9	**134**(131,139) N=10	**138** (132,144)N=19
Urinary Na(mmol/L)	**58** (46,77) N=23	**57**(51,122)N=9	**72** N=2	**68** (51,120) N=11
Plasma K (mmol/L)	**3.9** (3.7,4.3) N=25	**3.7**(3.2,4.5)N=11	**4.1** (3.7,4.6) N=10	**3.9** (3.6,4.6) N=21
Urinary K(mmol/L)	**13.1**(7.5,22.4)N=22	**12.4**(8.0,15.8)N=9	**6.3** N=2	**11.6**(7.6,14.7)N=11
Urinary Na:K ratio	**5.7** (3.0,7.2)N=22	**6.4**(4.0,9.3)N=9	**11.8** N=2	**8.4** (4.9,10.0)N=11
GFR (ml/min)	**92**(68,118) N=14	**133**(105,153)N=5	**138** N=1	**135** (117,146)N=6

Posmo – plasma osmolality, Uosmo – urinary osmolality, Na – sodium, K – potassium. Data are reported as median (interquartile range). N – number of women studied.

Table 4.11. Hormone concentrations in Hb SS women

	Non-pregnant	16 weeks pregnant	36 weeks pregnant	**All pregnant**
PRC (ng/ml/hr)	11.81 (7.18,18.20) N=23	21.63(12.39,35.17) N=10*	21.97(9.27,34.97) N=9	21.97(11.15,33.93) N=19*
Aogen (µg/ml)	0.98 (0.46,1.48) N=24	2.26(1.18,3.87) N=10***	3.17(1.37,4.46) N=8**	2.37(1.30,4.11) N=18****
ADH (pg/ml)	3.80 (3.40,4.73) N=22	3.60(3.40,4.68) N=6	3.17(1.37,4.46) N=4	3.65(3.40,4.28) N=10
Aldosterone (ng/ml)	62.7 (42.6,104.0) N=24	114.6(71.6,141.1) N=11*	186.3(126.4,253.4) N=5***	126.4(81.4,151.3) N=16***
Progesterone (ng/ml)	0.6 (0.3,1.0) N=23	22.9(19.0,33.8) N=11****	76.9(61.9,83.3) N=5***	31.1(19.4,65.1) N=16****
Prolactin (ng/ml)	35.0 (16.3,104.3)* N=25	126.6(74.6,168.2) N=11***	305.2(227.0,434.9) N=6***	162.1(83.9,291.1) N=17****

PRC – Plasma renin concentration, Aogen – angiotensinogen, ADH – arginine vasopressin. Data are reported as median (interquartile range).

*P<0.05, **P<0.01, ***P=0.005, ****P<0.001.

4.2.2.6. Association between hormones and plasma volume indices

There was significant correlation between plasma renin concentration and plasma volume per unit body surface area and between serum aldosterone concentration and plasma volume, both at 16 weeks gestation in Hb SS women (Figures 4.7 and 4.8). There was also a significant positive correlation between log10 aldosterone and PV in Hb SS pregnancy in general (Figure 4.9).

4.2.2.7. Association between plasma volume and measured indices in all pregnant women

As done in non-pregnant women, I also examined correlations between plasma volume and other parameters in all pregnant women, regardless of genotype. The only statistically significant one is shown in figure 4.10 below.

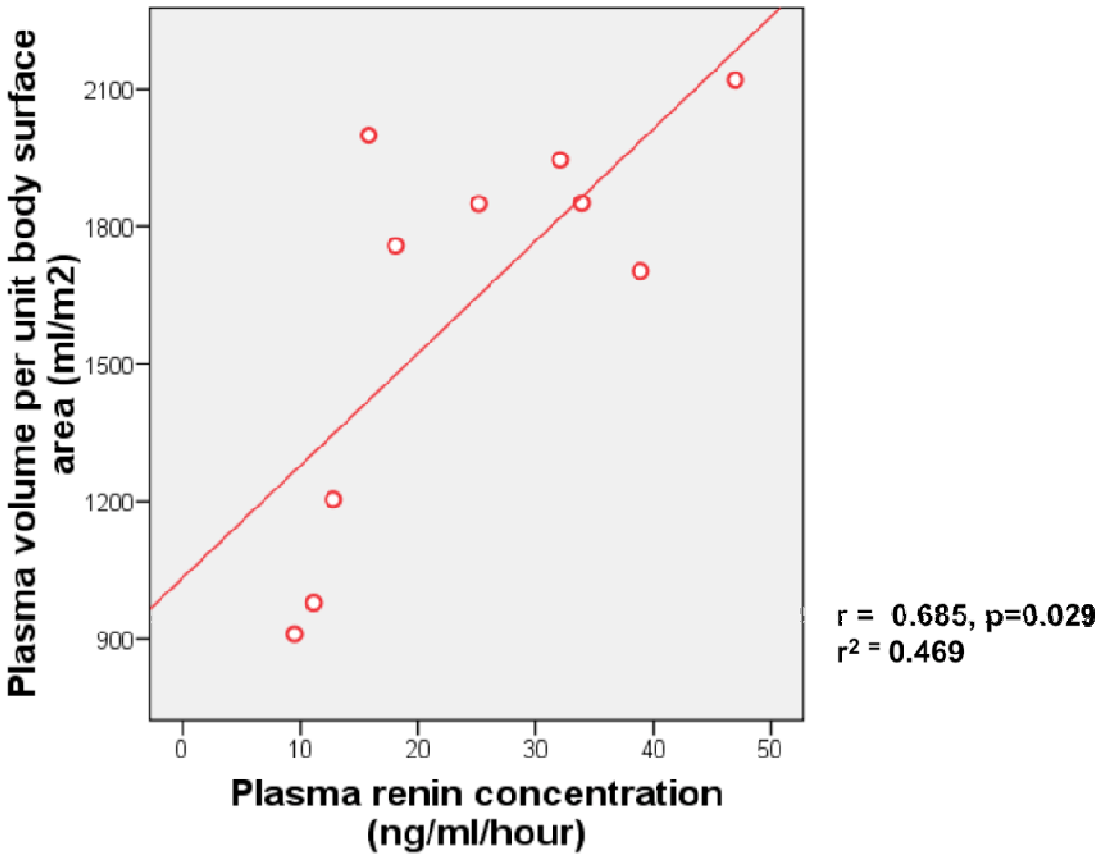


Figure 4.7. A scatter plot showing the relationship between plasma renin concentration and plasma volume per unit body surface area in 16 weeks' pregnant Hb SS (N=10) women. The computed best line of fit is displayed as in Fig 4.2 above.

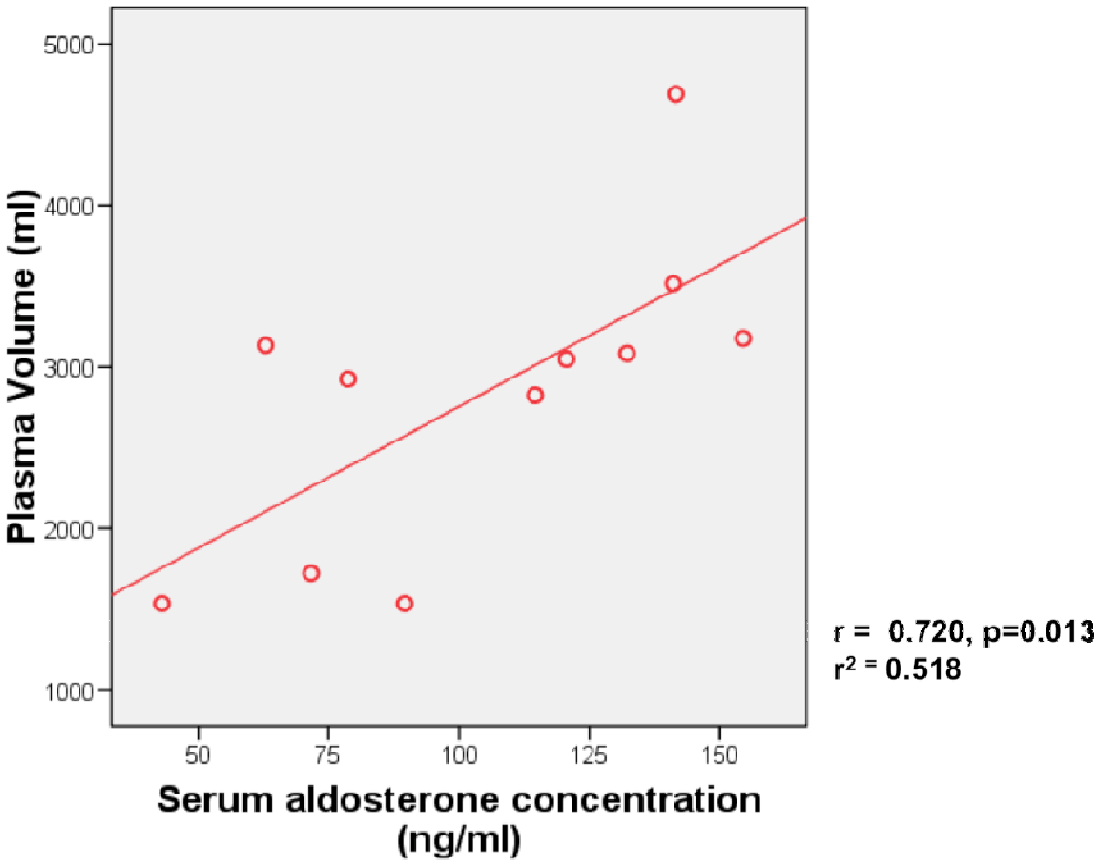


Figure 4.8. A scatter plot showing the relationship between serum aldosterone concentration and plasma volume in 16 weeks' pregnant Hb SS (N=11) women. The computed best line of fit is displayed as in Fig 4.2 above.

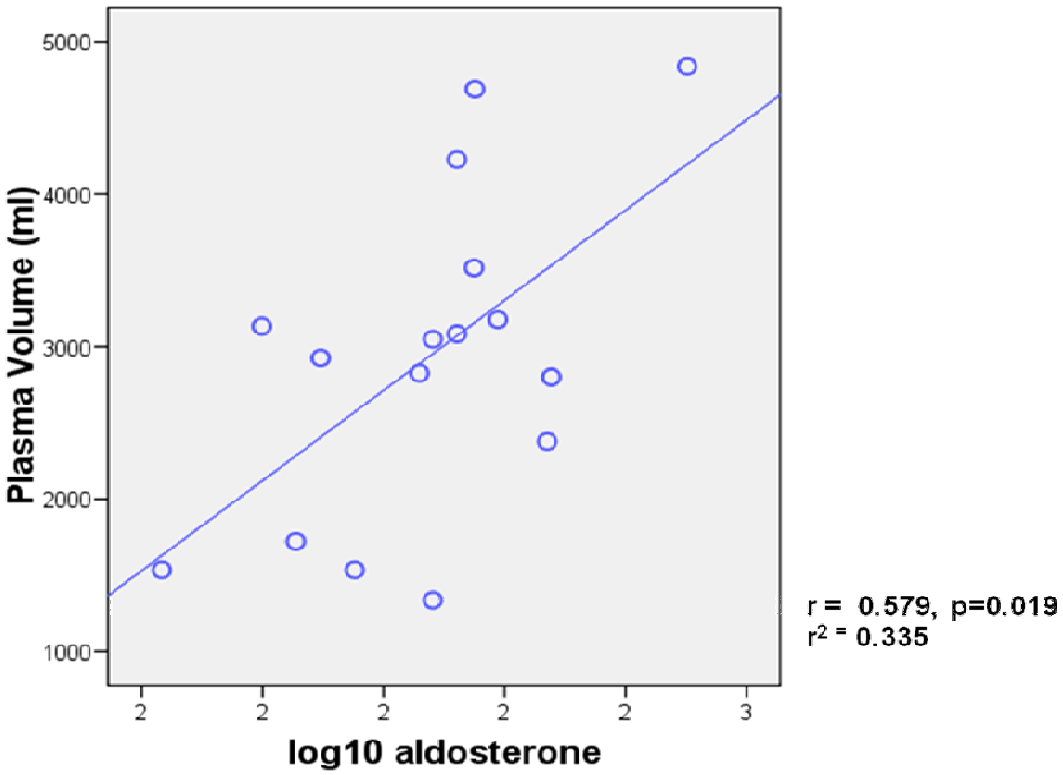


Figure 4.9. A scatter plot showing the relationship between aldosterone concentration and plasma volume in all pregnant Hb SS (N=16) women. The computed best line of fit is displayed as in Fig 4.2 above.

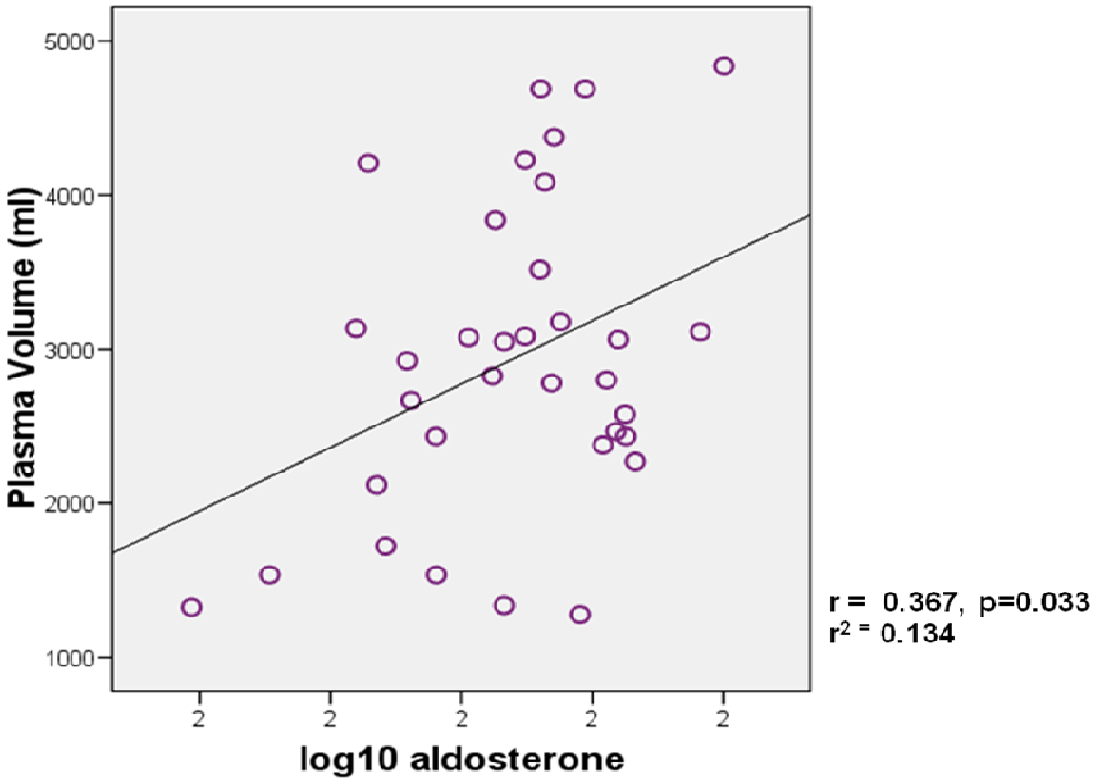


Figure 4.10. A scatter plot showing the relationship between aldosterone concentration and plasma volume, in all pregnant women (N=34). The computed best line of fit is displayed as in Fig 4.2 above.

4.3. BIRTH WEIGHT AND PLASMA VOLUME

Having examined the plasma volume indices and hormones and electrolytes known to affect them, I proceeded to examine birth weights of the babies between the genotypes and their association with plasma volume and other known determinants.

4.3.1. Birth weight and gestational age at delivery

As expected there was a significant difference in both birth weight and gestational age at delivery between Hb AA and Hb SS women (Table 4.12).

Table 4.12. Birth weight and gestational age at delivery between pregnant women

	Hb AA	**Hb SS**
Birth weight (kg)	3.3±0.4(N=19)	2.7±0.5 (N=23)*****
Gestational age at delivery (weeks)	38.7±1.3 (N=19)	37.4±2.0 (N=21) *
Babies below 2.5kg	0 (N=19)	26.1% (N=23)*
Birth weights of babies ≥ 37 weeks gestation at delivery	3.3±0.4 (N=18)	2.9±0.4 (N=16)***
Number of babies below 2.5kg in babies ≥ 37 weeks gestation at delivery	0 (N=18)	2 (N=16)

N = number of women studied in each group. Data are reported as mean ± standard deviation. *P<0.05, ***P<0.005, *****P<0.0001.

4.3.2. Plasma volume and birth weight

As birth weight is a single outcome and I was analysing it in relation to all my various other measurements, I used all the initial data I had i.e. including the repeated measurements of some of the women as shown in the figures below. Historically increase in plasma volume is known to correlate positively with birth weight thus I examined the relationship between the two parameters. As there is a difference in plasma volume expansion in both gestational age groups studied, each gestational age group was examined separately. There was no significant correlation at 16 weeks gestation but at 36 weeks' gestation, there was an unexpected significant negative correlation between birth weight and plasma volume measurements (Figures 4.11 and 4.12) in Hb SS women.

There was a significant negative correlation between birth weight and plasma potassium concentration in Hb AA women at 16 weeks gestation (Figure 4.13) but there was no significant correlation with any other haematological, hormonal or electrolyte parameters.

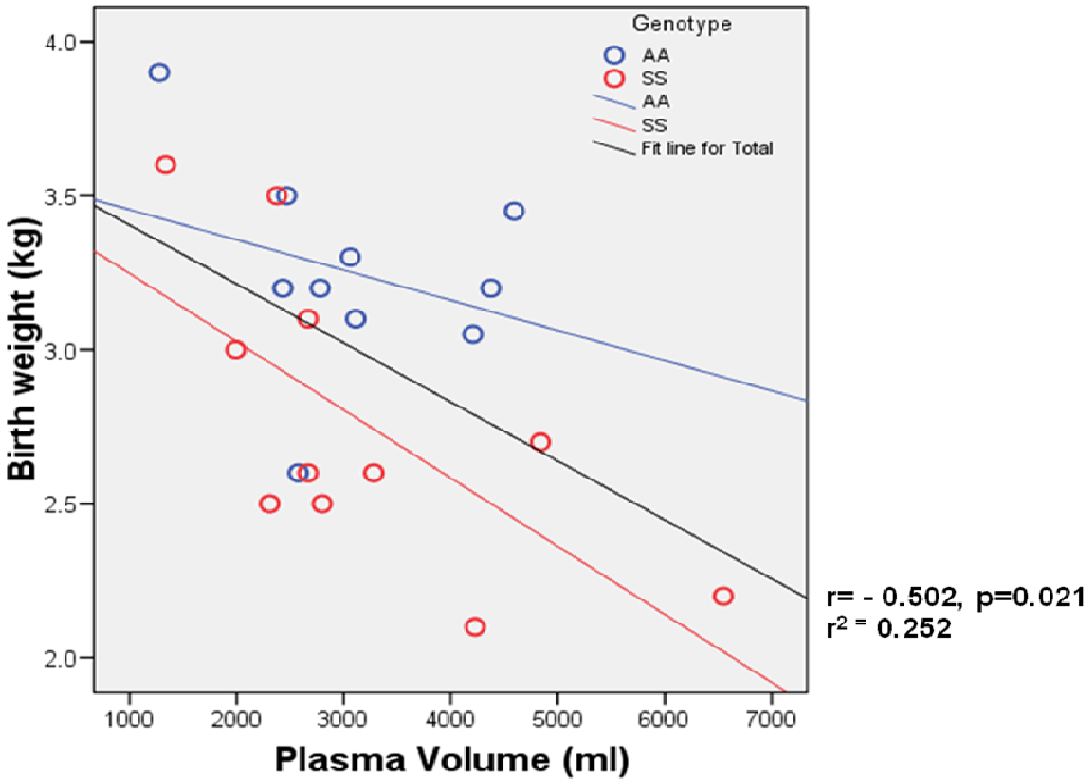


Figure 4.11. A scatter plot showing the relationship between birth weight and plasma volume, in 36 weeks pregnant Hb AA (N=13) and Hb SS (N=14) women. The computed best lines of fit are displayed as in Fig 4.2 above. There is a significant negative correlation overall as shown and a significant negative correlation in the Hb SS women as well (r= -0.583, P=0.029).

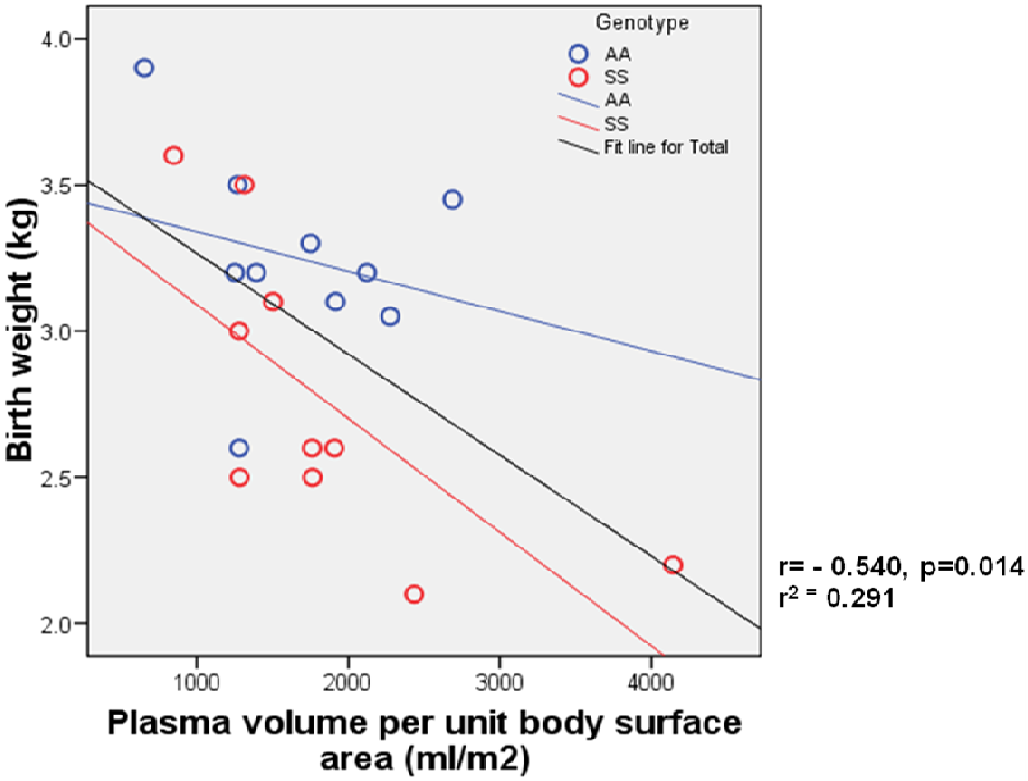


Figure 4.12. A scatter plot showing the relationship between birth weight and plasma volume per unit body surface area, in 36 weeks pregnant Hb AA (N=13) and Hb SS (N=14) women. The computed best lines of fit are displayed as in Fig 4.2 above. There is a significant negative correlation overall as shown and a significant negative correlation in the Hb SS women as well (r= -0.601, P=0.023).

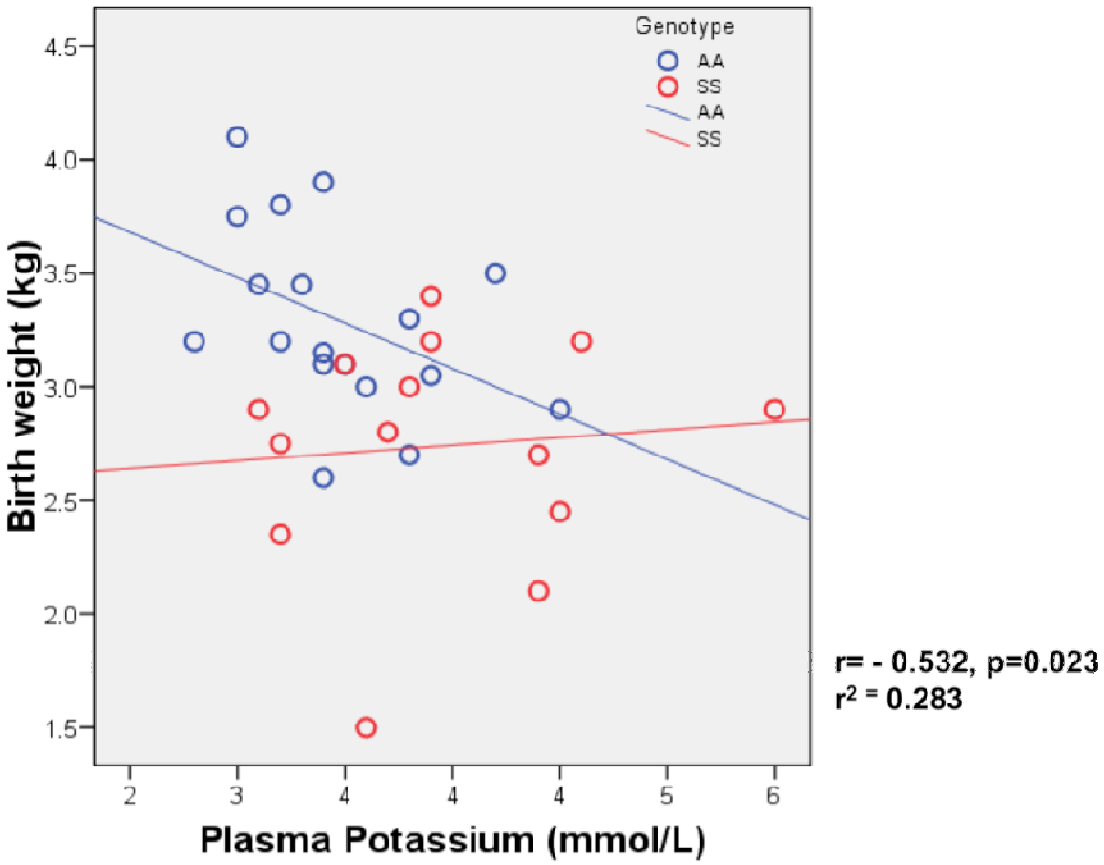


Figure 4.13. A scatter plot showing the relationship between birth weight and plasma potassium, in 16 weeks pregnant Hb AA (N=18) and Hb SS (N=14) women. The computed best lines of fit are displayed as in Fig 4.2 above. There is a significant negative correlation in Hb AA women as shown.

5. DISCUSSION

In this chapter, I will discuss the characteristics of all the women in the study then go on to discuss the results obtained from the non-pregnant women, followed by those from the pregnant women.

5.1. CHARACTERISTICS OF STUDIED WOMEN

Hb SS individuals are usually smaller, often in weight and sometimes height than their Hb AA counterparts, leading to a lower BMI (Table 3.2). The causes of this are unclear as despite their chronic anaemia, their oxygen carrying capacity is usually compensated for by a shift in the oxygen dissociation curve to the right, indicating a low oxygen affinity and thus a higher availability for use. However, a high demand for nutrients due to the constant breakdown of cells is usually not matched by appropriate intake and a small study on Hb SS children (Heyman *et al.*, 1985) showed a positive effect of dietary supplementation on their growth. Hb SS children with high levels of haemoglobin F (Hb F) have also been found to have a more normal weight gain pattern than their Hb AA counterparts (Lowry *et al.*, 1977).

It was noted also that the Hb SS women were younger which reflects their early health-seeking behaviour due to frequent illness and knowledge of their health condition. There is a sickle cell clinic in the hospital dedicated to the management of people with this disorder and they attend regularly from early childhood. This is in contrast to the poor health-seeking behaviour of the Hb AA population who seek medical attention mainly for serious or life threatening illness. The many Hb AA female nurses and house officers that were recruited to the non-pregnant AA would also have contributed to the difference in age, as they have undertaken professional

training and are therefore likely to be older than their non-pregnant counterparts. On the other hand, they were younger than the pregnant AA women as in recent times, these women tend to have completed tertiary education before starting their family (Odumosu *et al.*, 1999). The age groups for all non-pregnant women and for the pregnancy groups were all similar however (Table 3.2) and the differences found were not likely to be clinically significant.

5.2. NON-PREGNANT WOMEN

5.2.1. Haematological variables

The differences of lower haemoglobin levels and higher white cell counts that were seen in Hb SS women here were as expected. The known haematological variability of the disease was also seen from the greater degree of scatter, particularly in the white cell, neutrophil and lymphocyte counts of the Hb SS women.

5.2.2. Plasma volume measurements

The mean plasma volume of 2477ml found in the 44 non-pregnant women studied cannot be put in the context of previous studies, as it is a combination of Hb AA and Hb SS women. That of Hb AA women alone was 2165ml, which is very similar to data from the U.S.A. – 2036ml from 21 normal non-pregnant women with an average age of 29.7 years (Bernstein *et al.*, 2001) and from Nigeria – 2044 ml from 20 Hb AA non-pregnant women with an average age of 24.8 (Abudu & Sofola, 1988). A U.K. study found a mean of 2378 ml from 52 non-pregnant women in the reproductive age group (Whittaker & Lind, 1993) but this was still well below the 2714ml found in the 25 Hb SS women examined in this study.

Plasma volume is often quoted per unit weight or per unit body surface area as it has been found to depend on size (Hutchins, 1980). A study found it to be even better correlated with lean body mass than with body weight or body surface area (Boer, 1984). I did not use lean body mass however as it is a bit cumbersome to measure, is not widely used and would thus make comparison difficult. Using plasma volume per unit weight, the non-pregnant PVs of women with Hb SS of 51.1ml/kg is still much higher than the non-sickle cell female European figures of 42.7ml/kg (Boer, 1984) and the 46 ml/kg found in non-sickle cell postpartum Nigerian women (Harrison, 1966). A study done in the USA found 15 Hb SS adult females to have a mean plasma volume of 65ml/kg (Barreras *et al.*, 1966) (Steinberg *et al.*, 1977), which is even higher than my findings. A possible reason for this difference is the difference in climate. Nigeria, particularly the southern region where Lagos State is situated is a very hot and humid tropical country with an average annual temperature of 28^0C (Lagos, 2010) and more body fluids are lost from perspiration. Another U.S. study of young Hb SS men and women with a mean age of 27.4 which found a mean plasma volume of 55.0 ml/kg, used ^{51}Cr labelled autologous erythrocytes to estimate total blood volume and from this, with the haematocrit, the plasma volume (Steinberg *et al.*, 1977). They also felt that they probably underestimated the plasma volume of their population because they used a higher whole body-to-venous haematocrit ratio than they should have for their population, who were likely to be asplenic.

It can be seen from Fig 3.1 that the range of values was considerably higher in HbSS women, and an F test showed this to be statistically significant ($F = 5.092$, P

=0.029). This is most likely due to the variability of the severity of the manifestations of the disease but could also be due to different levels of hydration in the women studied.

As mentioned in the Introduction (1.1.1.1), the cause of the higher plasma volume in non-pregnant Hb SS individuals is unknown as it exceeds the expansion in other anaemias with similar red cell mass (Erlandson *et al.*, 1960; Steinberg *et al.*, 1977). We know that Hb SS women drink a lot of water although we did not measure their intake in this study. However, they also excrete large amounts of urine as shown in this study. Their urine is known to be dilute as well (Allon *et al.*, 1988) and in this study, the urinary osmolality in the Hb SS women was lower than in Hb AA although this did not reach statistical significance (Table 3.5). Thus urinary osmolality cannot explain their expanded PV.

The osmolar clearance in the Hb SS was significantly higher in the small number of women in whom I could calculate it, and as this suggests a smaller free water clearance for a given urine volume, it would appear that the Hb SS women clear less free water than their AA counterparts and this may be one of the causes of the supranormal plasma volume. However the free water clearance as calculated from the urine volumes and osmolar clearance was not significantly different from that of the Hb AA women (Table 3.6).

Although the difference in GFR is just short of clinical significance, it is close enough (p=0.051) to be biologically relevant. A beta (Type II) error is also a possible reason for it not to have been significant. As such, it is possible that the

increased plasma volume seen in the non-pregnant Hb SS women is as a result of a lower GFR in them. Normally GFR is known to be raised in childhood and normal by adolescence in Hb SS individuals. It then falls although it is not clear at what age group this fall occurs. A recent study found GFR to still be supranormal at age 20 in Hb SS individuals. However, when the women in the study were examined separately, their GFR was not significantly different from their Hb AA counterparts (Thompson *et al.*, 2007). If GFR were to be the cause of the fall, one would expect plasma volume to be normal or low in childhood and only begin to rise as GFR declines. As far as this postulation goes, there are no reported studies of low or normal plasma volume measurements specifically in Haemoglobin SS children; in fact a study of 16 Hb SS children aged between 2 and 14 years showed a higher plasma volume than non-sickle cell controls (Jenkins *et al.*, 1956). Also as there is no correlation between any plasma volume measurement and GFR in Hb SS, it would appear that there are other factors driving the plasma volume apart from just the GFR.

Another possibility would be that plasma volume is high from childhood with an accompanying high GFR and then the GFR begins to fall with age but the PV remains the same. This would also suggest that other factors apart from, or as well as, the GFR are responsible for the plasma volume increase.

Urinary sodium concentration was found to be significantly lower in the Hb SS women as would be expected in the presence of sodium retention and an increased plasma volume. It is therefore possible that renal conservation of sodium may be

partly responsible for their steady state supranormal plasma volume as it is in other anaemias.

In exploring relationships between plasma volume measurements and the different indices that could affect them, I found the Hb AA women behaved as expected with there being a positive relationship between plasma volume measurements and related variables such as plasma renin concentration (PRC) and serum prolactin and a negative one with serum progesterone (Schrier & Niederberger, 1994; Ganong, 2005). Plasma renin through its production of angiotensin II as well as aldosterone, leads to an increase in the reabsorption of sodium from the proximal nephron, which is known to increase plasma volume. The prolactin relationship upholds previous findings in mammals where it is expected to increase renal reabsorption of sodium and water (Horrobin, 1980; Campbell & MacGillivray, 1982). For the total urinary sodium however, it may be expected that the higher the amount of sodium excreted from the kidneys, the more the accompanying water loss would be and as such the lower the plasma volume. However, it is more likely that the higher the plasma volume is, the higher the total excreted sodium and water would be and that the plasma volume probably drives the urinary sodium output.

Regarding the negative relationships, as progesterone is known to be natriuretic, because it acts as a competitive inhibitor of aldosterone due to their marked structural similarity (Berl & Better, 1980), it was expected that an increase would increase sodium excretion and water loss. Also, although there are some exceptions, when GFR is increased plasma volume usually reduces and vice-versa (Ganong, 2005).

In the Hb SS women, the only relationship between plasma volume and all the appropriate indices examined against it was a negative one with ADH, which was seen with all the plasma volume measurements. This implies that ADH and PV are very tightly linked in non-pregnant Hb SS women. Normally, an increase in ADH should lead to an increase in plasma volume. As the relationship is inverse in the case of these women, it would appear that the plasma volume drives the ADH concentration in them. Volume control of ADH is very strong and overrides that of osmotic stimuli (Ganong, 2005) thus a decrease in ADH secretion would be expected in those with a supranormal plasma volume such as these women. It would therefore appear that ADH secretion in Hb SS women might be highly dependent on plasma volume. This implies, presumably, that some other stimulus (or stimuli), is driving the plasma volume expansion.

The finding of significantly lower prolactin concentration in the non-pregnant Hb SS women was unexpected. The reason for this is not immediately clear. One would have expected it to be higher in them, explaining the supranormal plasma volume since prolactin has been implicated in the retention of sodium and water in other mammals (Horrobin, 1980). If it was low due to haemodilution or because the high plasma volume was driving the process, one would expect some of the other hormones such as aldosterone to be low as well and this was not the case.

A more recent study found prolactin to be natriuretic in euvolaemic, anaesthetised rats (Ibarra *et al.*, 2005). As there have been many contradictory findings on the role

of prolactin in sodium regulation however, and there are no definitive confirmatory studies in humans, this particular result should be interpreted with caution.

It therefore appears that the mechanism responsible for the supranormal plasma volume in non-pregnant Hb SS women is one that involves a reduction in the excretion of sodium. However there is no correlation between PV measurements and any of the sodium controlling hormones measured in them. It is therefore possible that there are several factors controlling the PV, that there is a single factor that was not measured, perhaps ANP or prostacyclin, both of which are natriuretic (Pocock, 2004) or that the reduction in sodium excretion is primarily a renal compensation mechanism.

5.3. PREGNANT WOMEN

5.3.1. Haematological variables

The lower haemoglobin concentration, haematocrit and red cell count, and higher white cell count, neutrophils, lymphocytes and platelets when compared to Hb AA women, is as expected. However, unlike in the non-pregnant women, the degree of scatter in the Hb SS women was similar to that in the AA women except for platelet count in which the scatter was significantly higher than that in Hb AA. This suggests a relative haematological homogeneity in Hb SS women in pregnancy. This could be due to the physiological demands of pregnancy or that it is only those Hb SS women with certain haematological or physiological capabilities who can achieve pregnancy and thus manifest similar traits when pregnant.

5.3.2. Plasma volume measurements

The only other reported study of plasma volume measurement in sickle cell disease in pregnancy (Abudu & Sofola, 1988) had some similarities as well as differences from my findings. They found no significant difference in mean plasma volume between Hb AA and Hb SS women at 16 weeks, as I did. However, at 36 weeks gestation, I found no significant difference in mean plasma volume between the two genotypes whilst Abudu et al found a significant reduction in plasma volume in the Hb SS women, compared to the Hb AA.

What was clear with my findings was that women with SCD did not have a significant change in their plasma volume (PV) during pregnancy, or between the non-pregnant state and pregnancy. Hb AA women however behaved as expected

with a significant rise in PV and PV/BSA at 36 weeks and in PV at 16 weeks. All the hormone levels also rose in them as expected in pregnancy.

The reason for the constancy of PV in the Hb SS women is unclear. PRC did not rise at 36 weeks from the value at 16 weeks:

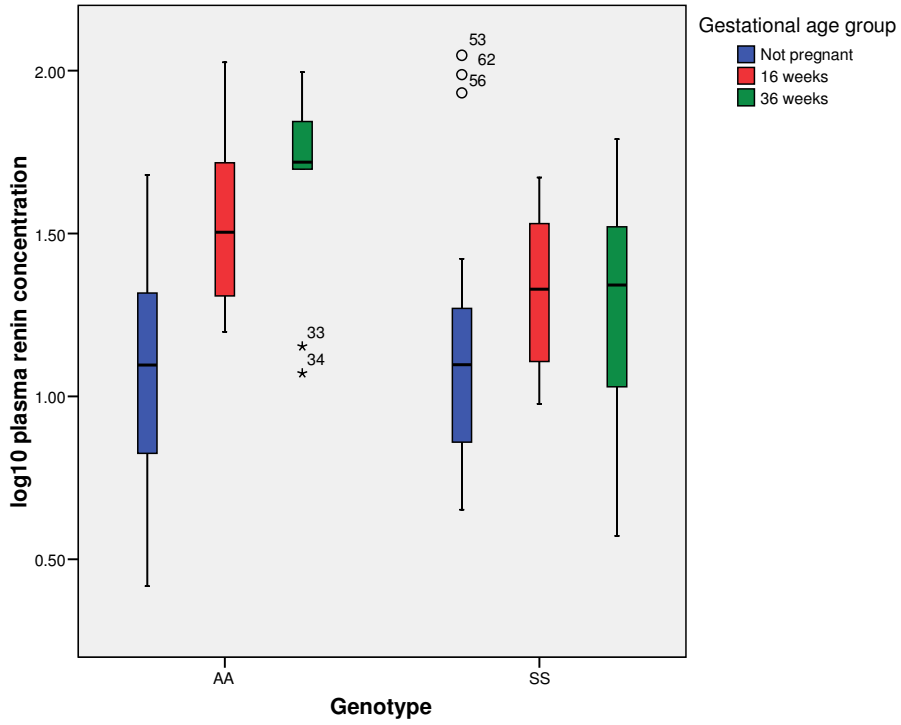


Figure 5.1. Boxplots of PRC across genotypes and in the gestational age groups.

whereas PRC continued to rise in HbAA women, and was also significantly lower at 36 weeks and in pregnancy overall than that in Hb AA pregnancy. It is possible that as Hb SS women enter pregnancy with an already raised plasma volume, the RAAS does not get activated as it usually would. This is because the usual response to a volume increase is a lack of activation of the RAAS (Ganong, 2005). If this were to be the case, I would expect PRC to be low from early in pregnancy and for there to

be no rise or perhaps even a fall in plasma volume in Hb SS pregnancy. However, although there is no change in plasma volume, there is a rise in PRC at 16 weeks of the sickle cell pregnancies, albeit a smaller rise than in the Hb AA pregnancies at the same gestational age. It may therefore be that the RAAS initially responds to the usual arterial under-filling of pregnancy, which we presume occurs in the Hb SS women in early pregnancy as it does in Hb AA women (Al Kadi *et al.*, 2005). However as the pregnancy progresses, PRC eventually falls in HbSS. A possible cause of this may be similar to that which occurs in pre-eclampsia, where there is thought to be a relative deficiency of prostacyclin (Fitzgerald *et al.*, 1987; Granger *et al.*, 2001) which leads to vasoconstriction and a reduction in renin concentration (Brown *et al.*, 1997). A deficiency of other vasodilatory substances such as nitric oxide has also been postulated in pre-eclampsia (Begum *et al.*, 1996; Savvidou *et al.*, 2003).

If vasoconstriction and a subsequent reduction in renin obtains, one would have expected aldosterone to be equally low or unchanged at 36 weeks. Renin is one of the main regulators of aldosterone secretion and changes in its concentration would be expected to be in the same direction as aldosterone concentration. However, in pregnancy, angiotensinogen, not renin, is rate-limiting in the synthesis of angiotensin II (Al Kadi *et al.*, 2005) and plasma angiotensinogen concentrations were not significantly different in HbAA and HbSS women (Table 4.4). In women with preeclampsia and idiopathic intrauterine growth restriction (IUGR), aldosterone as well as renin concentration is reportedly low (Brown *et al.*, 1992b). Although serum aldosterone was lower in Hb SS pregnant women, the difference was not statistically significant. Other authors have found aldosterone reduction in pre-

eclamptic patients to be proportionally less than renin reduction. This was thought possibly to be due to a non-Angiotensin II dependent route of stimulation of aldosterone synthesis or release such as plasma potassium or atrial natriuretic peptide (Brown *et al.*, 1997). Potassium increases aldosterone secretion by depolarizing the plasma membrane of zona glomerulosa cells and opening a voltage-gated calcium channel, with a resultant increase in cytoplasmic calcium and the stimulation of calcium-dependent processes (King, 2010). In this study, plasma potassium was significantly higher in pregnant Hb SS women, which might explain the non-significant reduction of aldosterone despite the lower PRC. Although some of the relative hyperkalaemia could be attributed to in vitro haemolysis in the SS women, a common phenomenon in them (Serjeant & Serjeant, 2001), they are also known to have potassium secretory problems that result in high potassium levels (DeFronzo *et al.*, 1979).

Continuing with this line of thought, I would expect there to also be a fall in PV in the Hb SS women as is the case with pregnancies where the RAAS is inhibited, especially as both PRC and serum aldosterone are significantly correlated with PV at 16 weeks gestation. A reduction in glomerular filtration could explain the lack of change in PV in them but an effect on GFR could not be shown due to limited available data. It is interesting to note that their urine volume did not change in pregnancy and in fact fell at 36 weeks from non-pregnant and 16 week values although this was not statistically significant. Meanwhile the urine volume of Hb AA women rose significantly in pregnancy as expected. As there is no increase in urine output, one can surmise that there is unlikely to be an increase in GFR either and it could explain the lack of change in PV despite the low PRC.

5.3.3. Plasma volume and birth weight

Many authors have found a significant correlation between plasma volume (Pirani *et al.*, 1973; Rosso *et al.*, 1992; Salas *et al.*, 1993), or at least an increment in plasma volume (Hytten & Paintin, 1963; Gibson, 1973) and birth weight especially in late pregnancy. However, a previous report on healthy pregnant women in my centre did not find any correlation in PV and birth weight at 30 or 36 weeks gestation (Abudu & Sofola, 1985). On analysing raw published data of healthy pregnant women in their third trimester from a study with a similar population to mine, I found no significant correlation either (Fig 5.1). In fact when I removed a possible outlier (circled) from the scatter plot, the relationship between plasma volume and birth weight was also negative though not significantly so.

I was however surprised to find a significant negative correlation between PV and birth weight in pregnant Hb SS women at 36 weeks gestation. If plasma volume reflected the haematocrit or haemoglobin concentration in them, it may explain this phenomenon as one would expect a low haematocrit with a high plasma volume as well as a low haematocrit with a low birth weight. However, there was no significant correlation between birth weight and haematocrit or haemoglobin concentration, or between plasma volume and haematocrit or haemoglobin concentration. It appears that for Hb SS women at least, a raised plasma volume in late pregnancy is not a positive sign as it is associated with a lower birth weight. The numbers are too small to determine the possible reasons for this now.

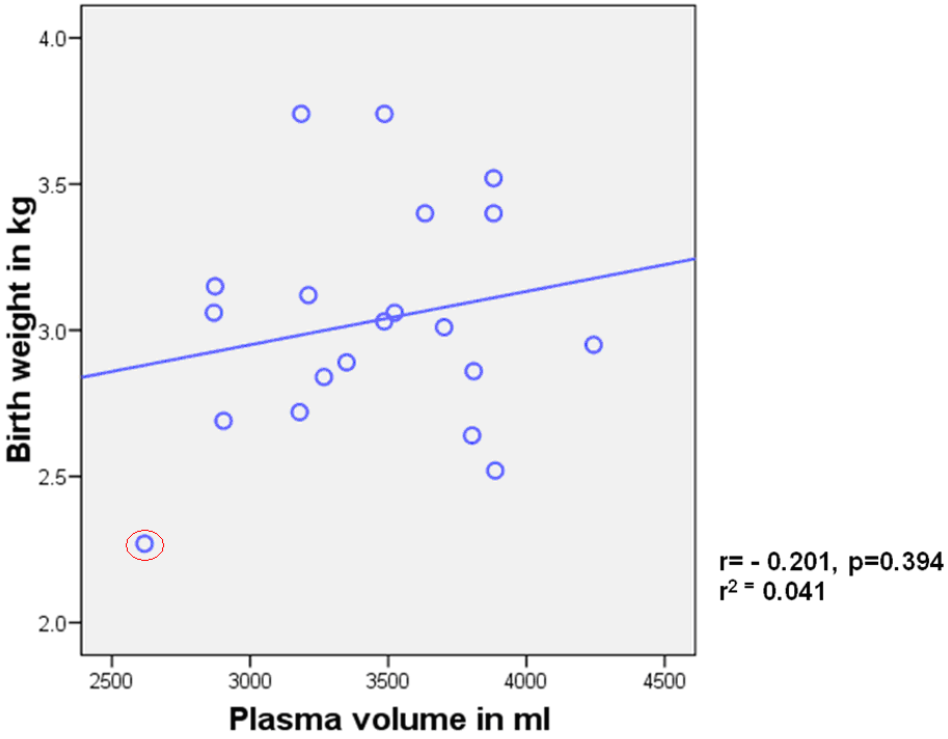


Figure 5.2. A scatter plot showing the relationship between birth weight and plasma volume in healthy pregnant Nigerian women in their third trimester (N=20) (Harrison, 1966).

In summary, it is possible that Hb SS pregnant women have a generalised increase in peripheral resistance in late pregnancy leading to a reduction in some components of the RAAS, but do not reduce their plasma volumes from their pre-pregnant supranormal levels as their urine output remains the same in pregnancy. There may be an abnormality at either the hypothalamo-pituitary level or in the renal sodium sensing system. To test this latter hypothesis I would need to measure vasodilatory substances such as prostacyclin and nitric oxide, and also explore the renal function of these women in late pregnancy. It might be possible also to study the effects of salt loading on renal function in non-pregnant HbSS women.

6. CONCLUSIONS

This thesis attempts to increase the knowledge base concerning plasma volume and its hormonal and electrolyte determinants in pregnant women with sickle cell disorder.

I was able to recruit and examine a considerable number of women with sickle cell disorder (49 in total), despite the invasive nature of the examination I had to carry out on them. This is only the second study ever to measure PV in pregnant women with SCD and the first to measure so many different variables in them – 24 different hormones, electrolytes and haematological parameters. I found that non-pregnant Hb SS women already had a supranormal plasma volume which did not increase significantly in pregnancy; thus unlike in uncomplicated pregnancy, pregnant women with sickle cell disorder did not expand their PV. I also found that although PRC rose in them at 16 weeks, it rose significantly less than it does in AA pregnancy and there was no further increase by 36 weeks unlike in AA pregnancy. Although aldosterone was increased in them as expected, the increase was to a lesser extent than in AA women and could have been due to non Angiotensin II dependent mechanisms. I surmised this lack of PV rise, therefore, to be due to a general vasoconstriction as pregnancy progresses in Hb SS women, caused by a deficiency of vasodilatory substances and similar to what occurs in pre-eclampsia, which prevents the RAAS and the PV in turn from rising. To support this hypothesis, I would need to prove that the aldosterone rise in SS women is significantly lower than that in AA women and that there is a relative deficiency of vasodilatory substances such as prostacyclin or nitric oxide in them.

I plan to research further on this subject by designing a study to examine plasma prostacyclin, nitric oxide and aldosterone concentrations. I would also like to measure GFR and other indices of renal function in both pregnant and non-pregnant women with sickle cell disorder.

Another prospective study will look at the outcome of pregnancies of women with sickle cell disorder including the incidence of pre-eclampsia and other maternal complications as well as intrauterine growth restriction, prematurity and other perinatal complications.

Finally, whilst carrying out the literature review, I found that there have been separate reports of enlarged cardiac chambers in pregnant and non-pregnant women with SCD respectively. I would like to carry out blinded echocardiographic measurements in a comparative study of both pregnant and non-pregnant women to see if their cardiac chambers are larger during pregnancy and if they revert to normal postpartum.

The ideal would have been to perform a longitudinal study and continue to measure the same women up to 8 weeks post-partum, which was my initial intention. However, due to several constraints (see section 2.9.6), I could only do a cross-sectional one. As a result of this change in methodology, a lot of data could not be used. Data from 34 women was not used for this reason mainly because, in order to avoid autocorrelation, which is the likelihood of all data from the same woman to behave in the same way, all repeated measures of the women had to be removed. This reduced the power of the study. If I were starting this study all over again, I

would carry out a cross-sectional study ab initio and thus ensure that I measure each woman only once and achieve recruitment of my full sample size with fewer resources and less time wasted. I would also prefer to use another method for measuring PV that is more accurate than Evans blue such as Dextran 70 (see section 2.9.4).

In conclusion, this thesis was the first to investigate reasons for the lack of PV rise in pregnant Hb SS women and to examine so many hormones and electrolytes in them. It showed that with perseverance and organisation, it is possible to obtain meaningful results from such a major study, in an environment with relatively poor infrastructure such as Nigeria. It also provides more ideas for research into the apparently saturated area of blood pressure in pregnancy.

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APPENDIX 1

RESEARCH INFORMATION SHEET FOR VOLUNTEERS

Title of Project

Plasma volume expansion and osmoregulatory hormones in pregnant patients with sickle cell disorder

Name of Investigator

Dr Bosede AFOLABI (Consultant Obstetrician and Gynaecologist).

Background

There is a blood condition known as sickle cell disorder, which is very common in Nigeria. Women with sickle cell disorder usually have more problems in pregnancy and smaller babies. We know that women whose plasma (the liquid part of blood) increases in pregnancy usually have good-sized babies. A previous study done in this hospital found that the plasma of women with sickle cell does not rise much in pregnancy.

We wish to try to discover the cause of this in the hope that we can correct the problem in future. We will need pregnant women and non-pregnant women, both with sickle cell disease and without i.e. Haemoglobin AA women.

What does the study involve?

We will admit you to a comfortable ward for 24 hours. For non-pregnant women, this will only be once while for pregnant women, you will need to be admitted 3 times – at 16 and 36 weeks of pregnancy, and 8 weeks after delivery.

The admissions will be at no cost to you.

To measure the plasma, a small amount of a substance called Evans blue will be injected into your veins. This dose is completely safe and has been used thousands of times in both pregnant and non-pregnant human beings.

Also, we will take a little blood from you and ask you to collect all your urine so we can test it as well.

The admission is because of the urine collection. If you would prefer not to be admitted, we can arrange for you to collect your urine at home and just come in with it for your blood tests.

What are the side effects of taking part?

The insertion of the small tube for the injection (just like when on 'drip') may be associated with slight pain. There are no other risks of taking part.

RESEARCH VOLUNTEERS CONSENT FORM

Title of Project:

Plasma volume expansion and osmoregulatory hormones in pregnant patients with sickle cell disorder

Please read this form and sign it once the investigator or her representative has explained fully the aims and procedures of the study to you:

I agree to take part in this study

- I confirm that I fully understand the attached information sheet.
- I authorise the investigator to disclose the results of my participation in the study but not my name.
- I understand I am free to withdraw from this study at any time without having to give a reason for withdrawing.
- I confirm that I have disclosed relevant medical information before the study.

Name:_____

Address:_____

_____

Telephone number:_____

Signature:_____

APPENDIX 2

INSTRUCTIONS TO VOLUNTEERS AND PATIENTS REGARDING 24-HOUR URINE COLLECTION

1. To begin your 24-hour collection, empty your bladder (pass urine) and flush urine down the toilet.

2. Record the date and time you emptied your bladder on the label attached to your 24-hour urine container.

3. For the next 24 hours, add **ALL** urine that you pass into the container. Refrigerate container between additions if possible.

4. When you are about to have your bath, pass urine before and after and add to the container. Ensure you do not pass urine away DURING your bath.

5. Even when you go to pass stool, collect the urine beforehand and add it to the container; don't let it run into the toilet.

6. End collection 24 hours after start time by emptying bladder at the same time you started but on the next day and adding the final sample to the collection container.

7. Record the ending date and time on the label attached to your 24-hour urine container.

APPENDIX 3

ALDOSTERONE ASSAY

Reagent preparation

All standards and controls were in ready-to-use solutions. Wash buffer concentrate was prepared by diluting 50ml concentrate in 450ml water (1:10 dilution). The Aldosterone-Avidin Horseradish-peroxidase (HRP) conjugate was also prepared as in the instructions. All reagents were allowed to equilibrate to room temperature before use.

About 250 l of wash buffer was dispensed to all wells and aspirated after mixing for 5 seconds. 50 l of standards and samples were then pipetted into appropriate wells immediately so the wells were not allowed to dry at any time. One well was saved for a blank and nothing was added. 100 l of (1:50) Avidin conjugate solution was added to each well except the blank. This was mixed gently for 10 seconds and incubated at room temperature for 60 minutes on a plate shaker set at 200 rpm.

The reaction mixture was removed and the plate washed three times with wash buffer, tapping the plates over paper towels between washings. 150 l of Tetramethyl benzidine (TMB) substrate solution was added to each well – blue colour developed. This was mixed gently for 10 seconds, the plate was covered and incubated at room temperature for 15 minutes on the plate shaker or until calibrator A attained the dark blue colour for the desired optical density.

The reaction was stopped by adding 50 l of Stop solution to the wells. This was mixed gently for 10 seconds whereupon the blue colour turned yellow. The absorbance was measured at 450 nm using an automated ELISA reader (Stat Fax 2100) reader within 15-20 minutes.

Calculation of results

All tests (i.e. standards and controls) were done in duplicate. The mean absorbance of standards and samples were calculated and the blank values are subtracted. The standard curve and concentrations of all samples were plotted and calculated automatically by the ELISA reader.

ARGININE-VASOPRESSIN ASSAY

Extraction procedure

In order to have accurate determinations of vasopressin, extraction of the samples is recommended. Ice-cold acetone of twice the sample volume was added to the sample, vortex-mixed and centrifuged at 12000g for 20 minutes. The supernatant was aspirated and transferred to a new tube and five times its volume of ice cold petroleum ether was added, vortex-mixed and centrifuged at 10000g for 10 minutes. The top ether layer was discarded and the remaining aqueous layer was transferred to a glass tube and dried down under gas. The sample was reconstituted with assay buffer at the time of assay.

Reagent preparation

All reagents were allowed to equilibrate to room temperature.

The Vasopressin Standard: Serial dilutions were made from a stock standard of 10,000pg/ml into 7 tubes using the assay buffer (Concentrations of vasopressin were 1000, 400, 160, 64, 25.6, 10.24 and 4.10pg/ml for tubes #1- #7). The wash

buffer was prepared by diluting 5ml of the concentrate with 95ml of deionised water. The maximum binding (Bo) well was 100% bound and had 0pg/ml of vasopressin; the non-specific binding (NSB) well was 0.00% bound. These were used in calculating the % bound and the results of the samples.

Assay procedure

All the standards and samples were run in duplicate. The appropriate number of wells was laid out and 100 l of assay buffer was pipetted into the NSB and the Bo wells. 100 l of standards and samples were pipetted into the appropriate wells.

50ul of assay buffer was pipetted into each of the NSB wells. Finally 50 l of the blue conjugate was added into each well except the Total Activity (TA) and Blank wells.

At this point all the used wells were green in colour except the NSB wells, which were blue. The Blank and TA wells were empty.

The plate was tapped gently, sealed and incubated at 4^0C for 24 hours. The plate contents were emptied and washed by adding 400 l of wash solution to every well; this was repeated twice for a total of 3 washes. After the final wash, the wells were emptied and the plate tapped dry on a lint free paper towel.

5ul of the blue conjugate was then added to the TA wells. 200 l of the p-NitroPhenyl Phosphate (pNpp) substrate solution was added to every well and incubated at room temperature without shaking. Finally, 50 l of Stop solution

was added to every well and the plate read immediately using an automated ELISA reader (Stat Fax 2100) with primary filter of 405nm and secondary filter of 570nm.

Calculation of results

The average net optical density (OD) for each standard and sample were calculated as follows:

Average Net OD = Average Bound OD – Average NSB OD. Percent Bound = Net OD/Net Bo OD x 100.

Using Log paper, the percent bound versus the concentration of AVP was plotted for the standards. A straight line was approximated through the points and the concentration of AVP in the samples was interpolated from the curve.

PROGESTERONE ASSAY

Principle

The progesterone EIA is based on the principle of competitive binding between progesterone in the test specimen and progesterone –HRP conjugate for a constant amount of rabbit anti-progesterone.

Reagent preparation

All reagents were allowed to equilibrate to room temperature before use. Working Progesterone-HRP conjugate reagent was reconstituted according to manufacturer's instructions.

Assay procedure

25 l of standards, specimens and controls were dispensed into the appropriate wells. 100 l of Working Reagent and 50 l of rabbit anti-progesterone reagent was dispensed into wells. The wells were vortex mixed for 30 seconds and

incubated at room temperature for 90 minutes. The microwells were rinsed and flicked 5 times with distilled water. 100 l of TMB reagent was dispensed into each well and gently mixed for 10 seconds. This was then incubated again at room temperature for 20 minutes and the reaction stopped by adding 100 l of Stop solution to each well and gently mixed for 30 seconds to ensure that all the blue colour changed to yellow completely. The absorbance was read at 450nm with a microtiter automated well reader (Stat Fax 2100) within 15 minutes.

Calculation of results

The microwell reader plotted the standard curve and gave concentrations of all samples analysed in ng/ml.

PROLACTIN ASSAY

Principle

The prolactin quantitative test kit was based on a solid phase ELISA. The assay system utilised an anti-prolactin antibody for solid phase immobilization and another mouse monoclonal antiprolactin antibody in the antibody-enzyme conjugate solution.

Reagent preparation

All reagents were equilibrated to room temperature before use. Lyophilized standards were reconstituted with distilled water and allowed to stand for 20 minutes before mixing.

Assay procedures

50 l of standard, specimens and controls were dispensed into appropriate wells. 50 l of enzyme conjugate reagent was also dispensed into each well, vortex mixed for 10 seconds and then incubated at 37^0C for 30 minutes. The incubation mixture was removed and the contents flicked into the sink. The microtiter wells

were rinsed and flicked 5 times with distilled water. 100 l of TMB solution was dispensed into each well and gently mixed for 5 seconds. The reaction wells were incubated at room temperature for 10 minutes and the reaction stopped by adding 100 l of Stop solution to each well. This was then gently mixed for 5 seconds until the entire blue colour changed to yellow completely. The absorbances were read at 450nm and concentration of samples calculated from a standard curve by an automated microtiter well reader (Stat Fax 2100).

OSMOLALITY MEASUREMENTS

The Wescor Vapor Pressure Osmometer (Wescor Inc, Utah, USA) was used for all the osmolality measurements.

Procedure

The instrument was allowed to warm up for about 20 minutes and then it was calibrated using the 290mmol/Kg and then the 1000mmol/Kg standards. The 100mmol/Kg standard was processed to determine the cleanliness of the thermocouple. A set of controls (low, normal and high) provided by the manufacturers was assayed with each batch of samples. 10 l sample of the plasma or urine was pipetted onto a small solute-free paper disc that was then inserted into a sample chamber and sealed. The dew point temperature depression, which is a function of solution vapour pressure, was then measured and displayed.

CREATININE

Reagents

1. Base – Disodium Phosphate (6.4mmol/l) and Sodium hydroxide (150mmol/l)
2. Dye – Sodium dodecyl sulphate (0.75mmol/l) and picric acid (4mmol/l) at pH 4
3. Creatinine Standard – 2mg/dl (177umol/l)

Working reagent: equal volumes of base and dye.

Procedure

Standards and samples were not measured in duplicates as the method had been validated and in use in our laboratory for many years. Each batch was run with controls and this guided the reporting of results. The Westgard rule of 2 SD was used to accept or reject runs.

ELECTROLYTES ASSAY (SODIUM AND POTASSIUM)

Principle

Plasma separated from lithium heparin tubes was used.

Equipments and Reagents

- Jenway Flame Photometer by Barloworld Scientific
- 10ml Bijou Bottles
- Adjustable micropipette (100-1000ul)
- Deionised water
- Sodium/Potassium commercial standard

- Sodium/Potassium commercial control

Procedure

The flame of the photometer was ignited and adjusted. 1:100 dilution of standard/control/samples were made into bijou bottles and mixed gently. The standards were assayed and so were the samples and controls. The concentration of sodium and potassium were displayed on the readout apparatus. As with creatinine, standards and samples were not measured in duplicate as the method has been validated and been in use in our laboratory for many years.

PLASMA ANGIOTENSINOGEN (AOGEN) CONCENTRATION (PAC) ASSAY

Determination of endogenous (sample) Aogen in the presence of excess exogenous Renin using an indirect radioimmunoassay (RIA) for Angiotensin-I (A1)

Molar excess of human renin was added to highly diluted plasma samples (containing Aogen). Under these conditions the amount of Aogen substrate is the rate-limiting factor in the production of A1. Thus the amount of A1 produced over a given period of time is directly relative to the starting amounts of Aogen substrate. Relative concentrations of sample Aogen were determined by comparing the concentrations of A1 produced in a set period (usually 2hrs) in each sample.

Materials:
Acetone (Fisher Scientific – A/0600/PB17)
Acid washed activated charcoal (Sigma – C5510)
Angiotensin-I ^{125}I (NEN, Perkin Elmer)
Bovine Serum Albumin (BSA) (Sigma – A7030)
Dextran (Sigma – D4876)
Dimercalprol
EDTA (disodium, Sigma – E5134)
8-Hydroxyquinoline (hemi) sulphate (8-HQS)(Sigma – H6752)
Disodium hydrogen (ortho) phosphate [dibasic sodium phosphate] (Sigma – S3264)
Sodium dihydrogen (ortho) phosphate [monobasic sodium phosphate] (Sigma – S3139)
Sodium Chloride (Fisher Scientific – S/3160/63)

Preparation:
Sufficient quantities of Buffer 'B' (Buffer 'A' containing 0.3% BSA) were determined and made up. 0.6ml eppendorfs, LB3 and LP5 tubes were labelled as appropriate. Water bath was set at 37°C. The centrifuge was pre-cooled and all procedures performed at 4°C or less.

Procedure

1. The samples were diluted as follows: 10µl in 10ml Buffer 'A'.

2. One tube of human renin (5ml) was added to 5ml buffer 'A'. Angiotensinase inhibitors were added to the 10 ml as follows: 0.1ml/ml (1ml) 0.3M EDTA; 0.05ml/ml (0.5ml) 8-HQS; 0.01ml/ml (100µl) dimercalprol injection.

3. To 400µl of each diluted sample, 100µl of the prepared human Renin/Buffer 'A' (see 2. above) was added and mixed well.

4. 100µl (0hr time point) was added to a fresh 0.6ml eppendorf. The remaining 400µl was placed into a water bath preheated to 37°C and incubated for 2 hours.

5. An equal volume (100µl) of ice-cold acetone was immediately added to each 100µl (0hr time point from 3. above).

6. This was mixed and centrifuged at 2500rpm for 10 minutes at 4°C.

7. 100µl of the supernatant was transferred to LP3 tubes containing 400µl of Buffer 'B' (Buffer 'A'/0.3% BSA, see appendix 'A'), and held under 'cling-wrap' at 4°C until the 2hr time point samples had reached the same stage.

8. Standard Curve preparation.
The A1 serial dilutions were prepared as below:-

1st dil: 10µl of 1mg/ml A1 was added to 50ml of 0.9% Saline ($NaCl_2$) [200.0pg/µl].
2nd dil: 1ml of above dilution was added to 4ml Buffer 'A'/0.3% BSA [40.0pg/µl].
Seven LP3 tubes 'A' to 'G' were labelled and 1ml Buffer 'A'/0.3% BSA was added to each.

Std.		**Tubes.**
'A':	1ml of 2nd dilution into 1ml Buffer 'B' [20.0pg/µl].	25,26,27.
'B':	1ml of 'A' into 1ml Buffer 'B' [10.0pg/µl].	22,23,24.
'C':	1ml of 'B' into 1ml Buffer 'B' [5.0pg/µl].	19,20,21.
'D':	1ml of 'C' into 1ml Buffer 'B' [2.5pg/µl].	16,17,18.
'E':	1ml of 'D' into 1ml Buffer 'B' [1.25pg/µl].	13,14,15.
'F':	1ml of 'E' into 1ml Buffer 'B' [0.625pg/µl].	10,11,12.
'G':	1ml of 'F' into 1ml Buffer 'B' [0.3125pg/µl].	7, 8, 9.

The first 27 tubes were utilised for the standards.

1-3 Non-specific binding (NSB)
4-6 Nil Standard
7-27 Standards as above

To tubes 1 – 3 (NSBs), 450µl Buffer 'B' was added, to tubes 4 – 6 (Nil stds), 400µl Buffer 'B' was added, and to tubes 7 – 27, 300µl Buffer 'B' and 100µl of the relevant standard were added (as above).

N.B. The total amounts of A1 in each 100µl of standard was 'A' – 2000pg, 'B' - 1000pg, 'C' – 500pg, 'D' – 250pg, 'E' – 125pg, 'F' – 62.5pg, 'G' – 31.25pg, and Nil stds – 0.00pg.

5ml of Buffer 'B' was placed into a universal tube and 5ml of ice-cold acetone was added. This was mixed well and centrifuged at 2500rpm for 10 minutes at 4°C. Then 100µl was added to all the Standard curve tubes (1 – 27 inclusive).

The standard curve tubes were held under 'cling-wrap' at 4°C while Aogen samples completed their 2hr incubation.

9. After 2hrs, 300µl of the incubated Aogen samples was transferred to fresh 0.6ml eppendorfs and equal volumes (300µl) of ice-cold acetone were added.

10. These were mixed and centrifuged at 2500rpm for 10 minutes at 4°C.

11. 100µl of the supernatant was transferred to three separate LP3 tubes containing 400µl of Buffer 'B'.

12. 50µl of freshly prepared Angiotensin I -^{125}I was added to every tube (standards and samples).

13. 50µl of freshly diluted antiserum was added to all tubes except 1, 2 and 3.

14. These were mixed well and incubated under 'cling-wrap' at 4°C for at least 48 hours.

15. After incubation – 500µl of Dextran coated charcoal suspension (freshly diluted 1part + 3parts Buffer 'B') was added and centrifuged at 2500rpm for 15 minutes at 4°C. Free A1 (both hot and cold) was trapped in the pellet while the antibody 'bound' A1 remained in solution in the supernatant.

16. The supernatant was decanted into another LP3 tube and radioactivity of both the pellet and the supernatant was counted using a Gamma counter.

Calculations

The gamma counter on board software was utilised to calculate the % of total sample radioactivity in each fraction and this was used to derive from the standard curve the quantity of A1 in each of the fractions.

The mean amount of A1at the 2-hour time point was calculated and the zero time point amount was subtracted from it. This gave the amount of A1 in pg for each sample (X).

To correct for the original dilution and convert to µg of A1 per ml, the following formula was used: ('X'/1000) x 25.

PLASMA RENIN CONCENTRATION (PRC) ASSAY

Determination of endogenous renin in the presence of excess exogenous Angiotensinogen (Aogen) substrate using an indirect radioimmunoassay (RIA) of Angiotensin-I production

Molar excess of sheep angiotensinogen (Aogen) substrate was added to undiluted plasma samples (containing renin). Under these conditions the amount of renin available is the rate-limiting factor in the production of A1. Thus the rate of A1 production over a given period of time is directly relative to the specific activity of renin and thus to the amount of active Renin. Therefore relative amounts of sample renin were determined by comparing the rates of A1 production over a set period (usually 2hrs) in each sample.

Materials:
Acetone (Fisher Scientific – A/0600/PB17)
Acid washed activated charcoal (Sigma – C5510)
Angiotensin-I ^{125}I (NEN, Perkin Elmer)
Bovine Serum Albumin (BSA) (Sigma – A7030)
Dextran (Sigma – D4876)
Dimercalprol
EDTA (disodium, Sigma – E5134)
8-Hydroxyquinoline (hemi) sulphate (8-HQS)(Sigma – H6752)
Disodium hydrogen (ortho) phosphate [dibasic sodium phosphate] (Sigma – S3264)
Sodium dihydrogen (ortho) phosphate [monobasic sodium phosphate] (Sigma – S3139)
Sodium Chloride (Fisher Scientific – S/3160/63)

Preparation:
Sufficient quantities of Buffer 'B' (Buffer 'A' containing 0.3% BSA) were determined and made up. 0.6ml eppendorfs, LB3 and LP5 tubes were labelled as appropriate. Water bath was set at 37^{o}C. The centrifuge was pre-cooled and all procedures performed at 4^{o}C or less.

1. One tube of Sheep substrate (Aogen, 5ml) was added to 5ml buffer 'A'. Angiotensinase inhibitors was added to the 10ml: 0.1ml/ml (1ml) 0.3M EDTA; 0.05ml/ml (0.5ml) 8-HQS; 0.01ml/ml (100µl) dimercalprol injection.

2. 400µl of the prepared sheep Aogen/Buffer 'A' (see 1. above) was added to 200µl of each undiluted sample, and mixed well.

3. 100µl (0hr time point) was transferred to a fresh 0.6ml eppendorf. The remaining 400µl was placed into a water bath preheated to 37°C and incubated for 2 hours.

4. An equal volume (100µl) of ice-cold acetone was immediately added to each 100µl (0hr time point from 2. above).

5. This was centrifuged at 2500rpm for 10 minutes at 4°C.

6. 100µl of the supernatant was transferred to LP3 tubes containing 400µl of Buffer 'B', and held under 'cling-wrap' at 4°C until the other time point samples had reached the same stage.

7. Steps 2 – 5 were repeated after a further 30min, 1hr and 2hrs of incubation.

8. Standard Curve preparation.
Prepare A1 serial dilutions as below:-

1st dil: 10µl of 1mg/ml A1 was added to 50ml of 0.9% Saline ($NaCl_2$) [200.0pg/µl].
2nd dil: 1ml of above dilution was added to 4ml Buffer 'A'/0.3% BSA [40.0pg/µl].
Seven LP3 tubes 'A' to 'G' were labelled and 1ml Buffer 'A'/0.3% BSA was added to each.

Std.		**Tubes.**
'A':	1ml of 2nd dilution into 1ml Buffer 'B' [20.0pg/µl].	25,26,27.
'B':	1ml of 'A' into 1ml Buffer 'B' [10.0pg/µl].	22,23,24.
'C':	1ml of 'B' into 1ml Buffer 'B' [5.0pg/µl].	19,20,21.
'D':	1ml of 'C' into 1ml Buffer 'B' [2.5pg/µl].	16,17,18.
'E':	1ml of 'D' into 1ml Buffer 'B' [1.25pg/µl].	13,14,15.
'F':	1ml of 'E' into 1ml Buffer 'B' [0.625pg/µl].	10,11,12.
'G':	1ml of 'F' into 1ml Buffer 'B' [0.3125pg/µl].	7, 8, 9.

The first 27 tubes were utilised for the standards.

1-3 Non-specific binding (NSB)
4-6 Nil Standard
7-27 Standards as above

To tubes 1 – 3 (NSBs), 450µl Buffer 'B' was added, to tubes 4 – 6 (Nil stds), 400µl Buffer 'B' was added, and to tubes 7 – 27, 300µl Buffer 'B' and 100µl of the relevant standard were added (as above).

N.B. The total amounts of A1 in each 100µl of standard was 'A' – 2000pg, 'B' - 1000pg, 'C' – 500pg, 'D' – 250pg, 'E' – 125pg, 'F' – 62.5pg, 'G' – 31.25pg, and Nil stds – 0.00pg.

5ml of Buffer 'B' was placed into a universal tube and 5ml of ice-cold acetone was added. This was mixed well and centrifuged at 2500rpm for 10 minutes at 4°C. Then 100µl was added to all the Standard curve tubes (1 – 27 inclusive). The standard

curve tubes were held under 'cling-wrap' at 4^{o}C while Aogen samples completed their 2hr incubation.

9. 50µl of freshly prepared Angiotensin I -^{125}I was added to every tube (standards and samples).

10. 50µl of freshly diluted antiserum was added to all tubes except 1, 2 and 3.

11. These were mixed well and incubated under 'cling-wrap' at 4^{o}C for at least 48 hours.

12. After incubation, 500µl of Dextran coated charcoal suspension (freshly diluted 1part + 3parts Buffer 'B') was added and centrifuged at 2500rpm for 15 minutes at 4^{o}C. Free A1 (both hot and cold) was trapped in the pellet while the antibody 'bound' A1 remained in solution in the supernatant.

13. The supernatant was decanted into another LP3 tube and radioactivity of both the pellet and the supernatant was counted using a Gamma counter.

Calculations

The gamma counter on board software was utilised to calculate the % of total sample radioactivity in each fraction and this was used to derive from the standard curve the quantity of A1 in each of the fractions. This was in pg for each sample.

'EXCEL' was used to determine (for each set of 4 time points – 0hr, 30min, 60min, 120min) the slope (m) and the intersection point of the line on the 'y' axis (c). Using the formula $y = mx + c$, y was deduced when x = 60min. This gave pg/hr, which was divided by 1000 to convert to ng/hr and multiplied by 60 (the correction factor for this assay) to give ng/ml/hr.

APPENDIX 4

WORKSHEET FOR PLASMA VOLUME EXPERIMENT

Materials

Evans blue dye – 15 ml
20ml syringes x 3
5ml syringe x 5
10ml syringe x 2
Pink intravenous cannula (20 gauge) x 2
3-way tap
Heparin saline (Hepsal) 1000IU in 100ml saline
Stopwatch
Prelabelled tubes – three plain (STANDARD-15ml), three plain (labelled 10, 20 and 30 minutes), one LiH (3ml), one cold EDTA (4ml), one EDTA (2ml)
Cotton wool
Spirit
Tourniquet
Plaster

Procedure

Put ice in flask or icebox and precool EDTA bottle.

Put 15 ml of Evans blue dye into a 20 ml syringe.

Fill 2 syringes with Hepsal, one 10ml syringe and one 5ml.

Pre label tubes.

2 kidney dishes – 1 by patient, 1 for syringes.

Insert cannula into arm. Take out 25ml blood and put gently into the specimen bottles as per volumes stated above. Attach 3-way tap, push in some Hepsal and stick down with plaster. Close 3-way tap. Set the stopwatch to 10 minutes.

Inject 15 ml of Evans blue dye into the cannula through the pink stopper of the cannula itself (not the 3-way tap). As you start the injection, start the stopwatch.

This should all go in within one minute. Then flush the cannula with hepsal through the pink covered outlet and through the 3-way tap, till all traces of dye are gone. Push in about one millilitre more of Hepsal and close 3-way tap.

When the stopwatch reads 1min 30 seconds, withdraw about 5ml of blood into the syringe with hepsal in it. Then position an empty 5ml syringe and start withdrawing 5ml of blood, slowly into it when the stopwatch reads 10 seconds. As soon as the stopwatch begins to beep, press the Start/Stop button, 3 times, while still continuing to withdraw blood into the 5ml syringe, gently with the other hand. Hand the 5 ml syringe to your assistant to empty gently into the 10 minutes plain bottle. Push in 5ml of the blood and hepsal mixture.

Repeat the cycle again 10 minutes after and another 10 minutes after, putting the blood into the 20 and 30-minute plain bottles, respectively. Re-inject all the blood/hepsal mixture after the 30-minute sample has been taken.

APPENDIX 5

PATIENT BIODATA FOR SICKLE CELL PLASMA VOLUME

RESEARCH

Name:

Date of birth:

Address:

Home:

Business:

Phone number (s):

Occupation:

Genotype (patient's account):

Genotype (confirmed):

Weight:

Height:

Body mass index:

Current medication and doses:

LMP: (first day of Last Menstrual Period):

Parity:

Pregnant patients:

Gestational age by LMP:

Gestational age by scan …….. done on……(date of scan).

Date:

APPENDIX 6

STUDY RAW DATA

subjno	age	parity	weight	height	BMI	genotype	gestage	plasvol	BSA2	PVBSA2	plvolwt	PVBMI	PRC	AOGEN
0130	37	1	62	1.7	21.45329	1	0	2460.88	1.711075	1438.207	39.69161	114.7088	41.06	0
0103	26	0	51.5	1.55	21.436	1	0	1112	1.489081	746.7695	21.59223	51.87534	2.613992	0.948234
0038	25	0	72	1.6	28.125	1	0	1977.4	1.788854	1105.4	27.46389	70.30756	16.91872	1.239812
0001	25	0	57.5	1.72	19.43618	1	0	2594.7	1.657475	1565.454	45.12522	133.4984	47.88	0.66
0046	25	0	45.5	1.64	16.91701	1	0	2168.2	1.439714	1505.993	47.65275	128.1668	7.839511	1.203786
0027	20	0	43.5	1.6	16.99219	1	0	1551.2	1.390444	1115.615	35.65977	91.28901		
0011	22	0	56.5	1.59	22.3488	1	0	2410.4	1.579689	1525.87	42.66195	107.8537	35.6092	0.461682
0024	20	0	61	1.7	21.10727	1	0	1845.4	1.69722	1087.308	30.25246	87.42961	6.48173	0.679636
0030	22	0	59.5	1.55	24.76587	1	0	2432.7	1.600564	1519.902	40.88571	98.22793	10.86614	1.847692
0018	25	0	59	1.59	23.33768	1	0	2449.6	1.614259	1517.476	41.51864	104.9633	6.891238	1.225662
0009	26	0	60	1.64	22.30815	1	0	2506.6	1.65328	1516.138	41.77667	112.3625	20.72693	0.334745
0012	18	0	60	1.7	20.76125	1	0	1977.4	1.683251	1174.751	32.95667	95.24477	20.81083	0.997589
0017	27	0	59	1.63	22.20633	1	0	2489.4	1.634438	1523.092	42.19322	112.1032		
0021	27	0	62.5	1.67	22.41027	1	0	2189.9	1.702735	1286.108	35.0384	97.71859	14.77	0
0048	29	0	62	1.48	28.30533	1	0	1336.87	1.596524	837.3629	21.56242	47.23032	4.220426	1.183588
0004	25	0	66.75	1.65	24.51791	1	0	3105.2	1.749107	1775.306	46.51985	126.6503	14.3122	
0010	31	0	59	1.66	21.41095	1	0	2355.5	1.649411	1428.086	39.92373	110.0138	3.70515	0.449688
0013	22	0	67	1.65	24.60973	1	0	1607.3	1.752379	917.2101	23.98955	65.31156	9.753348	1.142139
0005	25	0	85.1	1.72	28.76555	1	0	2565	2.016405	1272.066	30.14101	89.16917		
0068	25	0	67.5	1.65	24.79339	1	1	2432.72	1.758906	1383.087	36.0403	98.11971	23.26645	1.902966
0041	27	0	64.5	1.64	23.98126	1	1	3076.7	1.714157	1794.877	47.70078	128.296		
0003	37	2	60.6	1.64	22.53123	1	1	2118.8	1.661525	1275.214	34.9637	94.03836	106.0539	1.663572

subjno	age	parity	weight	height	BMI	genotype	gestage	plasvol	BSA2	PVBSA2	plvolwt	PVBMI	PRC	AOGEN
0045	24	0	71	1.71	24.28098	1	1	2270.3	1.836437	1236.253	31.97606	93.50119	42.50193	2.185199
0064	28	0	64.5	1.64	23.98126	1	1	4084.07	1.714157	2382.553	63.31892	170.3026	15.77626	1.518032
0042	24	0	74.5	1.57	30.22435	1	1	2667.4	1.802506	1479.829	35.80403	88.25335	34.90201	0.865847
0031	32	3	81	1.67	29.04371	1	1	4689.11	1.938427	2419.028	57.89025	161.4501	17.77761	3.308612
0006	27	2	87	1.7	30.10381	1	1	1324.6	2.026902	653.5095	15.22529	44.00108	29.17624	0.355314
0015	28	3	91.5	1.74	30.22196	1	1	2606.7	2.102974	1239.53	28.48853	86.25186		
0014	36	2	78	1.59	30.85321	1	1	3837.9	1.856071	2067.755	49.20385	124.3922	64.02482	2.082923
0114	31	0	90.5	1.69	31.68657	1	2	4376.14	2.061182	2123.121	48.35514	138.1071	98.87	4.85
0116	32	0	58	1.64	21.56455	1	2	3112.7	1.625491	1914.929	53.66724	144.3434	49.84763	3.772316
0124	39	1	84.1	1.62	32.04542	1	2	2466.56	1.945379	1267.907	29.32889	76.97075		
0115	36	2	87.5	1.67	31.37438	1	2	2576.86	2.014703	1279.027	29.44983	82.13263	14.24594	4.777789
0104	28	1	83	1.64	30.85961	1	2	2432.72	1.944508	1251.072	29.30988	78.83185	11.78471	2.090901
0092	30	0	85	1.69	29.76086	1	2	2779.9	1.997568	1391.642	32.70471	93.40791	52.3822	3.690103
0086	28	0	77	1.6	30.07813	1	2	4208.2	1.849925	2274.795	54.65195	139.909	51.21143	3.475439
0044	26	0	68.5	1.61	26.42645	1	2	3062.6	1.750278	1749.779	44.70949	115.8915	55.63234	2.68031
0076	32	2	85	1.62	32.38836	1	2	1276.7	1.955761	652.7895	15.02	39.41849	69.73	1.2
0036	22	0	67	1.57	27.18163	1	2	4594	1.70937	2687.54	68.56716	169.0112	71.75874	3.583383
0037	20	0	41	1.51	17.98167	2	0	1542	1.311382	1175.859	37.60976	85.75401	13.57833	1.097228
0091	20	0	55	1.49	24.77366	2	0	2891.3	1.508771	1916.328	52.56909	116.7086	7.18235	1.421391
0096	19	0	48	1.57	19.47341	2	0	2193.27	1.446836	1515.908	45.69313	112.629	6.139827	1.980001
0089	22	0	46	1.52	19.90997	2	0	2218.66	1.393636	1591.994	48.23174	111.4346	18.1986	0.309012
0099	21	0	57.5	1.6	22.46094	2	0	3278.5	1.598611	2050.844	57.01739	145.9645	4.985357	1.258894
0029	20	0	57.5	1.66	20.8666	2	0	3327.3	1.628309	2043.409	57.86609	159.4558		
0094	22	0	62	1.62	23.62445	2	0	3133.94	1.670329	1876.241	50.54742	132.6566	11.80584	0.241587
0098	20	0	49	1.58	19.62827	2	0	2070.58	1.466477	1411.941	42.25674	105.4897	10.75857	1.533319
0088	23	0	59	1.64	21.93635	2	0	3885.2	1.639444	2369.827	65.85085	177.1124	13.26	0.98
0055	26	0	57	1.59	22.54658	2	0	5322.2	1.586663	3354.335	93.37193	236.0536	17.96477	0.827179
0095	20	0	42.5	1.46	19.93808	2	0	1901.5	1.312864	1448.361	44.74118	95.37029	13.76032	1.690117
0022	32	1	57.3	1.6	22.38281	2	0	2259.9	1.595828	1416.13	39.43979	100.9659	4.492524	0.615771
0026	25	0	56.5	1.71	19.32219	2	0	2884.6	1.638215	1760.818	51.05487	149.2895	20.30433	0.436598

subjno	age	parity	weight	height	BMI	genotype	gestage	plasvol	BSA2	PVBSA2	plvolwt	PVBMI	PRC	AOGEN
0002	22	0	49	1.65	17.99816	2	0	2524	1.49861	1684.227	51.5102	140.2365		0.96327
0008	19	0	47	1.56	19.31295	2	0	2992.9	1.427118	2097.163	63.67872	154.9685	19.11163	0.604841
0016	23	0	52.5	1.64	19.51963	2	0	2793.1	1.546501	1806.077	53.20191	143.0918	8.012748	0.987795
0007	20	0	55.5	1.62	21.14769	2	0	4188.8	1.580348	2650.555	75.47387	198.0736	85.46859	1.234742
0028	19	0	55.5	1.69	19.43209	2	0	2547.4	1.61413	1578.187	45.8991	131.0924	5.946752	1.091239
0032	21	0	59	1.65	21.67126	2	0	1954.8	1.644435	1188.737	33.1322	90.20242	26.44	0
0063	19	0	50	1.62	19.05197	2	0	1002.5	1.5	668.3333	20.05	52.61922	8.782523	1.85515
0020	22	0	42.5	1.56	17.46384	2	0	2377.3	1.35708	1751.776	55.93647	136.127	16.48111	1.501491
0067	26	0	56	1.65	20.56933	2	0	3148.4	1.602082	1965.193	56.22143	153.0628	7.307226	0.306803
0079	28	0	61	1.68	21.61281	2	0	1406.3	1.687207	833.5078	23.0541	65.06789	97.25	0.52
0047	22	0	46.5	1.55	19.35484	2	0	3811.5	1.41495	2693.735	81.96774	196.9275	10.69012	1.658387
0051	26	0	69	1.73	23.05456	2	0	2193.27	1.820943	1204.469	31.78652	95.13388	6.525372	0.114257
0127	24	0	48.5	1.61	18.7107	2	1	4689.12	1.472762	3183.895	96.68289	250.6117		
0138	33	0	57	1.55	23.72529	2	1	2667.4	1.566578	1702.692	46.79649	112.4286	38.89	0.67
0052	27	0	65.5	1.56	26.91486	2	1	1534.9	1.684735	911.063	23.43359	57.02798	9.507903	3.792488
0109	32	1	68	1.56	27.94214	2	1	3177.5	1.716586	1851.058	46.72794	113.7171	33.9349	3.822622
0107	32	0	56	1.58	22.4323	2	1	1534.9	1.56773	979.0588	27.40893	68.42365	11.1549	2.237475
0074	37	0	56.5	1.65	20.75298	2	1	3133.94	1.609218	1947.492	55.46797	151.0115		
0106	30	1	46	1.49	20.71979	2	1	2924.77	1.379815	2119.683	63.58196	141.1583	46.98034	5.034665
0053	29	0	52.5	1.6	20.50781	2	1	2825.49	1.527525	1849.717	53.81886	137.7763	25.1622	1.403991
0062	31	0	55.5	1.63	20.88901	2	1	3083.8	1.585218	1945.347	55.56396	147.6279	32.09287	1.26998
0059	25	0	67.5	1.65	24.79339	2	1	3516.3	1.758906	1999.14		141.8241	15.82	4.03
0049	32	1	47.5	1.55	19.77107	2	1	1722.24	1.430084	1204.293	36.25768	87.10909	12.79916	0.922548
0087	29	0	66	1.64	24.53897	2	1	3048.5	1.733974	1758.1	46.18939	124.231	18.1	2.28
0133	27	0	55.5	1.62	21.14769	2	2	6547.7	1.580348	4143.201	117.9766	309.6177		
0122	34	1		1.58		2	2	4838.28					3.727331	2.467812
0121	23	0	66.6	1.63	25.06681	2	2	4227.57	1.73652	2434.508	63.47703	168.6521	10.71856	4.485159
0134	27	1	69	1.67	24.74094	2	2	2832	1.789087	1582.93	41.04348	114.4662	36.8	1.54
0139	33	0	68	1.67	24.38237	2	2	2667.4	1.776076	1501.851	39.22647	109.3987		
0131	36	0	69.5	1.68	24.62443	2	2	2307.3	1.800926	1281.174	33.19856	93.69962	23.34	3.88

subjno	age	parity	weight	height	BMI	genotype	gestage	plasvol	BSA2	PVBSA2	plvolwt	PVBMI	PRC	AOGEN
0123	24	0	69	1.71	23.597	2	2	2377.34	1.810387	1313.167	34.4542	100.7475		
0132	27	0	58	1.51	25.43748	2	2	1992.9	1.559736	1277.716	34.36035	78.34502	61.68	4.55
0135	33	0	54	1.53	23.06805	2	2	2667.4	1.514926	1760.746	49.3963	115.6318	33.13	
0097	26	0	54.5	1.65	20.01837	2	2	1336.9	1.58048	845.8823	24.53028	66.78367	11.45473	4.368936
0058	29	0	56	1.62	21.33821	2	2	2799.6	1.587451	1763.582	49.99286	131.2013	7.822756	1.311007
0137	22	0	65	1.64	24.16716	2	2	3281.6	1.720788	1907.033	50.48615	135.7876	21.97	0

subjno	pADH	aldos	progest	prolactin	posmolality	uosmolality	urinevol	psodium	ppotassium	usodium	upotassium	ucreat	pcreat
0130	3.4	185		15.4									
0103	4.5	55.1	3.8	30.1	274	701	0.805	145	3.8	146	41.9	4896	
0038		23.9	1.9	29.5	269	234	0.62	142	3.1	86	6.9	10384	
0001	3.4	72.4	0	166	298	413	0.866	138	3.9	183	43.1	19040	132
0046	4.1	86.6	4.1	35.8	268	357	1.72	138	3.6	110	12.2	2902.6	71.8
0027	3.8	100.1	1.2	36.3	283	102	1.13	142	3.6	53	6.6	3264	67.8
0011	4.1	96.7	0.9	148	285	506	0.53		3.8	135	30	6528	76
0024	5	66.6	2.5	80.1	287	409	0.87	140	3.8	54	8.7	4080	80
0030	4	118.1	1	45.3	267	115	1.8	145	3.7	51	15	4352	68
0018	4	55.8	0.1	146	277	251	1.2	150	4.9	75	7.1	4080	64
0009		95.1	0.6	108	276	210	1.8	139	3.7	77	7.1	1122.5	55
0012	3.4	80.1	0	101	284	362	0.73	147	3.8	53	5.3	4652.5	55
0017	5.1	80.1	0.2	106	282	506	1.18	134	3.9	97	40.7	8160	48
0021	3.6	30	1.9	31.6	276	414	0.802	144		89	4.2	5168	49
0048	4.6	97.1	3.8	23.4	276	757	0.6	138	3.6	126	15.6	13190	46
0004		72.3	0	138	283	235	0.999	150	3.9	79	7.8		51
0010	3.4	90.6	0.1	134	277	392	1.48		4.2	97	12.6	1632	42
0013	3.2	58.9	0	148	268	220	1.11	140	3.8	63	6.7	1632	48
0005	6.2	54	0.7	78	265	479	1.116					1904	46
0068	8.7	89.4	26.7	56.6	270	304	2.6	136	2.8	84	7.1	5546.6	130
0041	3.9	103.1	17.8	133	271	383	3.04	134.4	3	71	29.6	4624	85.6
0003	9.2	68.9		134	269	79	1.28		4.5	67	8.8		67

subjno	pADH	aldos	progest	prolactin	posmolality	uosmolality	urinevol	psodium	ppotassium	usodium	upotassium	ucreat	pcreat
0045	6.3	214.6	56.1		264	366	1.305	125.9	3.3	125	24.6	7072	80
0064	3.8	144.3	28.6	156.2	270	533	1.8	147	3.8	122	21.3	4896	57.8
0042	3.6	80.1	17.8	133.1	275	607	0.6	136	3	130	36.2	13751	67.8
0031	3.9	172.3	18	78.6	285	325	1.79	138	3.6	89	4.8	4209.5	58
0006	3.4	30.5		164	287	321	1	140	3.4	77	6.8	2592.2	56
0015	4.4		6.3	147	268	135	2.017	147	3.4	71	3.9	1403	45
0014	3.4	116.1	6.5	179	265	183	1.21	145	3.2	111	7.8	1632	33
0114	9.4	150.2	72	390.5				142	3.9				
0116	8.8	285.7	75	506.4				150	4.3	61	41.9		
0124	4.2	197.1	79.5	823			2.1						
0115	4	205.2	73.9	380.2				144	4.3				
0104	8.2	206	37.8	156.1		118	3.4	144	3.5	16	2.3		
0092	4	148.6	45	278.2	262	610	1	141	3.4	114	42	1088	
0086	3.5	66.3	50.6	312.2	262	353	1.6	149	3.4	43	11.5	3367.6	
0044	4.2	199.1		124.1	264	90	2	138.3	3.2	52	4.8	2176	63
0076		168.4	51.2	156.1	277	335	1.2	148	3.8	79	32.7	3648	68
0036	3.8			125.6	270	77	3.12	139	3.4	56	7.1	3264	41
0037	5.7		1.1	27.8	269				3.3				
0091	3.8	50	0.7	32	277	432		150	4.4				
0096	3.6	187.1	0.3	18.6	274	330	1.2	144	3.8	60	12	2806.3	
0089	3.9	52.4		3.2	266	232	2.2	153	3.9	53	6.6	2992	
0099	6.8	43.1	0.3	16.5	284	445	1.2	143	4.4	55	9.5	3264	
0029	3.4	49.3	0.4	43.5	245	182	2.3	140	4.1	58	8.4	3264	
0094	3.4	61.4	0.6	30.6	300	313	2.4	145	4	55	9.3	3536	
0098	4.8	34.5	1.9	16.1	272	299	1.2	150	4.4	77	19	4770.7	
0088	3.8	63.9		10.7	279	385	2	149	4.3	101	17.1	4770.8	
0055		102.1	1	35	274	256	1.5	125.1	3.7	65	41.6	5712	
0095	4.3	66.6	0.5	16.9	270	437	1.4	144	5.2	77	25.5	8432	
0022	3.7	156.1	1	180	273	98	2.559	137	3.4	77	11.1	1403	118.6
0026	3.9	107.1	0.7	44.5	268	108	1.88	143	4.3	45	15.7	2720	116

subjno	pADH	aldos	progest	prolactin	posmolality	uosmolality	urinevol	psodium	ppotassium	usodium	upotassium	ucreat	pcreat
0002	8.5	69.6	1.3	108	277	458	1.62		3.8	205	37.1	3087	98
0008	3.2	39.8	0	124	270	128	1.27	148	4.6	41	14.2	5331	84
0016		104.6	3.5	118	282	150	1.87	145	4	45	3.8	2176	90
0007	3.2	42.3	0.1	121	280	221	1.93		3.9	78		1088	78
0028	3.6	224.6	1	45.3	265	186	2.19	137	3.5	46	7.7	3264	77.3
0032		42.4	0.8	100.6	268	74	4.875	143	3.4	44	4.3	1632	78
0063	3.4	58.5	0.3	13.4	282	316	2.8	141	4	96	21.4	2806.3	65.4
0020	3.4	111.1	0.1	108	269	145	2.35	135	3.8	33	3.6	2720	54
0067	3.8	38.2	0.3	14.4	248	386	1.9	140	3.7	53	6.7	2992	58
0079	8	16.5	0.3	12.2	250	511	2.4	142	4	79	41.9	8704	56
0047	3.4	64.6	0.3	88.1	278	330	1.6	138	3.6	51	16	2448	38
0051	4.7	64.1	1.4	88.1	289	382	1.3	137	3	76	36.5	11093	43.5
0127		141.6	24.8	157.9			2						
0138						252	2.8		3.6				
0052	3.4	89.6	20.5	260	267	95	3.01	137	3.5	57	11.6	3367.6	
0109		154.5	19	93.1	261	229	2.4	142	3.9	134	12.7	3929	
0107		43	13.3	113.1	269	239	2.405	144	3.9	120	12.4	5332	
0074	3.8	62.9	22.9	64.1	264	354	1.405	150	5.5	51	16.8	5440	
0106		78.7	33.8	74.6	270	282	2.7	145	4.5	71	27.4	5612.6	
0053		114.6	16.5	162.1	257	233	1	128.8	3.2	124	14.7	4624	75
0062	3.4	132.2	19	126.6	273	232	3.2	137	3.7	52	5.8	1403	49
0059	3.4	141.1	36.8	168.2	277	216	2.4		3.1	51	8.4	1360	60.5
0049	4.5	71.6	50.7	15.9		250	1.3	148	3.2	49	7.6	2525.7	39
0087	5.2	120.6	28.4	200	267	289		147	4.6				45
0133								132.3	4.6				
0122	4.2	317.4	68.2	67.7			1.8	144	4.3				
0121	3.5	132.2	80	308.4			4	138	3.9				
0134								132	3.2				
0139					279	402	2.6						
0131								130	4.8				

subjno	pADH	aldos	progest	prolactin	posmolality	uosmolality	urinevol	psodium	ppotassium	usodium	upotassium	ucreat	pcreat
0123		186.3	76.9	566.4			1.4						
0132				391		370	1.2	131	4				
0135	4.2					51	1.2	128	4.1				
0097	3.4	120.6	55.6	302	268	220	4	140	4.7	68	5	3166.5	
0058		189.3	86.6	280.1	264	281	1.2	138	3.7	76	7.6	3264	
0137								135.5	3.6				57.8

subjno	totalUsod	totalUpot	GFR	FENa	Cosmo	FWC	FEK	WBC	RBC	hbconc	HCT	MCV	MCH	MCHC
0130								3.3	3.74	114	0.356	95.2	30.2	320
0103	117.53	33.7295			2.059507	-1.25451		4.3	4.16	119	0.366	88	28.6	325
0038	53.32	4.278			0.539331	0.080669		3.1	4.24	106	0.33	79	25	317
0001	158.478	37.3246	52.09849	0.919346	1.200195	-0.3342	7.661603	6.3	4.73	128	0.402	85	27.1	318
0046	189.2	20.984	75.79109	1.971745	2.291194	-0.57119	8.382906	3.6	3.3	109	0.354	107.3	33	308
0027	59.89	7.458	80.0708	0.775295	0.407279	0.722721	3.808211			88				
0011	71.55	15.9	91.23263		0.940982	-0.41098	9.191176			116				
0024	46.98	7.569	95.16	0.756303	1.239826	-0.36983	4.489164	5	3.84	118	0.365	95.1	30.7	323
0030	91.8	27	107.38	0.549569	0.775281	1.024719	6.334459	4.2	4.34	127	0.382	88	29.3	332
0018	90	8.52	110.2563	0.784314	1.087365	0.112635	2.272909	4.3	4.7	132	0.409	87	28.1	323
0009	138.6	12.78	129.3382	2.714265	1.369565	0.430435	9.402275			96				
0012	38.69	3.869	138.4145	0.426221	0.930493	-0.20049	1.648802			126				
0017	114.46	48.026	144.4517	0.425812	2.117305	-0.93731	6.138763	5.3	4	122	0.36	90	30.5	339
0021	71.378	3.3684	149.898	0.586005	1.203	-0.401		4.6	4.04	110	0.354	87.6	27.2	311
0048	75.6	9.36	155.593	0.318423	1.645652	-1.04565	1.511246	4	4.19	119	0.389	92.8	28.4	306
0004	78.921	7.7922	156.5353	4.9375	0.829558	0.169442	18.75	4	4.21	114	0.375	89.1	27.1	304
0010	143.56	18.648	159.2438		2.09444	-0.61444	7.720588	3.7	4.16	113	0.348	83.7	27.2	325
0013	69.93	7.437	171.2967	1.323529	0.911194	0.198806	5.185759			123				
0005			221.26		2.017223	-0.90122		4.1	3.88	107	0.334	86.1	27.6	320

subjno	totalUsod	totalUpot	GFR	FENa	Cosmo	FWC	FEK	WBC	RBC	hbconc	HCT	MCV	MCH	MCHC
0068	218.4	18.46	62.1	1.447628	2.927407	-0.32741	5.943152	4.4	3.7	100	0.306	82.7	27	327
0041	215.84	89.984	88.55187	0.977946	4.296384	-1.25638	18.26528	3.4		83	0.25			
0003	85.76	11.264	96.88764		0.375911	0.904089		9	4.2	121	0.363	86.4	28.8	333
0045	163.125	32.103	107.068	1.123135	1.809205	-0.50421	8.432744	4.7	3.27	97	0.306	93.6	29.7	317
0064	219.6	38.34	129.982	0.979781	3.553333	-1.75333	6.617325			94				
0042	78	21.72	132.5617	0.471303	1.324364	-0.72436	5.949531	5.1	4.11	93	0.324	78.8	22.6	287
0031	159.31	8.592	156.8607	0.888604	2.041228	-0.25123	1.837114	3.3	3.69	105	0.337	91.3	28.5	312
0006	77	6.8	182.5757	1.18818	1.118467	-0.11847	4.320654	2.3	3.9	118	0.361	92.6	30.3	327
0015	143.207	7.8663	236.8427	1.549159	1.016026	1.000974	3.679091		3.05	84	0.263	86.2	27.5	319
0014	134.31	9.438	255.6509	1.547921	0.835585	0.374415	4.928768			97				
0114								7.5	4.03	103	0.321	79.7	25.6	321
0116								8.6	3.24	95	0.295	91	29.3	322
0124								6.4	3.38	98	0.301	89.1	29	326
0115								4.9	3.33	106	0.312	94.9	31.08	335
0104	54.4	7.82						5.5	4.48	122	0.374	83.5	27.2	326
0092	114	42			2.328244	-1.32824		6.5	3.67	109	0.322	87.7	29.7	339
0086	68.8	18.4			2.155725	-0.55573		10.2	3.63	97	0.312	86	26.7	311
0044	104	9.6	128.9105	1.088586	0.681818	1.318182	4.342831	7.6	4.18	127	0.401	95.9	30.4	317
0076	94.8	39.24	140.4	0.994992	1.451264	-0.25126	16.04051	5.1	3.63	95	0.303	83.5	26.2	314
0036	174.72	22.152	200.5424	0.506066	0.889778	2.230222	2.62309							
0037								5.8	3.6	85	0.281	78.1	23.6	302
0091								8.9	2.46	68	0.205	83.3	27.6	332
0096	72	14.4			1.445255	-0.24526		9.1	2.83	80	0.24	85	29.3	345
0089	116.6	14.52			1.918797	0.281203		5.5	3.4	83	0.255	75	24.4	325
0099	66	11.4			1.880282	-0.68028		10.9	2.36	68	0.195	82.6	28.8	349
0029	133.4	19.32		0.209428	1.708571	0.591429	1.035689	8.4	2.91	75	0.235	80.8	25.8	319
0094	132	22.32			2.504	-0.104		10.6	3.5	89	0.263	75.1	25.4	338
0098	92.4	22.8			1.319118	-0.11912		6.8	2.53	69	0.21	83	28.7	348
0088	202	34.2			2.759857	-0.75986		9.9	2.44	73	0.216	88.5	29.9	338
0055	97.5	62.4		0.20012	1.40146	0.09854	4.330381	10.5	2.81	73	0.232	82.6	26	315

subjno	totalUsod	totalUpot	GFR	FENa	Cosmo	FWC	FEK	WBC	RBC	hbconc	HCT	MCV	MCH	MCHC
0095	107.8	35.7			2.265926	-0.86593		10	2.15	65	0.2	91	33.2	363
0022	197.043	28.4049	54.2659	4.751133	0.918615	1.640385	27.59759	11.2	2.55	69	0.213	83.5	27.1	324
0026	84.6	29.516	58.25345	1.34204	0.757612	1.122388	15.57114	6.3	2.99	96	0.297	99.3	32.1	323
0002	332.1	60.102	61.36		2.678556	-1.05856	30.99415			83				
0008	52.07	18.034	70.41048	0.436509	0.602074	0.667926	4.864085		3.33	86	0.285	85.6	25.8	302
0016	84.15	7.106	70.98	1.283595	0.994681	0.875319	3.929228		3.35	102	0.32	95.5	30.4	319
0007	150.54		88.8		1.523321	0.406679		5.2	2.57	83	0.26	101	32.3	319
0028	100.74	16.863	90.35084	0.795182	1.537132	0.652868	5.210172							
0032	214.5	20.9625	93.61333	1.470588	1.346082	3.528918	6.04455	8.4	2.47	85	0.256	103.6	34.4	332
0063	268.8	59.92	96.20795	1.586703	3.137589	-0.33759	12.46802	6.7	2.85	83	0.246	86.3	29.1	337
0020	77.55	8.46	96.58519	0.485294	1.266729	1.083271	1.880805	10.1	2.74	86	0.25	91.2	31.4	344
0067	100.7	12.73	114.4717	0.733862	2.957258	-1.05726	3.510262	7.4	2.04	76	0.206	101	37.3	369
0079	189.6	100.56	126.88	0.357938	4.9056	-2.5056	6.73943	9.1	2.47	71	0.217	87.9	28.7	327
0047	81.6	25.6	150.1705	0.573671	1.899281	-0.29928	6.899056	11.8	1.83	57	0.196		31.1	291
0051	98.8	47.45	188.0607	0.217537	1.718339	-0.41834	4.771027	4.4	3.82	97	0.308	80.6	25.4	315
0127								13.2	2.06	63	0.192	93.2	30.6	328
0138								9.5	2.21	75	0.216	97.7	33.9	347
0052	171.57	34.916			1.070974	1.939026		7	2.89	78	0.251	86.9	27	311
0109	321.6	30.48			2.105747	0.294253		8.8	2.95	87	0.262	88.8	29.5	332
0107	288.6	29.822			2.136784	0.268216		8.1	2.02	66	0.191	94.6	32.7	346
0074	71.655	23.604			1.883977	-0.47898								
0106	191.7	73.98			2.82	-0.12		11.2	2.18	67	0.192	88.1	30.7	349
0053	124	14.7	80.808	1.561526	0.906615	0.093385	7.450935	12.4	2.54	79	0.246	96.9	31.1	321
0062	166.4	18.56	128.3976	1.325627	2.719414	0.480586	5.474755	5.4	2.58	73	0.215	83.3	28.3	340
0059	122.4	20.16	133.438		1.87148	0.52852	12.05408	9.3	2.11	73	0.212	100.5	34.6	344
0049	63.7	9.88	136.8	0.511231			3.6673	8	2.26	88	0.26	115	38.9	338
0087			169.312					12	2.72	72	0.22	80.9	26.5	327
0133								13.2	2.15	57	0.191	88.8	26.5	298
0122								8.4	2.79	62	0.206	73.8	22.2	301
0121									2.39	70	0.214	89.5	29.3	327

subjno	totalUsod	totalUpot	GFR	FENa	Cosmo	FWC	FEK	WBC	RBC	hbconc	HCT	MCV	MCH	MCHC
0134								15	1.88	59	0.179	95.2	31.4	330
0139					3.746237	-1.14624		11.4	3.65	98	0.29	80.5	26.8	333
0131								16.1	1.88	58	0.173	92	30	335
0123								10.5	1.97	66	0.195	99	33.5	338
0132								10.1	2.29	78	0.238	103.9	34.1	328
0135								9.8	2.56	79	0.249	97.3	30.9	317
0097	272	20			3.283582	0.716418		8.8	2.24	66	0.198	88.4	29.5	333
0058	91.2	9.12		0.232843	1.277273	-0.07727	0.868442	9.9	2.49	68	0.209	83	27.3	325
0137			138.0069					10.8	3.26	99	0.31	95.1	30.4	319

subjno	PLT	LYM	NEUT	LBW	birthwt	GADelivery	Gender	Modelivery
0130	113	1.7	0.9					
0103	209	1.9	1.7					
0038	230							
0001	74	2.9						
0046	191	1.9	1.1					
0027								
0011								
0024	204	2.2	2.1					
0030	246	2.1	1.9					
0018	205	2.6						
0009								
0012								
0017	87	2.1	2.6					
0021	183	1.8	2.5					
0048	181	2.1	1.5					
0004	241	1.9	1.1					
0010	210	1.7	1.8					
0013								

subjno	PLT	LYM	NEUT	LBW	birthwt	GADelivery	Gender	Modelivery
0005	185	1.9	1.3					
0068	169	1.4	2.2	2	3.2	38	1	2
0041				2	4.1	41	1	2
0003	228	2.7	5.1	2	2.9	40	1	1
0045	148	1.3	2.8	2	3.45	39	1	1
0064				2	2.7	38		
0042	58	1.5	3	2	3.75	40	1	
0031	210	1.4	1.5	2	3	38	2	1
0006	99	1.7		2	3.9	39	1	1
0015	330							
0014				2	3.8	40	1	1
0114	198	1.5	5.6	2	3.2	38	1	1
0116	203	0.6	7	2	3.1	36		
0124	182	1.6	4.4	2	3.5	38		
0115	201	1.3	3.1	2	2.6	37	1	2
0104	250	2.2	2.9	2	3.2	40	1	1
0092	183	1.3	4.7	2	3.2	39	2	1
0086	228	1.6	7.7	2	3.05	38	1	1
0044	190	1.1	5.4	2	3.3	37	2	1
0076	190	1.7	2.9	2	3.9	39	2	1
0036				2	3.45	40	1	1
0037	588	2.9	2.3					
0091	368	4.5	3.9					
0096	346							
0089	311	2.6	2.4					
0099	264	3	6.6					
0029	373	2.7	4.6					
0094	637	3.8	6.1					
0098	336							
0088	334	3.6	4.8					

subjno	PLT	LYM	NEUT	LBW	birthwt	GADelivery	Gender	Modelivery
0055	283	4.2						
0095	368							
0022	317	4.4						
0026	289	2.4	2.9					
0002								
0008	470							
0016	132							
0007	392	3.9						
0028								
0032	309	3.4	3.3					
0063	472	3.5	2.4					
0020	206	4.5						
0067	375	2.8						
0079	283	2.9	5					
0047	753	6	5.1					
0051	230	2.1	1.8					
0127	562	3.9	8.3	1	2	36	1	2
0138	306	2.4	5.8	1	1.5	31	1	2
0052	365	2.2	3.9	2	3.1	38		
0109	393	2		2	3.4			
0107	401	2.1	4.8	2	3.2	40	1	1
0074				2	2.9	37	1	1
0106	535	3.2	6.9	1	2.45	37	2	1
0053	436	2.4	9.3	1	2.35	39	2	2
0062	196	1.9	3	2	2.8	36	1	
0059	379	1.9	7.1	2	2.9	38	2	1
0049	430	2.2	4.5	2	2.75	39	2	2
0087	281	3.3	7.7	2	3.2	38	1	1
0133	494	4.4	8.2	1	2.2	35	2	2
0122	344	2.1	5.3	2	2.7	37	2	2

subjno	PLT	LYM	NEUT	LBW	birthwt	GADelivery	Gender	Modelivery
0121	448			1	2.1	36	2	2
0134	533	2.5	10.6					
0139	525	3.3	7.2	2	3.1	37	1	2
0131	326	7.7	7	2	2.5	39	1	2
0123	722	3.4	6.1	2	3.5	39		
0132	232	3.9		2	3	39	2	2
0135	505	2.4	6.6	2	2.6	37		
0097	382	3	5.2	2	3.6	39	1	1
0058	395	2.8	5.6	2	2.5			
0137	672	3	6.4	2	2.6	39	2	1

Key:
Genotype: 1 = AA; 2 = SS
Gestage: 0 = non-pregnant, 1 = 16 weeks pregnant, 2 = 36 weeks pregnant.
P refers to plasma samples eg pcreat is plasma creatinine, whilst u refers to urinary samples.
Gender: 1 = male, 2 = female
Mode of delivery: 1 = vaginal delivery, 2 = emergency caesarean section, 3 = elective caesarean section