METHODS FOR THE SCREENING AND PREVENTION OF PREECLAMPSIA AND ITS COMPLICATIONS

by

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To Nick and my parents. For their unconditional support.

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Abstract

Preeclampsia is a serious disorder of pregnancy. The syndrome is the leading cause of maternal mortality in the United Kingdom and it also contributes significantly to perinatal morbidity and mortality. Maternal mortality is primarily due to cerebral complications.

Despite continued research, the aetiology of preeclampsia remains unknown. This has limited the development of screening, preventive and therapeutic measures to control the syndrome.

Present management is based on basic screening to detect early signs of the syndrome, observation of its progress and occasionally therapeutic intervention to control hypertension. Delivery is timed to prevent maternal and fetal complications, while simultaneously aiming to gain fetal maturity. Unfortunately, in many situations this control is not possible.

Owing to an increasing understanding of the pathogenesis of the disease, a new option for prophylaxis - low-dose aspirin - may soon be available. If prevention of preeclampsia becomes a reality, a simple but sensitive screening test will be required to select those women who will benefit from treatment.

This thesis is focused on the prevention of preeclampsia and its complications. It will involve examination of screening tests, the preventive therapy low-dose aspirin, and the preliminary assessment of a new technique to detect women at risk for developing cerebral complications from the disease.

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Abbreviations

5HT	serotonin
ABP	ambulatory blood pressure
AII	angiotensin II
AC	abdominal circumference
ANOVA	analysis of variance
AST	Angiotensin Sensitivity Test
AUK	active urinary kallikrein
BP	blood pressure
BPM	beats per minute
CLASP	Collaborative Low-dose Aspirin Study in Pregnancy
CV	coefficient of variation
EPD	effective pressor dose
FVW	flow velocity waveform
HMWK	high molecular weight kininogen
IUGR	intrauterine growth retardation
IUK	inactive urinary kallikrein
IUK/Cr	inactive urinary kallikrein / creatinine ratio
JRH	John Radcliffe Hospital
K5	Korotkoff Phase 5
MAP	mean arterial pressure
MAXA2	maximum angiotensin II dose
MCA	middle cerebral artery
NPV	negative predictive value
OD 405	optical densitiy 405 nM
PGE1	prostaglandin E1
PGE2	prostaglandin E2
PGI2	prostacyclin
PI	pulsatility index
PPV	positive predictive value
QCH	Queen Charlotte's and Chelsea Hospital
SD	standard deviation
SENS	sensitivity
SPEC	specificity
TCD	transcranial Doppler ultrasound
TXA2	thromboxane A2

CHAPTER I

Introduction

BACKGROUND

Preeclampsia is a condition unique to human pregnancy; a disorder which results in significant maternal and fetal morbidity and mortality. It is a syndrome which may present with one or a combination of cardiovascular, renal, cerebrovascular, hepatic, neurological or haematological complications. No presentation is characteristic, but using present definitions, hypertension is an essential component of the disorder. Progression to the tertiary pathological complications such as renal nephrotic syndrome, cerebrovascular complications and eclampsia is unpredictable and dangerous.

Significance of the Syndrome

The syndrome occurs in at least 5-6 percent (Chesley 1978) of the general pregnant population although the increased incidence in primiparous women of 11-12% (Redman and Jefferies, 1988) has led to the concept of preeclampsia as a disorder of primiparity. Other risk factors include history of preeclampsia, underlying medical disorders, multiple pregnancy and certain placental pathologies. Preeclampsia and eclampsia combined are the major cause of maternal mortality in Western countries (Redman 1988) and with the higher maternal mortality rates in developing countries, death rates due to eclampsia are also greater (Duley 1992). The maternal mortality rate secondary to hypertensive disorders of pregnancy in the United Kingdom is reported as 12.6 per million maternities (Department of Health, 1991). Although the number of deaths is not high, many may be preventable, and the morbidity associated with the disease is considerable. The eclampsia rate in the United Kingdom is unknown, but two reports from New Zealand and the USA have shown a rate of 0.06% of all births (Medical Research Council 1962; Saftlas 1990).

The perinatal morbidity and mortality results from intrauterine growth retardation (IUGR), placental abruption, intrauterine asphyxia and preterm delivery. Preeclampsia may only be directly responsible for a stillbirth rate of 1-2/1000 pregnancies but the problems secondary to preterm delivery, whether iatrogenic or not, are a major contributer to morbidity and mortality. Preeclampsia contributes to approximately 5% of

perinatal mortality (Edouard and Alberman 1984) and preeclampsia is the main indication for elective preterm delivery (Rush et al, 1976).

Substantial medical resources are allocated towards the management of preeclampsia. They include those for antenatal surveillance to diagnose early disease and to monitor its progress, those for hospitalisation for severe illness, specialised monitoring, operative delivery and those for neonatal services.

Definition

Various definitions and classifications have been formed to assist in the clinical management and study of preeclampsia, and yet, uncertainty arises over their real meaning because no "gold standard" sign is available to define the disease with certainty. This is because the cause of preeclampsia is unknown, and none of the signs of the syndrome are either specific or consistently present. Currently, all of these definitions use hypertension as the major criterion to diagnose the syndrome, although controversy exists as to whether hypertension is a mandatory component of the syndrome. Therefore when any one of these definitions are used, recognition of its limitations should be kept in mind. One definition for preeclampsia (Redman and Jefferies, 1988) may be more useful than others because it selects a high proportion of primigravidae who are characteristically more susceptible to the disorder. Furthermore, it is the only definition which has been developed from analysis of a large database (16,000 pregnancies) and then applied to another group of similar size (15,000 women). In both analyses a higher proportion of primigravid women were selected and the definition excluded many women, predominantly multiparous, who would have been included by the traditional definition of using a threshold of 90mmHg diastolic pressure alone (Nelson 1955). This definition which recognises both nonproteinuric and proteinuric forms of the syndrome was used in the present study and is described in Chapter II.

Aetiology and Pathogenesis

The aetiology of the disease is unknown. Immunological, genetic and infective origins

have been proposed but never proven. It is recognised that preeclampsia is a uteroplacental disease because the manifestations disappear rapidly following delivery. That placental tissue only is needed is demonstrated by the occurrence of preeclampsia with hydatidiform mole (Chun et al, 1964).

The Placenta

Poor placentation, occurring at approximately 6-18 weeks gestation, is considered to be a primary event in preeclampsia. The signs and symptoms of the disease develop later (characteristically in the third trimester) and are thought to be the maternal response to increasing placental ischaemia. The structural lesions causing ischaemia have been identified. The maternal blood supply to the placenta depends on the maternal spiral arteries and the interface between the villous lakes and trophoblast. During placentation, the spiral arteries are converted into open vessels which resist the contractile response to autonomic and hormonal stimuli. The process involves trophoblastic invasion of the spiral artery to replace the normal muscular and elastic tissue of the media with fibrin and connective tissue. The invasion occurs in two waves: the first in the decidual portion of the spiral artery in the first trimester; the second in the adjacent distal myometrial segment at approximately 14-18 weeks gestation (Robertson et al, 1975). This enables a high volume blood flow to the intervillous space (Brosens et al, 1967). In preeclampsia and idiopathic IUGR without preeclampsia, the second invasion does not occur, the spiral arteries remain maladapted for the needs of pregnancy, and therefore the blood supply to the placenta cannot expand normally in the second half of pregnancy (Brosens et al, 1972; Robertson et al, 1975).

The second defect in the spiral arteries identified is an obstructive lesion called "acute atherosis" (Zeek and Assali, 1950; Robertson et al, 1967). The histological findings include aggregates of fibrin, platelets and lipid-loaded macrophages which partially or completely block the arteries. These pathological changes underlie the increased incidence of placental infarction and abruption associated with these disorders (Brosens and Renaer, 1972).

Other Factors

Although hypertension has been emphasised as a major component in the pathophysiology of preeclampsia, it is probable that it is only one of a number of secondary disturbances which arise from generalised dysfunction of the maternal endothelium (Roberts et al, 1989). Endothelial cell injury reduces the synthesis of vasorelaxing agents [prostacyclin (PGI2) and endothelium derived relaxing factor (EDRF)], and conversely increases the release of potent vasoconstrictors such as endothelin (Loscalzo 1992). Reduction in prostacyclin synthesis and release also promotes increased platelet activation and aggregation; a feature of the disorder. It has been proposed that the ischaemic placenta may release toxic factor(s) into the systemic circulation that will injure endothelial cells (Taylor et al, 1990). As yet this factor has not been identified. However, there is evidence for increased uteroplacental traffic in preeclampsia, in particular, deportation of trophoblast into the maternal systemic circulation (Chua et al, 1991).

The coagulation system is activated in preeclampsia (Bonnar et al, 1971; Howie et al, 1971). The fibrin deposition in "acute atherosis" results from local activation whereas the tertiary clinical syndromes of disseminated intravascular coagulation (DIC) and the HELLP syndrome [haemolysis, elevated liver enzymes and low platelets] (Weinstein 1982) reflect systemic involvement. Laboratory investigations show increased Factor VIII coagulant consumption (Redman et al, 1977a), increased fibrin degradation products (FDPs) and decreased fibrinogen levels. In addition, platelet consumption may be an early feature of the disease (Redman et al, 1978). Increased platelet consumption is demonstrated by reduced platelet count (Bonnar et al, 1971; Howie et al, 1971), increased platelet volume (Giles and Inglis, 1981) and reduced platelet lifespan (Rakoczi et al, 1979).

Prostaglandins

Arachidonic acid metabolites of the eicosanoid series are involved in maintaining the integrity of the circulation. Two of the most important are prostacyclin (PGI2) and thromboxane A2 (TXA2), synthesised by the enzyme cyclo-oxygenase.

TXA2, produced by platelets predominantly, is a potent vasoconstrictor, and stimulus for platelet aggregation and uterine activity whereas PGI2, produced by the vascular endothelium, is a vasodilator, inhibitor of platelet aggregation and inhibitor of uterine activity.

In normal pregnancy it is thought that a balance exists between these two compounds (Walsh et al, 1986). This acts to maintain vascular patency and to allow platelet activation only in response to endothelial injury. An imbalance of these two compounds, favouring TXA2, would account for many of the pathophysiological and clinical signs of preeclampsia.

These highly labile compounds are measured by their stable metabolites TXB2 and 6-keto-PGF1α in serum or urine. Various tissues of human pregnancy are known to produce PGI2 including the placenta, uteroplacental vessels, amnion, chorion, decidua and myometrium. It is likely that this production contributes to the vasodilation and reduced peripheral resistance characteristic of pregnancy. Women with preeclampsia demonstrate lower PGI2 concentration in the amniotic fluid (Ylikorkala et al, 1981) and in the placenta (Walsh 1985; Walsh et al, 1985), and possibly enhanced platelet (Fitzgerald et al, 1990; Wallenburg and Rotmans, 1982) and placental (Walsh 1985) TXA2 concentrations. Not all investigators have shown this increase in TXA2 concentration (Ylikorkala et al, 1981; Benigni et al, 1989) but most agree that the relative activity of TXA2 to PGI2 is shifted towards the former.

Results of serum and urinary excretion levels need to be interpreted with caution because prostaglandins are locally acting compounds involved in a paracrine system of regulation. Therefore such measurements are unlikely to reflect their action at a cellular level. In addition, changes which occur in the fetal compartment may not be reflected in

the maternal compartment and vice versa. PGI2 infused into maternal vessels for the treatment of hypertension associated with preeclampsia reduced systemic blood pressure (excluding side-effects) but did not increase uteroplacental perfusion or fetal well-being (Jouppila at al,1985).

In conclusion, an imbalance of the vasoactive prostaglandins most likely contributes to the pathogenesis of preeclampsia, but whether this is a direct consequence of uteroplacental ischaemia, or alternatively an indirect response secondary to endothelial damage is not known.

Management

The management of preeclampsia remains empirical. Termination of the pregnancy is the only method available which allows resolution of the disease process. Delivery is, whenever possible, expedited at the stage when the risks of preterm delivery are less than the risks for the mother or fetus "in utero". Control of moderate hypertension by drug therapy has not been shown to delay the onset of preeclampsia (Redman et al, 1976a; Sibai et al, 1990); antihypertensive treatment is used to protect the mother from the cerebral complications of severe hypertension [$\geq$ 170/110 mmHg] (Kyle and Redman, 1992).

The reason for this style of management has been twofold. Apart from determining "at-risk" groups from the medical history, no tests are available which reliably predict which patients will develop serious disease, and no methods for prevention or therapeutic intervention have been shown definitively to alter the course of the disease. However, evidence is accumulating that the disease may be prevented or ameliorated by antiplatelet therapy (Beaufils et al, 1955; Wallenburg et al, 1986; Imperiale and Petrulis et al, 1991). If proven, this will have a major impact on the disorder.

A NEW PREVENTIVE TREATMENT?

Low-dose Aspirin

Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDS) inhibit the enzyme cyclo-oxygenase that catalyses one stage of the conversion of arachidonic acid to TXA2 and PGI2. Aspirin inhibits the enzyme irreversibly by acetylation.

A dose of aspirin between 30-80 mg daily effectively inhibits cyclo-oxygenase in platelets while having minimal effect on the enzyme's action in the endothelium (Weksler et al, 1983). This is because new production of cyclo-oxygenase cannot occur during the platelet lifespan of up to 10 days as these cells are nonnucleated. In contrast, the enzyme can be resynthesised by nucleated endothelial cells within four hours of aspirin exposure (de Swiet and Fryers, 1990). Overall, this leads to reduced TXA2 but normal PGI2 production (Thorp et al, 1988), a result which favours decreased platelet aggregation. This differential effect may also be secondary to the degree of aspirin exposure: there are many more endothelial cells than platelets in the vascular system (Walsh et al, 1990), and much of the aspirin is metabolised in the liver before reaching the systemic circulation (Pedersen and Fitzgerald, 1984).

Knowledge of this mechanism stimulated investigators to administer low-dose aspirin to prevent thrombotic disease. Large-scale trials have demonstrated its efficacy in preventing transient ischaemic attacks (TIA) and thrombotic cerebrovascular accidents; extension of myocardial infarctions and their secondary occurrence (Antiplatelet Trialists Collaboration, 1988); and to maintain patency in arterio-venous grafts (Lorenz et al, 1984). The success of these trials and the recognition that increased platelet aggregation is a component of preeclampsia and IUGR led to clinical trials to assess the effectiveness of low-dose aspirin to prevent preeclampsia.

Clinical Trials

Following an initial case report of aspirin in the treatment of preeclampsia (Goodlin et al, 1978) and a retrospective study intimating a potential effect of aspirin to prevent the

disease (Crandon et al, 1979), the first prospective controlled trial was published in 1985 (Beaufils et al, 1985). This involved 102 high-risk women selected because of a history of obstetric or medical complications. The subjects were randomised to treatment from 12 weeks onwards (150 mg aspirin and 300 mg dipyridamole) or to none (controls). The pregnancy outcome was significantly improved with treatment, including a lower incidence of preeclampsia and IUGR. However, although this study was encouraging, it could be criticised because there was no placebo controlled group, the numbers were small, and the subjects had a diverse range of problems. If the groups were not totally randomised these underlying disorders may have biased the outcome.

The following year, the results of a small randomised double-blind placebo-controlled trial were reported. The trial involved a group of primigravid women considered to be at risk for preeclampsia because they showed increased pressor sensitivity to intravenous angiotensin II at 28 weeks gestation (Wallenburg et al, 1986). The 23 women on treatment (60 mg aspirin) developed significantly less preeclampsia compared to the 23 women assigned to the placebo group. This study was important for two reasons. Not only was it the first randomised placebo-controlled trial to show a beneficial effect from low-dose aspirin in pregnancy, but it also indicated that low-dose aspirin might prevent or ameliorate preeclampsia when commenced late in pregnancy.

Subsequently, many studies of variable size (Wallenburg and Rotmans, 1987; McParland et al,1990; Uzan et al, 1991) and their overviews [Imperiale and Petrulis, 1991; Collins 1992] have provided increasing evidence that low-dose aspirin is effective in preventing proteinuric preeclampsia and IUGR. Two other studies have shown that introduction of low-dose aspirin in the third trimester may prevent proteinuric preeclampsia (Schiff et al, 1989) or ameliorate IUGR (Trudinger et al, 1988). No effect has been demonstrated using low-dose aspirin for the treatment for established mild preeclampsia (Schiff et al, 1990). In contrast to most of these positive trials in high-risk women, two recent studies have shown no beneficial effect of low-dose aspirin to prevent preeclampsia in women of low to moderate risk (Italian Study of Aspirin in Pregnancy,

1993; Sibai et al, 1993). Therefore at present, the efficacy of low-dose aspirin to prevent preeclampsia remains unclear. Furthermore, none of the above trials have been large enough to allow a comment on the perinatal mortality rate, a much more significant clinical endpoint. In addition, although only one of the trials reported an adverse pregnancy complication in the treated group (Sibai et al, 1993), the numbers have been too small to identify rare but serious side-effects.

The Collaborative Low-Dose Aspirin Study in Pregnancy - CLASP

To address the questions of the potential effect of low-dose aspirin therapy on the perinatal mortality rate and the incidence of adverse side-effects, a large international multi-centre trial has been established in the United Kingdom to assess the efficacy of low-dose aspirin to prevent proteinuric preeclampsia, IUGR, and to reduce perinatal mortality in high-risk pregnancies (CLASP coordinating committee protocol, 1988). The aim is to recruit 10,000 women; a number calculated to allow potential outcome differences between the two allocation groups (aspirin or placebo) to be interpreted with statistical confidence. Follow-up of the children at 18 months age will provide more reliable information concerning potential treatment related complications.

Two entry groups have been defined.

1.The *preventive* arm of the trial involves women who have a compromised obstetric or medical history which predisposes them to develop preeclampsia and/or IUGR.

2.The *treatment* arm involves women recognised to have early or established preeclampsia and/or IUGR before 32 weeks gestation.

The study design involves double-blind randomisation to 60 mg aspirin daily for treatment or a similar placebo tablet for controls, both commencing between 12 and 32 weeks gestation. Computer randomisation is performed using a stratification procedure to balance the entry groups. The study requires no further intervention and clinical

management is left to the individual obstetrician and/or physician. In many centres the tablets are discontinued at approximately 38 weeks gestation to avoid possible problems with epidural insertion, a theoretical risk which as yet has not been confirmed in practice. Following delivery, a review is obtained from the notes once the patient and infant have been discharged from hospital. A paediatric follow-up is performed at 18 months of age using a letter questionnaire to the parents of the child and the general practitioner.

The Potential Adverse Effects of Aspirin in Pregnancy

Before low-dose aspirin can be considered for widespread use in pregnancy, the range and severity of potential side-effects have had to be evaluated.

Safety combined with efficacy must always be considered when any drug is introduced into clinical practice and caution is required particularly in pregnancy, because both mother and fetus are involved. Fetal effects may include teratogenicity, diminished fetal growth, and maladaptation of a specific organ for function in extrauterine life. There need be only one adverse effect for the medication to be unacceptable.

The imputed therapeutic effect of low-dose aspirin in pregnancy is secondary to reduced TXA2 production and platelet aggregation. Such a nonspecific mechanism could potentially predispose to fetal and/or maternal haemorrhage. Therefore the degree to which such complications may occur must be determined before the therapy is introduced into general obstetric practice.

Aspirin Metabolism

Aspirin is absorbed predominantly from the small intestine and then is rapidly converted to salicyclic acid by hepatic metabolism. Following ingestion of low-dose aspirin the bioavailability to the systemic circulation is low. Salicyclic acid has different effects to aspirin, specifically, the enzyme cyclo-oxygenase is not inhibited by the levels achieved

in the human circulation following metabolism of routine doses of aspirin. There is no evidence that salicylate affects platelet aggregation (de Swiet and Fryers, 1990).

The degree to which low-dose aspirin crosses the placenta and affects the fetus is not fully known. Fetal PGI2 production was reduced with maternal ingestion of 500 mg but not 100 mg aspirin (Ylikorala et al, 1986). Some have reported partial inhibition of fetal TXB2 production (Ylikorala et al,1986; Benigni et al, 1989) or fetal platelet aggregation (Forestier et al, 1985) with maternal doses less than 100 mg, but others have observed no reduction in fetal TXB2 production (Ritter et al, 1987; Sibai et al, 1989). In addition, the effect appears to vary between individuals (Ritter et al, 1987). Therefore, the available evidence suggests that doses of 35-75 mg may partially but not consistently interfere with fetal platelet function. This may be of no clinical consequence, particularly as no clinical trial has reported an adverse fetal outcome using aspirin at a dose less than 150 mg. Nevertheless, caution is still required until more reliable evidence becomes available from the paediatric follow-up in the CLASP trial.

The potential adverse maternal and fetal effects from aspirin exposure are now reviewed.

Haemorrhage

Pregnancy, labour and delivery are associated with a high risk for haemorrhage that can cause significant maternal and perinatal morbidity and mortality. Intracranial haemorrhage occurs more frequently in the preterm infant less than 34 weeks gestation.

The initial clinical trials using aspirin in doses of 150 mg or less have not shown an increased incidence of maternal or fetal haemorrhage although the extent to which these events were defined or followed up were not reported.

Previous reports which have indicated that aspirin may increase the risk for haemorrhage have involved therapeutic doses of aspirin used for analgesia or, more often, larger quantities associated with aspirin abuse.

Maternal Effects

Women known to have a high salicylate consumption in pregnancy (Collins et al, 1975) were found to have an increased incidence of peripartum haemorrhage, complicated deliveries, infants with low birth weights and increased perinatal mortality compared to non-aspirin takers. The incidence of congenital abnormalities was not increased. However, most of these subjects were ingesting high doses of aspirin-containing compounds as part of a habit of analgesic abuse endemic in the Australian population at that time. The relevance of this study to the present clinical situation is probably small, because much of the poor outcome may have been attributable to secondary features of the drug abuse such as analgesic nephropathy.

An increased incidence of intrapartum blood loss has been reported, if greater than five grams of aspirin were taken within five days of delivery (Stuart et al, 1982). Outside this period, the blood loss was similar to that of the control population of non-aspirin takers.

The development of an epidural haematoma following insertion of an epidural is a potential complication of aspirin therapy (MacDonald 1991), but as yet, no association has been reported despite aspirin being an ubiquitous drug in the community. Epidural haematomas, either spontaneous or iatrogenic, are rare (Kane 1981). Nevertheless, the extent of this risk needs to be known because of the increasing use of epidural analgesia and anaesthesia in present-day obstetrics, particularly in primigravid and high-risk pregnancies, the groups which are most likely to benefit from low-dose aspirin therapy. A preliminary report from the CLASP study has shown that of 1069 women who had an epidural, there was no increase in bleeding related to insertion of the epidural in women treated with aspirin in comparison to the placebo group (de Swiet and Redman, 1992).

Fetal Effects

Haemorrhage

A significant increase in the incidence of intraventricular haemorrhage was found in

preterm neonates (born less than 34 weeks gestation) of mothers who had taken aspirin in the week prior to delivery (Rumack et al, 1981). The information regarding aspirin intake was collected before the computerised tomographic (CT) scan was performed to make the diagnosis. The minimum dose of aspirin to cause this effect was one aspirin tablet (300 mg) within seven days of delivery. An increased incidence of neonatal bleeding has also been reported in infants of mothers exposed to aspirin (5-10 g) within five days of delivery (Stuart et al, 1982). Manifestations included petechial haemorrhages over the presenting part, cephalhaematoma, subconjunctival haemorrhages, or microscopic haematuria.

Therefore, there is a definite risk for abnormal bleeding in neonates exposed to large doses of aspirin but the risk associated with low-dose aspirin exposure remains unknown; low-dose aspirin was not used therapeutically at the time of these reports. These risks and their long-term complications need to be evaluated in paediatric follow-up studies.

Abruption

One study has shown an increased rate of abruption in the women taking low-dose aspirin (0.7%) as compared to placebo (0.1%) for the prevention of preeclampsia (Sibai et al, 1993). This complication has not been shown in any of the previous studies, and so the clinical impact of this finding awaits the results of the CLASP trial.

Teratogenicity

Although animal studies suggest that high doses of aspirin may induce a variety of congenital abnormalities (Warkany and Takacs, 1959), the problem has not been confirmed in epidemiological studies of human pregnancy. In a review of 50,282 pregnancies (the Collaborative Perinatal Project), women with heavy and intermediate aspirin exposure were identified (5128 and 9736 respectively) prior to the birth of the infant (Slone et al, 1976). No single malformation, nor the total number of malformations was greater in the aspirin-treated group compared to the controls. A

similar result was observed in a cohort of 144 pregnant women who were known to be heavy aspirin takers [greater than 1-2 grams per day] (Turner and Collins, 1975).

Two retrospective studies have shown an increased risk for certain malformations in aspirin takers during pregnancy (Richards 1969; Nelson and Forfar, 1971). However, there was the potential for bias associated with recall in a retrospective study and, in addition, the type of malformation was not consistent.

An increased incidence of specific congenital heart disorders associated with aspirin ingestion in the first trimester of pregnancy has been suggested (Zierler et al, 1985), but not confirmed in a larger study comparing those infants with congenital heart disease to a large control group of children with other congenital malformations (Werler et al, 1989).

The conclusion from these large and thus powerful epidemiological studies is that in therapeutic doses aspirin is unlikely to be teratogenic. Nevertheless, to avoid any possible risk, no woman is entered into the CLASP trial before 12 weeks gestation.

Premature Closure of the Ductus Arteriosus

Aspirin inhibits prostaglandin production and so may induce significant side-effects in situations where prostaglandin levels are critical for normal function.

The ductus arteriosus maintains patency in the fetal circulation by the action of the prostaglandins PGE1 and PGI2. This knowledge has been applied therapeutically for two complications in the neonate. With persistent patency associated with fetal compromise, the duct can be closed by indomethacin (Gersony et al, 1983) and conversely, when continued patency is desired prior to surgery with certain congenital heart defects, PGE1 or PGE2 can be infused (Lang et al, 1979).

The analogous situation in the in-utero environment implies a potential risk that aspirin may induce premature closure of the ductus arteriosus with associated pulmonary hypertension in early neonatal life.

Primary pulmonary hypertension in the neonate has been reported following maternal ingestion of indomethacin and aspirin (Levin et al,1978). Histological sections showed

smooth muscle proliferation in the pulmonary arteries and a reduced number of arteries per cm^3 in lung tissue. Premature closure of the ductus arteriosus in-utero was considered responsible for the clinical and histological findings.

However indomethacin, another cyclo-oxygenase inhibitor, has been used to suppress labour in pregnancies less than 34 weeks gestation with no adverse fetal or neonatal effects. In 50 women treated with indomethacin for preterm labour, four of the 12 perinatal deaths resulted from respiratory complications within 48 hours of birth but these were attributed to lung immaturity (Zuckerman et al, 1974). A subsequent trial showed no fetal or neonatal effects from indomethacin therapy (Niebyl et al, 1980).

Likewise, no abnormalities were shown in a prospective study of 27 neonates whose mothers had taken large doses of aspirin daily (1 gram) as prophylaxis against thromboembolism following insertion of prosthetic cardiac valves (Nunez et al, 1983). This endorses the findings of the large Collaborative Perinatal Project where no increase in the perinatal morbidity was observed in aspirin takers (Shapiro et al, 1976). Furthermore, it has been suggested that the ductus arteriosus may be less sensitive to aspirin than to indomethacin (MacLennan et al 1988).

In all of these cases the doses of aspirin and indomethacin were large. No report of an adverse effect on the neonatal pulmonary vasculature from low-dose aspirin ingestion in pregnancy is available. It is unlikely because the effect of low-dose aspirin on nucleated cells is minimal as these cells can resynthesise cyclo-oxygenase rapidly.

Intellectual Function

Concern that low-dose aspirin in pregnancy could have a detrimental effect on the later intellectual function of the infants has also been expressed (Streissguth et al, 1987). Cerebral haemorrhages could potentially impair cerebral function. However, in a follow-up study from the Collaborative Perinatal Project evaluating 19,226 pregnancies (Klebanoff et al, 1988), no reduction in the intelligence quotient of infants exposed to aspirin during pregnancy was shown at four years of age. This result from such a large study makes it unlikely that aspirin could have any adverse effect on intelligence.

Conclusion

At present, there is only one report of a significant complication from low-dose aspirin therapy in pregnancy (Sibai et al, 1993). This complication, abruption, and the evaluation of all other potential complications will only be available from the follow-up results of large multi-centre trials such as the CLASP trial. If side-effects are demonstrated, then the selection of women for treatment will depend upon a balance between risks for developing serious disease and those from the side-effects.

A SCREENING TEST FOR PREECLAMPSIA

Many women can be identified as being at increased risk for developing preeclampsia by their medical and/or obstetric history. Therefore they would be suitable for preventive therapy. However primigravid women and those with multiple pregnancy constitute significant risk groups and yet it is unlikely that all women in these groups will wish to take low-dose aspirin, particularly until potential side-effects have been fully excluded. A more satisfactory and acceptable approach would be to screen the women in these groups and to use preventive therapy in those women identified to be at increased risk.

The Philosophy of Screening

Screening has been defined as "the identification among apparently healthy individuals of those who are sufficiently at risk of a specific disorder to justify a subsequent diagnostic test or procedure, or in certain circumstances direct preventive action" (Cuckle and Wald, 1984). Not only must a screening test have high specificity and sensitivity, but it must also be simple, inexpensive, convenient and noninvasive to be useful in clinical practice.

Screening for Preeclampsia

The purposes of a screening test for preeclampsia are to enable more antenatal care to be directed towards a small group of high-risk women and, second, to be able to use preventive therapy if it is available. Low-dose aspirin, if proven to be effective, would be such a therapy.

Although the signs and symptoms of preeclampsia develop characteristically in the third trimester of pregnancy, poor placentation is thought to be a primary event in the disease occurring at 6-18 weeks gestation. It therefore seems logical that if the development of the disease commences early in pregnancy, physiological deviations from normal (biochemical, haematological or biophysical) should be detectable prior to the appearance of the conventional signs and symptoms.

History of Screening Tests

Many potential screening or early diagnostic tests for preeclampsia have been described (Table 1.1) but most have not been validated.

Tests of Altered Vascular Reactivity or responsiveness were initially investigated but most were unreliable and so are of historical value only. The pressor response to angiotensin II infusion is the exception although it is impractical for routine clinical use. It is described on page 21 because its use is central to this thesis. The high predictive value of the rollover test, described by Gant et al, 1974, has not been confirmed, and yet it is still used to try to select women at risk for developing preeclampsia (Schiff et al, 1989).

Biochemical Tests are altered in established disease. Examples are serum uric acid (Redman et al, 1976b) and urinary calcium excretion (Taufield et al, 1987), and the latter test may be useful for screening (Rodriguez et al, 1988; Sanchez-Ramos et al, 1991). In accordance with the hypothesis that preeclampsia is an endothelial cell disorder, markers which reflect endothelial cell damage have been and are being assessed. Cellular

Vascular Responsiveness

Mean arterial pressure (second trimester)
Cold pressor test
Isometric exercise test
Rollover test
Infusion of vasopressin
Infusion of catecholamines
Angiotensin Sensitivity Test (AST)

Biochemical Tests

Plasma uric acid
Urinary calcium excretion
Plasma fibronectin
Plasma placental proteins (PAPP-A)
Plasma deoxycytidylate deaminase

Haematological Tests

Haemoglobin / haematocrit / plasma volume
Platelet count / platelet volume
Plasma ß-thromboglobulin
Plasma Von Willebrand factor
Plasma antithrombin III
Platelet angiotensin II receptors
Platelet intracellular calcium response to arginine-vasopressin

Biophysical Studies

Doppler ultrasound of the uteroplacental circulation

Table 1.1
Tests for the early identification of preeclampsia.

fibronectin appears to be increased in women destined to develop preeclampsia (Lockwood and Peters, 1990; Taylor et al, 1991), but the predictive value of the test has not been determined.

Haematological Markers are considered to be altered early in the disease process, although most indices of increased coagulation such as factor VIII-related antigen (Redman et al, 1977a) and antithrombin III (Weiner and Brandt, 1982) are only elevated in established disease. However, several tests of platelet function, activation or receptor status are currently being investigated as potential screening tests. These include: platelet angiotensin II receptor levels (Baker et al, 1992), platelet free intracellular calcium levels and their response to arginine-vasopressin (Zemel et al, 1990; Zemel et al, 1992), and binding of monoclonal antibodies to specific antigens expressed at platelet activation (Janes et al, 1991). Initial reports have appeared encouraging, but require confirmation by others.

Biophysical Studies It seems plausible that Doppler flow studies of the uteroplacental circulation may detect abnormalities in pregnancies with preeclampsia and IUGR (Campbell et al, 1983) or those destined to develop these complications (Campbell et al, 1986) because abnormalities of the trophoblastic invasion of the spiral arteries is involved in the pathogenesis of these disorders. Quantitative examination of the flow velocity waveforms (FVW) is usually performed; estimation of flow volume is seldom used owing to the inaccuracy of the technique. Variable results are reported when this technique is used for screening in the second trimester (Campbell et al, 1986; Hanretty et al, 1989; Jacobson et al, 1990; Steel et al, 1990) but the different studies applied very different methods. A reference range for recordings standardised for placental and uterine site has recently been given which may contribute to greater accuracy (Bewley et al, 1989). A further study from this group of 977 women showed that although those with an abnormal flow velocity waveform developed more proteinuric preeclampsia and IUGR, the predictive values for the test were low (Bewley et al, 1991). The FVW used for analysis was an average of readings from the uterine and arcuate arteries on both

sides of the uterus. Nevertheless, with refinement of the technique, particularly with colour Doppler ultrasound to identify specific vessels, the predictive values may increase.

Currently, screening for preeclampsia in general antenatal care depends on the detection of blood pressure increments, and the development of proteinuria and excessive weight gain. The mean arterial pressure (MAP) in the second trimester is increased in those women who later develop preeclampsia (Page and Christianson, 1976; Gallery et al, 1977), but this has not shown sufficient sensitivity and specificity to be valuable as a predictive test (Villar et al, 1989).

THE ANGIOTENSIN SENSITIVITY TEST (AST)

In normal pregnancy blood pressure and total peripheral resistance fall during the first trimester returning to prepregnancy levels around term, a change which has been described as the "vascular inertia of pregnancy". For women who develop preeclampsia, the fall in blood pressure may not occur or does not persist, secondary to a rise in total peripheral resistance. The increase in the vascular resistance is considered to be the fundamental haemodynamic abnormality that occurs in the disease. Recognition of this altered physiology stimulated the investigation of the vascular response to certain endogenous hormones when infused in pregnancy.

Infusion of Pressor Agents

The pressor effects of extracts from the posterior pituitary gland [vasopressin] (Dieckman et al, 1937) and catecholamines (Raab et al, 1956) have been shown to be greater in women with preeclampsia than in normal pregnant women. No attempts have been documented for using these hormones as a predictive test for preeclampsia. Subsequent to these investigations, angiotensin II was found to cause significantly different vascular effects between nonpregnant, normotensive pregnant and preeclamptic women.

The Renin-Angiotensin System and the Effects of Exogenous Angiotensin II

Angiotensin II (AII) is the major active component of the renin-angiotensin system, one of the vasoactive hormonal systems of the circulation. The involvement of the renin-angiotensin system in the pathogenesis of preeclampsia has been assessed, particularly because overactivity may result in various forms of renal hypertension. Angiotensin II, an octapeptide produced from conversion of angiotensin I by converting enzyme in lung tissue, is a potent vasoconstrictor with a short half life of 1-2 minutes. The effect of a bolus dose is brief.

In 1961, it was demonstrated that the blood pressure response to a bolus dose of AII in a pregnant woman was much less than that observed in the same woman following delivery (Abdul-Karim and Assali, 1961). It was concluded that normal pregnant women were refractory to AII when compared to nonpregnant women. Subsequently, it was shown that pregnant women who develop preeclampsia require a lower dose of AII to elicit a pressor response; they appear to lack the resistance to AII of normal pregnancy (Talledo et al, 1968).

In 1973, Gant at al, confirmed that women at risk for developing preeclampsia lose the diminished response to AII of normal pregnancy. They studied 192 primigravid teenage women successively throughout pregnancy. During the infusion, the effective pressor dose (EPD) was defined as the AII infusion rate in nanograms per kilogram per minute (ng/kg/min) which would induce a diastolic blood pressure elevation of 20 mmHg from the baseline (Phase V Korotkoff sounds). Of the pregnancies studied, 72 (37.5%) developed preeclampsia - defined as a persistently raised blood pressure of 140/90 or more, with a rise in the diastolic pressure of at least 20 mmHg from a blood pressure that was normal in early pregnancy and also at six weeks postpartum. A statistically significant difference in the EPD after 23-28 weeks gestation was observed between women who developed preeclampsia and those who did not. Furthermore, the increased sensitivity to AII antedated the clinical signs of the disease. Ninety percent of the women shown to have an EPD less than 8 ng/kg/min in at least one infusion between 28 and 32

weeks gestation developed preeclampsia whereas ninety-one percent of the women with an EPD greater or equal to 8 ng/kg/min remained normotensive.

Because of these strong predictive values, and the apparent safety of the procedure, these investigators suggested that the AII infusion may be a suitable screening test for preeclampsia. Only six other studies have assessed the performance of the Angiotensin Sensitivity Test (AST) in other populations (Table 1.2). These have shown varying results.

Most of the studies have not reproduced the high positive and negative predictive values found by Gant et al, 1973. The majority have shown the test to have a high negative predictive value, with the false negative cases developing mild preeclampsia only (Oney and Kaulhausen, 1982), but in addition they have shown a low positive predictive value. The reduced incidence of preeclampsia in the subsequent studies (10-15% versus 37.5%), in addition to a less angiotensin sensitive nonblack population (the EPD needed to be increased from 8 to 10 ng/kg/min) may have contributed to the disparity. In addition, discrepancies between results of the later studies do occur, the reasons for which are unclear. The numbers involved in most of the studies were small but it seems unlikely that minor alterations in technique such as automatic blood pressure readings, or using an alternative method to define the response to infused AII [for example slope of the diastolic pressure response SLOD (Baker et al, 1992)] would have been responsible.

Therefore, although the original study of Gant et al, 1973 demonstrated the AST to be a discriminant predictor for preeclampsia, described as the "gold standard" of screening tests for preeclampsia, none of the subsequent studies have reproduced this finding. Furthermore, because of the complexity of the procedure, the AST cannot be recommended as a routine screening test.

The AST has been used in a research setting to select women at high risk for developing preeclampsia (Wallenburg et al, 1986), but the above review of the literature challenges the concept that the test is an efficient screening procedure.

Authors	Patient No.	Prevalence Hypertension	EPD (ng/kg/min)	Sens (%)	Spec (%)	PPV (%)	NPV (%)
Gant et al (1973)	153	37.5	<8	90	87	78	95
Morris et al (1978)	26	12	<8	33	39	7	82
			<10	67	30	11	87
Orozco et al (1979)	33	27	<8	89	79	62	95
Oney et al (1982)	231	15	<10	76	83	45	95
Nakamura et al (1986)	48	21	<10	20	97	75	82
			<10	80	82	53	94
			<12	100	74	50	100
Dekker et al (1990)	90	13	<8	75	99	90	96
			<10	92	86	50	99
Baker et al (1992)	34	29.4δ	SLOD*	60	63	40	79

δ subjects selected at 28-32 weeks with diastolic pressure at 80-85 mmHg
* slope diastolic pressure response to the angiotensin II infusion

Table 1.2
Predictive value of the Angiotensin Sensitivity Test (AST) performed between 28-32 weeks in reported studies.

The Mechanism of the AST

The mechanism for the increasing vascular resistance to the pressor effects of AII found in normal pregnancy has been investigated but is still not well understood. A similar vascular response is not found on exposure to other pressor hormones such as noradrenaline (Ramsay et al, 1992).

The endogenous plasma levels of renin, renin activity, AII and aldosterone are increased in normal pregnancy despite the fall in total peripheral resistance. By contrast, in women who have established preeclampsia, the circulating levels of these hormones are reduced almost to nonpregnant levels.

This altered response to exogenous AII could be due to:

1. Changes in the endogenous circulating levels of AII
2. An increase in the rate of metabolism of AII
3. Modification of the vascular response to the hormone

An attempt to assess the influence of these three factors has been made (Gant et al, 1974; Cunningham et al, 1975). Acute volume loading with normal saline, dextran or packed red blood cells produced no effect on the pressor response to infused AII. Therefore, it was assumed unlikely that the endogenous concentration of AII is the controlling factor. However, these experiments only demonstrated the effect of an acute reduction in the circulating AII concentration; chronic changes may have more influence. Angiotensinase levels are not elevated in pregnancy and so it is doubtful that increased metabolism of AII is the mechanism for the decreased pressor response. It was therefore inferred that altered vessel wall reactivity underlay the modified response to AII in pregnancy.

The vascular response to a hormone is dependent on the vessel receptor number; the receptor affinity to the hormone; and the biochemical steps subsequent to the receptor activation.

The density and affinity of AII receptors in the vascular smooth muscle may change during pregnancy but these receptors are difficult to study in human pregnancy because

of limited access to the vessels and low receptor number (Broughton Pipkin, 1988). In rat studies, the up or down-regulation of these receptors may be in response to circulating endogenous AII levels (Gunther et al,1980). An increased circulating level, as found in pregnancy, may cause down-regulation of receptors and a diminished response to infused AII. Furthermore, sodium loading or depletion modifies the response to AII by changing the receptor density in vascular smooth muscle. Changes in the sodium concentration alter the circulating AII levels (Aguilera at al,1981). However, knowledge of this mechanism remains incomplete because another detailed investigation of rat tissue showed conflicting results (Paller, 1984). Neither the receptor number nor affinity changed in vascular smooth muscle during pregnancy. These investigators suggested that the vascular refractoriness to AII is more likely to be a post-receptor phenomenon, possibly mediated by paracrine factors such as prostaglandins.

Modification of the Angiotensin II Response

Other hormones or drugs can modify the vascular response to infused AII. Indomethacin and aspirin, both potent cyclo-oxygenase and therefore prostaglandin inhibitors, increase the pressor response to AII (Everett et al, 1978a). The increased vascular sensitivity following administration of these drugs implies that vascular reactivity may be prostaglandin mediated. Moreover, infused PGE2 (Broughton-Pipkin et al, 1982) and PGE1 (Broughton-Pipkin et al, 1987) attenuates the vascular response to AII, as does low-dose aspirin (Sanchez-Ramos et al, 1987; Spitz et al, 1988). Another study has shown that an infusion of 5α-dihydroprogesterone diminishes the pressor response to AII (Everett at al, 1978b) indicating that progesterone may also contribute to the decreased vascular response present in pregnancy.

The cellular response may be secondary to changes in cyclic AMP and intracellular calcium levels. The AST showed a decreased pressor response following a dose of oral theophylline given to women with mild preeclampsia (Everett et al, 1978c). Theophylline inhibits phosphodiesterase, an enzyme which controls cAMP levels, and the ensuing rise

in cellular cAMP increases the ratio of unbound to free intracellular calcium culminating in smooth muscle relaxation.

At present the AST provides some information about the mechanism of blood pressure control in pregnancy. However, the renin-angiotensin-aldosterone system is only one of many neural and hormonal systems influencing blood pressure control and vascular reactivity during pregnancy. Therefore, much is still to be understood about vascular reactivity and its involvement in preeclampsia.

THE PRESENT TRIAL

Increased vascular reactivity to angiotensin II has been shown to be present in some women destined to develop preeclampsia. The Angiotensin Sensitivity Test (AST) has been described as the reference screening test for preeclampsia, and therefore was used to select a high risk group of women for assessment of preventive therapy.

At the time the CLASP trial was conceived, the Wallenburg et al, 1986 study was the only randomised controlled trial available assessing the value of low-dose aspirin as a preventive agent. As described above, Wallenburg et al, used the AST to select patients for aspirin or placebo therapy. The numbers were small and therefore it was considered important to repeat the trial using larger numbers to confirm with greater statistical confidence that low-aspirin could prevent preeclampsia when commenced at the beginning of the third trimester.

In the present trial, a cohort of 495 healthy primigravid women were screened at 28 weeks gestation by the AST to select those at increased risk for developing preeclampsia. The angiotensin II sensitive women were then invited to enrol into the CLASP trial.

This trial provided an opportunity to investigate preeclampsia in nulliparous women in the following areas.

1. The prevention of preeclampsia with low-dose aspirin commenced at 28 weeks gestation (Chapter III).

2. Assessment of the predictive value of the Angiotensin Sensitivity Test in greater numbers than performed previously (Chapter III).

3. The assessment of several new screening tests on their own and in comparison to the Angiotensin Screening Test (AST) (Chapters IV, V, VI, VII).

4. The assessment of the maternal cerebral circulatory response associated with an acute rise in blood pressure during the angiotensin II infusion (Chapter VIII).

CHAPTER II

Patients and Study Design

Design of the Study

This thesis is based on a prospective trial using a cohort of 601 healthy nulliparous women recruited from the antenatal clinics during the early second trimester, the patient's "booking" visit. The investigations listed in Table 2.1 were performed in subgroups of this cohort at defined gestational ages. The aims of these investigations were first to assess screening for preeclampsia, and second, the maternal cerebral response to an acute blood pressure rise.

The Structure of the Main Trial

The Angiotensin Sensitivity Test (AST) is one of the few recognised screening tests for preeclampsia (Gant et al, 1973), and therefore it was chosen as a method to select a group of high-risk women for entry into the placebo-controlled double-blind aspirin trial - the CLASP trial (Collaborative Low-dose Aspirin Study in Pregnancy). Because of the complicated nature of the procedure, it was recognised at the outset of the study that the number of subjects screened would be limited (to what degree was unknown in the designated population), and therefore, the numbers entered into the CLASP trial would form a small subset of this large trial.

Calculation of Numbers

From previous European studies assessing the predictive value of the AST, 40-55% of women demonstrating a positive response went on to develop preeclampsia (Oney and Kaulhausen, 1982; Dekker et al, 1990). The numbers required for this study, using a power of 0.9 and an alpha of 0.05, and assuming a preeclampsia rate of 40% in the untreated group (placebo) with a reduction to 10% in the treatment group (aspirin), would be 45 subjects in each group. Previous studies have shown that approximately 20% of nulliparous women are angiotensin II sensitive (Oney and Kaulhausen, 1982; Nakamura et al, 1986; Dekker et al, 1990), which indicated that 450 women would be required for screening. It was recognised that not all women would wish to participate

Investigation	Gestation performed (weeks)
Screening	
1. Angiotensin Sensitivity Test	28
2. 24 hour ambulatory blood pressure	18 & 28
3. Platelet intracellular calcium response to arginine-vasopressin	28
4. Inactive urinary kallikrein	28
5. Haematocrit	12-20 & 28
6. Fetal abdominal circumference	28
Assessment of the maternal cerebral circulation	
Transcranial Doppler ultrasound of the maternal middle cerebral artery (MCA)	28

Table 2.1
The table lists the investigations and their timing that were performed during the study.

in every aspect of the study (particularly the AST and 24 hour ambulatory blood pressure study), and therefore the smaller subsets would require separate analyses. Furthermore, the numbers in the other studies were reduced because the development of the methodology for two of the screening tests (platelet free intracellular calcium response to arginine-vasopressin stimulation, and 24 hour ambulatory blood pressure measurement) occurred following the commencement of the AST screening.

Site of the Study

Recruitment and all the main procedures associated with the trial (unless specified separately) were performed at the

John Radcliffe Maternity Hospital, Oxford, England

Queen Charlotte's and Chelsea Hospital, London, England

Recruitment

A part-time midwife dedicated to subject recruitment was based in the antenatal clinic of each hospital. Suitable women were approached following review of the antenatal notes. The criteria for patient selection were:

- *nulliparous*
- *no medical history including hypertension, asthma, renal or cardiovascular disease*
- *no medication (excluding Iron/Folate supplementation)*

The women were provided with oral and written information describing the background and design of the trial. If interest was shown, the woman was contacted the following week to determine whether she wished to enter one or more of the trials. At that time or by later telephone conversation, an appointment was given for her to return to the hospital to undergo the screening procedure under study at the required gestational age.

Prior to the procedure, consent was obtained as required by the Central Oxford Hospitals and Queen Charlotte's Research Ethics Committees.

The Numbers Involved in Each Study

The number of women who participated in each study is listed in Table 2.2. Distinction is made between those who participated in the designated test in parallel to the AST and those who did not. Due to the variation in the availability of the different tests and in subject consent, none of the studies contained all of the same subjects. Additional subjects (controls and established preeclamptics) were used for the assessment of platelet free intracellular calcium and both the validation of this investigation (Chapter VI) and transcranial Doppler of the middle cerebral artery (Chapter VIII). Precise details of their recruitment are provided in these chapters.

Follow-up

Follow-up of the pregnancy details and outcome was performed by the investigator. The information was obtained from the "flagged" hospital notes following delivery.

Information Database

All clinical and study information was transferred from a datasheet to a computer database (DBASE 1V version 1.1 Ashton Tate) for later analysis. Details of the statistical analysis will be described with each study. Although some of the patients were involved in the CLASP trial, all analyses for the thesis were performed by the investigator independently.

Direction of Analysis

Assessment of a Screening Test. Although the AST has been regarded as one of the best predictors of whether preeclampsia will develop later in the pregnancy, it is an invasive and time-consuming procedure which is impractical for routine clinical use. Therefore the potential screening tests listed above, all of which are simpler and less invasive than the AST, were analysed first in relation to the results of the Angiotensin Sensitivity Test,

	Numbers Involved in Each Study	
	In parallel to AST*	Total
Angiotensin Sensitivity Test	495	495
Ambulatory blood pressure	56	162
Platelet intracellular calcium response to Arg-vasopressin	74	74
Inactive urinary kallikrein	459	459
Haematocrit	346	346
Fetal abdominal circumference	492	492
Transcranial Doppler MCA	101	101

Table 2.2
The number of women who were involved in each investigation during the study. The numbers who underwent an Angiotensin Sensitivity Test (AST) in addition to the relevant test are listed separately.* Due to the variation in the availability of the different tests and in the subject consent, none of the studies contained all of the same subjects.

and second to the pregnancy outcome. The principal endpoints used for pregnancy outcome were decided before the commencement of the trial and are as follows:

1. Incidence of preeclampsia (proteinuric and nonproteinuric*). The definition used for preeclampsia in this study was the same as in the main CLASP study. This requires an increase in diastolic pressure from the booking reading in the first half of pregnancy by at least 25 mmHg, to a maximum of 90 mmHg or more (Redman and Jefferies, 1988). Proteinuric preeclampsia was distinguished by the new occurrence of at least '+' noninfective proteinuria on two or more successive urine samples, or greater than 0.3 grams per 24 hour speciman.

2. Gestational age at delivery. All the subjects' dates were based on either a sure last menstrual period (LMP) or an early ultrasound scan before 22 weeks gestation.

3. Birthweight. When birthweight was corrected for gestational age and sex, standard Oxford tables were used (Yudkin et al, 1987).

4. Blood Loss. Routine estimation of the blood loss at delivery was made by the attending medical staff.

Decoding of the CLASP Trial Allocation

The CLASP steering committee gave permission that the trial allocation of treatment should be prematurely revealed for women who entered this study with a positive Angiotensin Sensitivity Test.

Computers

A Viglen PC 386 Genie Professional 33SX was used for data storage, analysis, and word processing. The programmes used for the study included DBASE IV v:1.1 (Ashton-Tate); Minitab PC Statistical Package Version 8.2 (Minitab Inc); Confidence Interval Analysis (CIA 1.1) British Medical Journal; Wordstar 7.0; Reference Manager version 5.0 (Research Information Systems Inc.). For the 24 hour ambulatory blood pressure analysis, additional analysis was performed on the Apple MacIntosh SE 31 computer using the programmes Excel 3.0 spreadsheet (Microsoft Inc.), and Statview SE + Graphics (1988 Abacus Concepts).

*This definition recognises both nonproteinuric and proteinuric forms of preeclampsia based on the precept that proteinuria is a late manifestation of preeclampsia. It seeks to define a subgroup of women with a poor outcome who exhibit hypertension without proteinuria.

CHAPTER III

The Angiotensin Sensitivity Test (AST) and Low-Dose Aspirin as a Preventive Agent for Preeclampsia

INTRODUCTION

This chapter describes the use of the Angiotensin Sensitivity Test (AST), as one of the few recognised screening tests for preeclampsia (Gant et al, 1973) [Chapter I p21], to select a group of high-risk women for entry into the placebo-controlled double-blind aspirin trial - the CLASP trial (Collaborative Low-dose Aspirin Study in Pregnancy) [Chapter I p10]. The methods and results for using both the AST as a screening test, and low-dose aspirin for the prevention of preeclampsia are presented and discussed.

The Angiotensin II Infusion

In order to permit comparison with other studies, the blood pressure response to intravenously infused angiotensin II (angiotensin-amide Hypertensin: Ciba-Geigy, Horsham Sussex, UK) was assessed at 28 (range 27.1 - 29.9) weeks gestation using a protocol similar to that described by others (Gant et al, 1973; Wallenburg et al, 1986).

Hypertensin was supplied as a sterile freeze-dried preparation; 2.5 mg per ampoule. A new ampoule was opened and reconstituted with 0.9% saline immediately prior to each screening session. One ampoule was used for a maximum of two subjects screened consecutively; the chemical is stable in solution for up to six hours and the screening time per subject was 60-90 minutes. During the trial, the Hypertensin ampoules came from two batches (038400, 038500) that remained within expiry dates throughout the testing period.

Preparation and Administration of the Angiotensin II Infusion

Two mls 0.9% saline were withdrawn from a 500 ml bag and added to the ampoule containing the freeze-dried angiotensin II. Following thorough mixing to dissolve the powder, the contents of the ampoule were then returned to the bag of 0.9% saline, providing an angiotensin II concentration of 5 μg/ml. The solution was then diluted to 1 μg/ml in a 50 ml syringe (10 mls AII solution : 40 mls saline), which then enabled administration through a continuous flow pump (Secura perfusor syringe pump; B. Braun, Melsungen, Germany). The rate of infusion (ng/kg/min) was set as an infusion rate (mls/min) once the woman's weight was measured. Tables were formed for this

conversion to cover the expected range of weights, to allow easy execution of the screening procedure.

All the infusions were performed during the morning or early afternoon. The women were asked to refrain from smoking or caffeine ingestion for at least 8 hours prior to the infusion.

On arrival, the woman was weighed and a midstream sample of urine was provided to exclude proteinuria and for subsequent kallikrein analysis (see Chapter VII p125). A data sheet was completed from information obtained from the woman verbally on her relevant medical, obstetric and social history in addition to review of the current hospital notes and antenatal card. Subsequently, a full explanation of the CLASP study, the AST test and the purpose of screening for preeclampsia were provided for the subject by the investigator (PMK), and then oral or written consent (as per the relevant hospital research ethic's committee protocol) was obtained. The infusion was performed at ambient temperature in a quiet room with soft background music.

An intravenous (IV) catheter was inserted into a right antecubital vein, and blood was drawn for Full Blood Count (FBC) (see Chapter IV), serum creatinine, urate, serum store, and in some subjects, platelet function tests (see Chapter VI). The intravenous catheter was then attached to a 0.9% saline IV line to keep the vein open. Blood pressure was measured every 5 minutes in the left arm by a standard mercury sphygmomanometer*, with the woman lying in the semirecumbent position on her left side. Muffling of the Korotkoff sounds (phase IV) was recorded as the arterial diastolic pressure. The heart rate was measured manually from the radial pulse taken over 60 seconds after each blood pressure measurement. The woman rested until a stable blood pressure (diastolic pressure within 5 mmHg) had been recorded for at least 15 minutes and the heart rate was averaged over this period. These values were taken as the 28 week gestation measurements. The test was stopped at this point if the systolic or diastolic pressures were greater than 140 or 85 mmHg respectively. The line from the perfusor pump was then connected and an infusion of angiotensin II was commenced at 4 ng/kg/min. The blood pressure was then measured at 5 minute intervals, and the infusion

was increased stepwise by 2 ng/kg/min if the diastolic pressure had not increased by 20 mmHg. This procedure was followed up to a maximum of 12-14 ng/kg/min (see results section p46) or until diastolic pressure increased by 20 mmHg. If the latter occurred, the infusion was stopped and the blood pressure was repeated at 5 minute intervals until the pressure returned to the previous baseline value. At that point, the infusion was recommenced at 2 ng/kg/min and repeated using the same criteria. Only when the Effective Pressor Dose was repeated in duplicate was the AST taken to be positive. After stopping the infusion(s), the blood pressure was taken 15 minutes later to confirm a return to baseline level. Transcranial Doppler ultrasound of the maternal middle cerebral artery was performed in a subset of the women before, during and after the infusion (see Chapter VIII), and an ultrasound scan of the fetus to estimate fetal size was performed immediately preceding or following the infusion (see Chapter IV). Both these investigations were performed by PMK.

*__Blood Pressure Measurement__ The blood pressure measurements were taken by one midwife at each centre (DB and JK). Both of these midwives had been trained in the measurement of blood pressure, with previous involvement in studies on pregnancy hypertension (observational and validation), in addition to blood pressure taking in hypertension clinics. The investigator (PMK) was present at all infusions and was available to check any uncertain measurements.

Definition for AST Result

The Effective Pressor Dose (EPD) is the dose of angiotensin II required to induce a 20 mmHg increment in diastolic pressure. The thresholds described in previous European studies of the AST as a screening test (Oney and Kaulhausen, 1982; Wallenburg et al, 1986) were used in this study. A POSITIVE test was defined as an EPD $\leq$ 10 ng/kg/min, and a NEGATIVE test as an EPD $>$ 10 ng/kg/min. As will be described later, the subset who showed a BORDERLINE response of 12 and 14 ng/kg/min was also assessed, but this group was not used in the main analysis for the CLASP trial.

CLASP Trial

For the purposes of the CLASP trial, only those subjects with a POSITIVE AST were asked to participate further. It was explained to the woman that she was considered to be at increased risk for developing preeclampsia, and therefore was eligible for entry into

the CLASP trial; to assess the value of low-dose aspirin to prevent preeclampsia. The double-blind nature of the trial was emphasised. If accepted, the woman gave consent to enrol into the trial. Randomisation to either 60 mg aspirin or identical placebo tablet was performed by a telephone call to the CLASP randomisation centre (Clinical Trials Unit, Oxford, U.K.). From there, the woman was allocated a coded calendar pack of either aspirin or placebo tablets held in the respective hospital pharmacy (JRH or QCH). The woman was advised to take one tablet daily until 38 weeks gestation, and to discard the remaining tablets. In addition, she was asked to avoid aspirin-containing compounds for the rest of the pregnancy. A letter was forwarded to her family doctor explaining the trial, which included a name of a reference person to contact if problems arose. Hospital antenatal notes were labelled to document entry into the CLASP trial.

Following distribution of the tablets, no formal follow-up of the woman or assessment of pill compliance was made. Antenatal care was left up to the individual obstetrician, with care shared between the hospital and general practitioner as appropriate. At the time of delivery, the date when the woman stopped was recorded in the notes. Follow-up information was obtained from the notes after delivery as described in Chapter II p33.

Statistical Tests

Intergroup differences between measured variables were assessed by the 2-sample Student's t-test, one-way analysis of variance (ANOVA) and categorical data by the Chi-squared test. Statistical significance was taken at $p < 0.05$ (two-tail).

RESULTS

Angiotensin Sensitivity Test (AST)

495 women underwent the AST at 28 weeks gestation without complication. 99 infusions demonstrated an EPD $\leq$ 10 ng/kg/min but only 80 of these were confirmed in duplicate. Therefore 80 (16%) women were taken to have a positive AST. All these women were randomised into the CLASP trial (36 placebo and 44 aspirin). [The unequal numbers were secondary to the CLASP trial's randomisation system].

The clinical features of those women demonstrating a positive and negative response to angiotensin II are listed in Table 3.1. Women who demonstrated a positive response were heavier and slightly younger than those who did not, both at booking and immediately before the test at 28 weeks gestation ($p < 0.005$). No other differences were observed between the groups. The clinical features of those with a positive AST randomised to aspirin or placebo were similar (Table 3.2).

In the early stages of the study, it appeared that the incidence of positive tests was extremely low in comparison to previous studies (Table 3.3). Three potential causes were considered.

1. Incorrect method of administration of the drug and application of the test.
2. Decreased potency of the chemical compound Hypertensin (Angiotensin-amide) produced by Ciba-Geigy.
3. Decreased sensitivity to angiotensin II in the current study population.

Each of these potential problems was investigated.

Incorrect application of the test. The details of the AST procedure were reviewed in detail, and compared to the methodology described previously. No differences were found that could explain the observed decreased sensitivity to angiotensin II.

Decreased potency of the drug. Hypertensin was supplied initially from batch "038400" with expiry date June 1993. Following discovery of the results discrepant to previous studies, Ciba-Geigy was contacted and the samples were recalled immediately. Analyses, using both chromatographic and radioimmunoassay methods, were performed in several laboratories to compare the samples to the company's standard control (Table 3.4). In addition, samples were sent to the University of Nottingham (Professor F. Broughton-Pipkin), to assess the potency of the samples (radioimmunoassay method using antibodies raised in-house in New Zealand rabbits). All the results showed the samples to be within the company's accepted potency range, and therefore our doubts were not confirmed. The remainder of the samples were returned to us.

Before the results of the above analysis were available, it was considered that if the drug was less potent, women with a borderline negative result may exhibit an increased

	AST NEGATIVE (N=415)		AST POSITIVE (N=80)	
Booking 12-20 wks				
Age (years)	28.7	(4.8)	27.3	(4.5)*
Height (cm)	164.7	(6.9)	165.0	(6.5)
Weight (kg)	65.5	(10.1)	70.3	(13.6)t**
Body Mass Index (kg/m^2)	24.1	(3.6)	25.8	(4.9)t**
Systolic BP (mmHg)	115.0	(11.8)	116.8	(12.4)
Diastolic BP (mmHg)	68.2	(7.8)	68.0	(7.9)
Mean BP [MAP] (mmHg)	82.9	(8.1)	83.4	(8.1)
No. twin gestations	6.0	(1.5%)	2.0	(2.5%)
28 weeks gestation				
Weight (kg)	71.8	(10.5)	76.6	(13.8)t**
Weight Gain (kg)	6.3	(2.9)	6.2	(3.1)t
Systolic BP (mmHg)	108.2	(8.0)	110.1	(8.5)
Diastolic BP (mmHg)	67.7	(6.6)	66.7	(5.9)
Mean BP (MAP) (mmHg)	80.3	(6.2)	80.3	(5.6)
Heart Rate (bpm)	80.6	(9.2)	79.7	(8.2)

* $p=0.015$, ** $p<0.005$ (2-sample t-test)
t Includes twin gestations as results not affected by their inclusion.

Table 3.1
Comparison of the clinical characteristics of those women with a negative and positive response to the Angiotensin Sensitivity Test (AST). Statistical comparisons performed using the 2-sample t-test. Figures shown are the mean (SD) or number of cases (%) as appropriate.

	PLACEBO (N=36)		ASPIRIN (N=44)	
Booking 12-20 wks				
Age (years)	26.8	(4.0)	27.8	(4.8)
Height (cm)	166.8	(6.3)	163.5	(6.4)
Weight (kg)	72.4	(13.7)	68.7	(13.2) t
Body Mass Index (kg/m^2)	26.0	(4.8)	25.7	(5.0) t
Systolic BP (mmHg)	116.0	(11.6)	117.4	(13.0)
Diastolic BP (mmHg)	68.8	(6.6)	67.4	(8.9)
Mean BP [MAP] (mmHg)	83.7	(7.1)	83.2	(9.0)
No. twin gestations	0.0	(0.0%)	2.0	(4.5%)
28 weeks gestation				
Weight (kg)	78.8	(12.4)	74.8	(13.6) t
Systolic BP (mmHg)	109.9	(7.8)	110.2	(9.0)
Diastolic BP (mmHg)	67.1	(5.5)	66.3	(6.3)
Mean BP [MAP] (mmHg)	80.6	(4.8)	80.1	(6.2)
Heart Rate (bpm)	79.6	(7.9)	79.7	(8.6)

t Includes twin gestations as the results were not affected by their inclusion.

Table 3.2
Comparison of the clinical features of those AST Positive women entered into the placebo or aspirin arms of the CLASP trial. Figures are represented as mean (SD) or number of cases (%) as appropriate. No statistical difference in these features were found between the two groups (2-sample t-test).

AST STUDY Number subjects	No. Positive AST	(%)
The present study (Kyle et al, 1993)		
30	2	(7%)
210	23	(11%)
495	80	(16%)
Oney and Kaulhausen 1982		
231	58	(25%)
Wallenburg et al, 1986		
207	46	(22%)
Dekker et al, 1990		
90	22	(24%)

Table 3.3
The incidence of Positive Angiotensin Sensitivity Test (AST) results found during the progress of the present study and compared with other reported European studies.

Laboratory/ Method	Standard	Sample	HYPERTENSIN declared (mg/ampoule)	HYPERTENSIN AS FOUND in % of the declaration	in mg per ampoule
University Lausanne	Production	Batch 038100	2.5	103.7	2.59
RIA	Standard 82	Batch 038400	2.5	103.7	2.59
Basle (Ciba-Geigy)	Batch 038100	Batch 038400	2.5	1. 102.6	2.57
RIA				2. 96.6	2.41
pH = 4.631				mean = 99.6	mean = 2.49
Basle (Ciba-Geigy)	Production	Batch 038400	2.5	102.2	2.55
HPLC	Standard 82	Batch 038100	2.5	103.9	2.60
pH = 4.625					
University of Nottingham				Assay Conditions set to provide EC_{50} between 120-140 pg/ml	
RIA	Nottingham	Prod Standard 82	2.5	133	
anti-serum raised	Standard	Batch 038100	2.5	116	
in NZ rabbits		Batch 038400	2.5	128	

Table 3.4
The results of the analyses to assess potency of Hypertensin (angiotensin-amide, Ciba-Geigy) from three different laboratories. Both radioimmunoassay (RIA) and high-performance liquid-chromatography (HPLC) methods were used. The batch in question (038400) was tested against the production standard (82), another batch (038100) and the Nottingham standard. All results were within the company's (Ciba-Geigy) specification for adequate potency.

tendency to develop preeclampsia (i.e. they would have shown a positive result if the drug had been exhibiting full potency). Therefore, for the remainder of the trial, the angiotensin infusion was run to 14 ng/kg/min before discontinuing the infusion. The women demonstrating a rise in the diastolic pressure at either 12 or 14 ng/kg/min were marked as having a borderline test and were analysed separately when determining the incidence of preeclampsia.

Outcome

One subject who had a negative AST was lost to follow-up (she moved to another country) which left 494 subjects for the overall analysis. Follow-up of the 80 subjects randomised to CLASP was complete. The outcome assessment is described for AST NEGATIVE, AST POSITIVE (combined) [Table 3.5], AST POSITIVE (aspirin) and AST POSITIVE (placebo) [Table 3.6]. 53 (12.8%) of the AST negative women developed preeclampsia (15 proteinuric, 38 nonproteinuric) whereas 15 (18.8%) women in the AST positive (combined) group developed preeclampsia (5 proteinuric, 10 nonproteinuric) [$p > 0.05$ X^2 test]. Of the AST positive (aspirin) group, more women developed preeclampsia 11 (25%) (5 proteinuric, 6 nonproteinuric), compared to 4 (11%) (0 proteinuric, 4 nonproteinuric) in the AST positive (placebo) group although these differences were not statistically significant ($p > 0.05$ X^2 test). The mode of delivery, birth weight and gestation at delivery were similar in all groups; in particular the outcome was no worse in the AST positive (placebo) group.

The booking and 28 week clinical features of those women who later developed preeclampsia or not are listed in Table 3.7. A significant increase in blood pressure (systolic, diastolic and mean) was observed in those women who developed preeclampsia (nonproteinuric and proteinuric) [$p < 0.001$]. Those women who developed proteinuria were shorter than the others although this did not reach statistical significance. As expected in the analysis of pregnancy outcome (Table 3.8), the blood pressure level and incidence of proteinuria were higher in the preeclampsia group ($p < 0.0001$).

	AST NEGATIVE (N=414)		AST POSITIVE (N=80)	
No. Preeclampsia	53	(12.8%)	15	(18.8%)
Proteinuric	15	(3.6%)	5	(6.3%)
Nonproteinuric	38	(9.2%)	10	(12.5%)
Birthweight (grams)	3374	(485)	3289	(461)
Birthweight (excluding twins)	3385	(478)	3311	(446)
Centile < 10	23	(5.6%)	4	(5.0%)
Centile < 3	6	(1.5%)	2	(2.5%)
Delivery Gestation (days)	279	(10.8)	278	(13.1)
Fetal/neonatal death	1		1	
Mode Delivery				
Vaginal	349	(84.0%)	67	(84.0%)
LSCS	65	(16.0%)	13	(16.0%)
Blood Loss (mls)	331	(205.0)	339	(221.0)

Table 3.5
Comparison of the outcome of those with a negative and positive Angiotensin Sensitivity Test (AST). No significant differences were found in any of the outcome variables between the two groups (Chi-squared test and 2-sample t-test, $p > 0.05$). The figures shown are mean (SD) for continuous variables or number of cases (%) for frequency values.

	PLACEBO (N=36)		ASPIRIN (N=44)	
No. Preeclampsia	4	(11.1%)	11	(25.0%)
Proteinuric	0		5	
Nonproteinuric	4		6	
Birthweight (grams)	3335	(449)	3252	(472)
Centile < 10	1		3	
Centile < 3	1		1	
Delivery Gestation (days)	278.4	(13.4)	278.3	(12.9)
Fetal/Neonatal Death	0		1	
Mode Delivery				
Vaginal	32		35	
LSCS	4		9	
Blood Loss (mls)	322	(201)	352	(238)

Table 3.6
Comparison of the outcome of those women randomised to aspirin or placebo. No significant differences were found in any of the outcome variables between the two groups (Chi-squared test and 2-sample t-test as appropriate, $p > 0.05$). Figures are shown as mean (SD) for continuous variables or number of cases (%) for frequency values.

	A NORMAL N=426	B PREECLAMPSIA (proteinuric) N=20	C PREECLAMPSIA (nonproteinuric) N=48	D PREECLAMPSIA (combined) N=68
Booking 12-20 wks				
Age (years)	28.4 (4.7)	30.5 (4.8)	26.4 (4.5)	29.0 (4.7)
Height (cm)	164.8 (6.9)	161.7 (7.8)	165.4 (5.7)	164.3 (6.6)
Weight (kg)	65.9 (10.6)	64.4 (13.8)	68.7 (10.1)	67.4 (11.5)t
Body Mass Index (kg/m^2)	24.3 (3.7)	24.7 (5.3)	25.3 (4.1)	25.1 (4.5)t
Systolic BP (mmHg)	114.9 (11.8)	114.5 (9.9)	119.1 (12.5)	117.7 (11.9)
Diastolic BP (mmHg)	68.0 (7.8)	68.2 (8.2)	69.0 (8.4)	68.8 (8.3)
Mean BP [MAP] (mmHg)	82.2 (8.0)	82.7 (7.9)	84.9 (8.5)	84.2 (8.3)
No. twin gestations	3.0 (0.7%)	0.0 (0.0%)	5.0 (14.6%)	5.0 (7.4%)
28 weeks gestation				
Weight (kg)	72.2 (10.9)	70.2 (13.0)	75.8 (10.6)	74.0 (11.6)t
Systolic BP (mmHg)	108.0 (7.9)	112.1 (8.8)	111.7 (8.4)	111.8 (8.4)**
Diastolic BP (mmHg)	66.9 (6.2)	69.7 (6.9)	72.0 (6.7)	71.3 (6.8)***
Mean BP [MAP] (mmHg)	79.8 (5.8)	82.9 (6.7)	84.4 (6.6)	83.9 (6.6)***
Heart Rate (bpm)	80.0 (8.8)	84.4 (8.3)	83.3 (10.8)	83.6 (10.1)*

* $p < 0.05$ ** $p < 0.001$ *** $p < 0.0001$

t Includes twin gestations as results not affected by their inclusion.

Table 3.7
Clinical characteristics of those women who had a normal outcome or developed preeclampsia (listed as proteinuric, nonproteinuric and combined). Results are represented as mean (SD) or number of cases (%). * Statistical comparison between Groups A and D was performed by Student's 2-sample t-test.

	A NORMAL N=426		B PREECLAMPSIA (proteinuric) N=20		C PREECLAMPSIA (nonproteinuric) N=48		D PREECLAMPSIA (combined) N=68	
Systolic BP (MAX)	125.6	(10.9)	155	(14.2)	145.7	(12.2)	148.4	(13.4)****
Diastolic BP (MAX)	77.7	(7.6)	103.8	(9.4)	100.4	(7.1)	101.4	(7.9)****
Birthweight (grams)	3381	(472)	3137	(534)	3275	(523)	3235	(526)*
Birthweight (excluding twins)	3388	(465)	3137	(534)	3334	(506)	3271	(519)
Centile < 10	21	(5.0%)	2	(10.0%)	4	(8.3%)	6	(8.8%)
Centile < 3	6	(1.4%)	0	(0.0%)	2	(4.2%)	2	(2.9%)
Delivery Gestation (days)	280.0	(11.1)	274.0	(11.2)	276.4	(10.8)	275.6	(10.9)**
Fetal/Neonatal death	2	0	0	0				
Mode Delivery								
Vaginal	368	(86%)	14	(70%)	34	(71%)	48	(71%)***
LSCS	58	(14%)	6	(30%)	14	(29%)	20	(29%)
Blood Loss (mls)	320	(194)	483	(301)	374	(246)	406	(267)*

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$

Table 3.8
Comparison of the delivery outcomes between those women who remained normal (A) or developed preeclampsia. The latter are grouped as proteinuric (B), nonproteinuric (C) or combined (D) preeclampsia. Results are shown as mean (SD) or number of cases (%). * Significant results of the statistical comparison between Groups A and D are shown (Student's 2-sample t-test or Chi-squared test as appropriate).

Furthermore, the blood loss and incidence of caesarean section were higher in this group whereas the birthweight, and gestation at delivery were lower. The two perinatal deaths were in the normal outcome group. Those with proteinuric preeclampsia had a slightly poorer outcome compared to those in the nonproteinuric preeclampsia group.

Angiotensin Potency

Because of the initial doubts about the potency of Hypertensin (angiotensin-amide) used in the study, the details of a positive (EPD $\leq$ 10 ng/kg/min), borderline (EPD >10, $\leq$ 14 ng/kg/min) and nonresponsive (EPD $\geq$ 14 ng/kg/min) AST result are shown (Table 3.9). Overall, as the trial progressed, the proportion of AST positive results increased, so that the final figure of 16% was not significantly different from the previous European studies. The incidence (percentage) of subjects developing preeclampsia (proteinuric and nonproteinuric) were slightly greater in the AST borderline group compared to the AST positive group (21.3% vs 18.8%), and both these levels were higher than those in the AST nonresponsive group.

The AST as a Screening Test

To assess the value of the AST as a screening test for preeclampsia in this population, the sensitivity, specificity, positive and negative predictive values (PPV and NPV respectively) were calculated. To perform this, it was necessary to include those women randomised to aspirin or placebo. It was recognised that the natural history of the syndrome may have been modified with aspirin or possibly placebo intake, but because the incidence of preeclampsia was increased in the aspirin versus placebo group (although not statistically significant), this inclusion was considered justified.

In an expanded version of Table 1.2 (Table 3.10), the numbers from this study are compared to those of previous studies. The sensitivity and PPV were particularly low at 22.1% and 18.8% respectively, with a specificity of 84.7% and a NPV of 87.2%. By raising the Effective Pressor Dose (EPD) to 14 ng/kg/min these values were only marginally altered (see discussion).

	Number (%)	Incidence Preeclampsia
AST Result		
Effective Pressor Dose		
≤ 10 ng/kg/min	80 (16.2%)	15 (18.8%)
>10, ≤ 14 ng/kg/min	61 (12.3%)	13 (21.3%)
> 14 ng/kg/min	353 (71.3%)	40 (11.3%)
TOTAL	494	68 (13.8%)

Table 3.9
Division of the Angiotensin Sensitivity Test (AST) results into Positive (EPD ≤ 10 ng/kg/min), Borderline (EPD >10, ≤14 ng/kg/min) and No Response (EPD >14 ng/kg/min). The numbers and incidence of preeclampsia in each group are shown.

Authors	Patient No.	Prevalence Hypertension	EPD (ng/kg/min)	Sens (%)	Spec (%)	PPV (%)	NPV (%)
Gant et al (1973)	153	37.5	<8	90	87	78	95
Morris et al (1978)	26	12	<8	33	39	7	82
			<10	67	30	11	87
Orozco et al (1979)	33	27	<8	89	79	62	95
Oney et al (1982)	231	15	<10	76	83	45	95
Nakamura et al (1986)	48	21	<10	20	97	75	82
			<10	80	82	53	94
			<12	100	74	50	100
Dekker et al (1990)	90	13	<8	75	99	90	96
			<10	92	86	50	99
Baker et al (1992)	34	29.4δ	SLOD*	60	63	40	79
Kyle et al (1993)	494	13.8	≤10	22	85	19	87
Kyle et al (1993)	494	13.8	≤14	41	74	20	89

δ subjects selected at 28-32 weeks with diastolic pressure at 80-85 mmHg
* slope diastolic pressure response to the angiotensin II infusion

Table 3.10
Predictive value of the Angiotensin Sensitivity Test (AST) performed between 28-32 weeks in reported studies and in the present study. The Effective Diastolic Pressure (EPD) used for the analysis is listed.

DISCUSSION

Low-dose aspirin

In contrast to the results of a previous study (Wallenburg et al, 1986), the present study does not confirm that low-dose aspirin can prevent preeclampsia when commenced at 28 weeks gestation in high-risk nulliparous women. Moreover, the number of women who developed preeclampsia (proteinuric and nonproteinuric) was higher in the aspirin versus placebo group, although these results were not statistically significant.

There are several factors to be considered which may contribute to the divergent results between the two studies even though they were performed in a similar manner. First, the numbers randomised to aspirin or placebo in both studies were small, and so although the results Wallenburg et al (1986) obtained were statistically significant at the $p = 0.01$ level, the different results could solely be explained by a Type I statistical error. Both studies recruited the required numbers derived from power calculations assuming that the AST was an efficient screening test for preeclampsia. However, as the positive predictive value of the AST was very low in the present study, and was only inferred rather than calculated in the Wallenburg study (no comment was made on the outcome of the AST negative subjects), the straightforward structure of the trial enabling such small numbers to be studied to evaluate low-dose aspirin may have been incorrect.

In the present trial, the participants were asked to stop the tablets (aspirin or placebo) at 38 weeks gestation, whereas in previous studies they were taken until delivery. The procedure was consistent with the CLASP coordinating committee's policy which was introduced to resolve the unanswered question of whether low-dose aspirin administration can increase the risk of epidural haematoma associated with catheter insertion. Although this risk is likely to be negligible, it is unknown, and hence many anaesthetists are unwilling to offer epidural/spinal analgesia or anaesthesia to those who may have been exposed to aspirin in the preceding few days. The cutoff gestation of 38 weeks was an arbitrary time to maximise the potential beneficial effects of low-dose aspirin while not

jeopardising the chances for a woman to have epidural analgesia. It was considered that discontinuing the tablets at 38 weeks gestation would not have an effect on the incidence of severe early-onset preeclampsia (the most important) but, it could conceivably have an effect on the number of women developing preeclampsia (possibly mild, as was found in this study). This potential effect will have to be considered in the analysis of the CLASP trial.

The second major difference between the two trials is that no compliance assessment was performed in the present trial. Wallenburg et al, 1986, measured serum malondialdehyde levels at 36 weeks gestation, a stable metabolite of platelet thromboxane production which should exhibit 90% inhibition following daily low-dose aspirin ingestion. In their trial, two subjects were considered to be noncompliant - one from her volunteered history and the other from an uninhibited malondialdehyde level. Furthermore, both women (assigned to aspirin treatment) were excluded from further analysis. In the present study, all analyses were performed on an "intention to treat basis".

It could be criticised that no attempt to assess compliance formally was made (when seen at later antenatal clinic visits, patients were asked whether any problems were incurred while taking the trial tablets). However, this was not a component of the trial protocol because it was considered that, first, the women would be highly motivated to take the trial tablets after they were found to have an increased risk for developing preeclampsia, and, second, it was not part of the main CLASP trial protocol since its purpose was to assess the efficacy of low-dose aspirin in routine clinical practice where such checks are not made consistently. It was also felt to be necessary not to interfere with the third trimester obstetric care, which was predominantly under general practitioner and community midwife care.

Although three studies have shown that low-dose aspirin prevents preeclampsia and intrauterine growth retardation (IUGR) when started in the third trimester of pregnancy (Wallenburg et al, 1986; Schiff et al, 1989; Trudinger et al, 1988), another has shown no

effect on the course of mild preeclampsia (Schiff et al, 1990). If it is considered that preeclampsia is a placental disease derived from inadequate trophoblast invasion of the intramyometrial portions of the uterine arteries in the early second trimester, the preventive effect of low-dose aspirin is more likely to occur when commenced early in pregnancy.

In this study the findings of Wallenburg et al, 1986, were not confirmed, but because of the small sample size, the question has not been answered with confidence. It will be addressed further in the analysis of the CLASP trial, specifically in the group entered with early onset preeclampsia (< 32 weeks gestation), the therapeutic arm of the CLASP trial.

The Angiotensin Sensitivity Test

The present study is the largest ever reported using the AST as a screening test for preeclampsia. The results, a sensitivity and PPV of 22% and 19% respectively, indicate that the AST is an ineffective screening procedure at least in this population. From the outset, it was recognised that an assessment of the screening test would be limited because an intervention was being performed in one of the AST outcome groups; either low-dose aspirin or placebo was prescribed to the AST positive women which could affect the natural history of their pregnancy. However, it was considered important to follow the outcome of all patients entered into the study (AST positive and negative) to, first, make some assessment of the efficacy of the test, and second, to enable comparison of new potential screening tests to this reference test (Chapters IV, V, VI, VII). Once the randomisation groups had been uncoded, it was observed that both the AST positive (aspirin) and AST positive (placebo) groups had enough cases of preeclampsia to make us question whether the natural histories of these subjects had been altered. Although this cannot be confirmed, it was considered worthwhile to assess the value of the AST as a screening test in the usual way.

In assessing the outcome of the AST negative women, it was a surprise to discover

that 11% of these women developed preeclampsia (3% proteinuric) and, furthermore, that some of these women developed the most severe disease of the whole study population. This finding contrasts to those of previous studies (Gant et al, 1973; Oney and Kaulhausen, 1982), where not only the number of false negative cases were small, but in addition these cases were more likely to develop mild disease only.

The reasons for these discrepancies are not clear, but certain points can be commented upon. First, in comparison to previous studies of the AST, different responses to the hormone were noted very early into the trial. The incidence of positive tests was much lower than those from previous European trials; a surprising finding as it was expected the study populations would be similar. Following the verification of drug potency (performed by Ciba-Geigy, Basle, Switzerland and the University of Nottingham, U.K. [Table 3.4]) the study was continued, ultimately resulting in an incidence of positive tests of 16%, which was lower than expected (previous studies 20-25%), but a difference which could have been accounted for by chance alone. An alternative explanation is that this study population was less sensitive to angiotensin II although the incidence of preeclampsia was similar (13-15%) to that of the other studies. By identifying those women who showed a "borderline" response to the AST it was found that the incidence of preeclampsia in this group was similar to the AST positive group, but higher than the AST "nonresponsive" group. One would expect a continuum in the response to the AST so that the threshold for a positive test is to some extent, arbitrary, chosen to detect the maximum number of cases of preeclampsia and exclude most that remain normal. However, the high incidence of preeclampsia in the AST negative group is surprising, and would suggest that either the present study population was atypically less sensitive to angiotensin II than others, or that the AST is not a good screening test despite the results of previous studies. If the threshold for a positive test is altered to $\leq$ 14 ng/kg/min, the efficacy of the AST as a screening test is only marginally improved over the established threshold of $\leq$ 10 ng/kg/min (Table 3.10).

The AST positive women were heavier than their negative counterparts. This

association has not been reported before. According to the present and previous protocols for performing the test, the amount of angiotensin II infused is based on the subject's weight (kg); therefore heavier women receive an increased absolute amount of the drug, but the effective concentration is equivalent. If this assumption concerning the pharmacokinetics of the drug is incorrect, it may have contributed to the poor efficacy of the test in the present study. However, such a problem ought to have been recognised before. A formal study of the pharmacokinetics of angiotensin II would be required to answer this question.

None of the subjects in this trial developed preeclampsia before 32 weeks gestation, and the majority developed mild disease in the last few weeks of pregnancy. A very large trial would be needed to include enough cases of proteinuric preeclampsia to assess whether the AST was effective in predicting these cases. However, this seems unlikely as the few severe cases of proteinuric preeclampsia were not predicted by the AST and therefore an expansion of the study seems unwarranted. The definition for preeclampsia used in this study is different from previous studies, with the criteria having a stronger ability to exclude those cases of chronic hypertension (Redman and Jefferies, 1988). However, such cases seemingly had little impact in this study of nulliparous women because the incidence of preeclampsia was similar to that of other studies (Oney and Kaulhausen, 1982; Dekker et al, 1990).

In conclusion, a review of the accuracy of the AST shows widely discrepant results ranging from the initial encouraging reports in black, predominantly teenage women (Gant et al, 1973) to more recent, but less consistent results in European women (Dekker et al, 1990; Baker et al, 1992). The results of the present study, which is the largest ever to have been reported, show that the efficacy of the test is questionable. Therefore, to employ this time-consuming and invasive test, even in a research setting to select women at increased risk for developing preeclampsia, does not seem to be advisable.

The poor results of the AST in the population has major implications for the remainder of the study in which the new screening tests were to be compared to the

outcome of the AST. Therefore, for the analysis of haematocrit, fetal abdominal circumference, 24 hour ambulatory blood pressure and platelet calcium response to vasopressin stimulation as screening tests, the emphasis will be placed on comparison to pregnancy outcome rather than to the AST response.

To summarise, in this study population the Angiotensin Sensitivity Test was not an effective screening test for preeclampsia, and, low-dose aspirin, when commenced late in pregnancy, did not prevent the clinical manifestations of the syndrome.

CHAPTER IV

Screening: Measurement of the Fetal Abdominal Circumference and Maternal Haematocrit

BACKGROUND

Two tests which are used almost routinely during pregnancy may have potential to predict the later onset of preeclampsia. These are second trimester maternal haematocrit and ultrasound measurement of the fetal abdominal circumference (AC). If either test was shown to be effective when performed and interpreted at a certain stage of gestation, it would provide a simple and inexpensive screening test for preelampsia.

Fetal Abdominal Circumference

Preeclampsia is a common cause of fetal growth retardation (Gruenwald 1966). The association is stronger for women with early onset disease (Baird et al, 1957; Long et al, 1980), significant proteinuria (Acien et al, 1990), and raised urate levels (Redman et al, 1976b); all these features indicating more severe disease. However, it is by no means uniform as women developing eclampsia near term may deliver a fetus of normal size. In the context of preeclampsia, fetal growth impairment is thought to reflect altered maternal-fetoplacental blood flow dynamics secondary to placental involvement of the disease. Because abnormal placental development is considered to be an integral component of preeclampsia (see Chapter I p4), signs of early growth impairment could be potentially apparent by the beginning of the third trimester.

Clinical assessment of fetal size is an inaccurate and subjective method of measurement. In contrast, ultrasound measurement of the fetal abdominal circumference shows a strong correlation with fetal weight (Campbell and Wilkin, 1975) although even then the measurement has a wide 95% confidence interval of up to $\pm$ 15%. However, the measured abdominal circumference, can be relied upon to detect significant deviations in fetal growth when plotted upon fetal centile charts for the abdominal circumference (Manning and Hohler, 1991).

Maternal Haematocrit

It has been reported that the plasma volume is reduced in women with established

preeclampsia (Freis and Kenny, 1948; Cope 1961) and that this reduction may antedate the clinical signs of the disease (Gallery et al, 1979; Hays et al, 1985). The reduced plasma volume is secondary to both vasoconstriction and reduced intravascular oncotic pressure (Redman 1984) and may be reflected in a rise in haemoglobin concentration or haematocrit (Sagen et al, 1982). One study has reported that haemoglobin levels of 13.2g/l or greater measured between 13-19 weeks gestation are significantly related to the subsequent development of preeclampsia (Murphy et al, 1986). It appears that confirmation of the predictive value of a early second trimester haemoglobin or haematocrit has not been performed prospectively.

Therefore, the present study's aim was to determine whether estimation of fetal size by ultrasound measurement of the fetal abdominal circumference at 28 weeks gestation and measurement of haematocrit levels in the early second trimester and 28 weeks gestation are useful predictive tests for preeclampsia. Both sets of measurements were compared to the Angiotensin Sensitivity Test (AST) and the pregnancy outcome. If those women who later develop preeclampsia demonstrate reduced fetal growth, and/or a raised haematocrit value by 28 weeks gestation, then it may be possible to calculate a threshold level to enable those women at increased risk to be identified.

METHODS

Fetal Abdominal Circumference

Either immediately prior to or following the AST (see Chapter III p37), an ultrasound scan of the fetus was performed by the investigator (Hitachi EUB340 [Diasonics Sonotron Ltd., Bedford, U.K.] or ATL Ultramark 4 [Advanced Technology Laboratory UK Ltd, Letchworth, Herts]). Abdominal circumference (AC) was measured in triplicate at the level of the stomach and intrahepatic portion of the umbilical vein using 2 orthogonal abdominal diameters measured on the same image, one anteroposterior and the other transverse (AD1 and AD2). Assuming the dimensions of a circle, the AC was calculated as follows: **AC = 3.142 X 0.5(AD1 + AD2)**. The average of three measurements was taken. The intraobserver coefficient of variation was 2.5% calculated from measurements on 6 women before the commencement of the study.

Adjustment to 28 weeks gestation. Because the AST test and therefore ultrasound scan was performed in the interval between 27.1 and 29.9 weeks gestation, it was decided to standardise the AC measurement to that of 28 weeks gestation to remove this known cause of variation from the results. Assuming linear growth of the AC during these weeks, it was calculated from the currently used fetal growth charts (Campbell and Wilkin, 1975) that the daily expected AC increment would be 0.157 cm. This value, multiplied by the difference in days between the test date and 28 weeks gestation, was either added (test performed before 28 weeks gestation) or subtracted (test performed after 28 weeks gestation) to the original AC measurement. This corrected AC measurement was then compared to the AST result and the pregnancy outcome. For this analysis, twin measurements were excluded because too many other factors may influence their growth pattern.

Maternal Haematocrit

Haematocrit levels were available on 344 women both at booking (12-20 weeks) and 28 weeks gestation. The numbers were less than the main study because not all "booking" values were taken between 12-20 weeks gestation and in addition some results were unobtainable. The booking values were obtained from the hospital and general practitioner records, whereas those at 28 weeks gestation were taken immediately before the AST at insertion of the intravenous catheter (see Chapter III p38). The venous blood was aspirated into EDTA anticoagulant, and Full Blood count (FBC) and haematocrit measurements were made (STKR T890, Coulter Electronics, Luton, Bedfordshire). The change in haematocrit was calculated by determining the difference between the two values.

Statistical Analysis

Comparisons were performed using the Student's 2-sample t-test. Statistical significance was taken at $p < 0.05$.

RESULTS

Abdominal Circumference

Comparison to the AST result

484 singleton fetal AC measurements were available for analysis. No significant differences were present between the corrected AC measurement of women with a negative compared to a positive test - mean (SD) [23.44 (1.40) vs 23.33 (1.40), $p > 0.05$].

Comparison to pregnancy outcome

The numbers in Table 4.1 show that no significant difference was observed in the corrected fetal AC measurement between those who later developed preeclampsia and those who did not. Furthermore, no significant difference was apparent when those women who later developed proteinuric preeclampsia were analysed separately despite there being a significant difference in birthweight. The analysis was performed twice to include and then exclude those randomised to aspirin. No difference was found between the results.

Maternal Haematocrit

Comparison to the AST result

No significant differences were observed in the "booking", 28 weeks gestation, or the change in the haematocrit between the women who demonstrated a positive or negative response to the AST (Table 4.2).

Comparison to pregnancy outcome

No significant differences were observed in the haematocrit values at both of these gestations between women who showed a normal pregnancy outcome and those who later developed preeclampsia (Table 4.3). The difference in the values between the two gestations was similar. A separate analysis of those who developed proteinuric preeclampsia compared to normal pregnancy outcome did not contribute further information. In addition, randomisation to aspirin had no effect on the analysis. Table 4.4,

		Abdominal Circumference (cm)	Birthweight (grams)
A: Aspirin Cases Included			
Normal	(N=420)	23.3 (1.4)	3390 (466)
Preeclampsia(TOTAL)	(N= 63)	23.4 (1.5)	3271 (519)
Preeclampsia(PROTEIN)	(N= 20)	23.1 (1.3)	3137 (534)*
B: Aspirin Cases Not Included			
Normal	(N=388)	23.3 (1.4)	3395 (464)
Preeclampsia(TOTAL)	(N= 53)	23.4 (1.4)	3294 (552)
Preeclampsia(PROTEIN)	(N= 15)	22.9 (1.1)	3166 (603)

* $p < 0.05$

Table 4.1
The corrected fetal abdominal circumference following standardisation to 28 weeks gestation (see text) and the birthweight for each of the outcome groups; normal, preeclampsia (TOTAL), preeclampsia (proteinuric) are listed. The values represented are the mean (SD). Part **A** includes those subjects randomised to aspirin treatment, whereas Part **B** does not. The birth weight of those who developed proteinuric preeclampsia was significantly lower than the normal outcome group ($p < 0.05$, 2-sample Student's t-test).

	AST NEGATIVE (N=300)	AST POSITIVE (N=44)
Booking Haematocrit	0.360 (0.027)	0.362 (0.023)
28 week Haematocrit	0.342 (0.028)	0.345 (0.024)
Change in Haematocrit	-0.018 (0.025)	0.017 (0.027)

Table 4.2
The haematocrit values are listed, mean (SD), for those women who showed a negative or positive response to the Angiotensin Sensitivity Test (AST). No significant differences were observed between the two groups (2-sample student's t-test).

	NORMAL	PREECLAMPSIA (total)	PREECLAMPSIA (proteinuric)
A: Aspirin cases included	N=297	N=47	N=14
Booking Haematocrit	0.360 (0.026)	0.361 (0.030)	0.354 (0.031)
28 week Haematocrit	0.342 (0.024)	0.344 (0.028)	0.336 (0.040)
Change in Haematocrit	-0.018 (0.025)	-0.017 (0.027)	-0.018 (0.022)
B: Aspirin cases not included	N=277	N=4	N=11
Booking Haematocrit	0.360 (0.026)	0.360 (0.030)	0.349 (0.026)
28 week Haematocrit	0.342 (0.024)	0.342 (0.028)	0.327 (0.038)
Change in Haematocrit	-0.018 (0.024)	-0.018 (0.027)	-0.021 (0.022)

Table 4.3
The haematocrit values, mean (SD), at booking (12-20 weeks gestation), 28 weeks gestation, and the difference between the two values are listed for those who remained normal or later developed preeclampsia (total, and proteinuric). No significant differences were observed (2-sample Student's t-test).

	NUMBER		
	Subjects	PREECLAMPSIA	(%)
Booking Haematocrit			
(≤ 0.30)	5	0	**0.0**
(0.31 - 0.35)	149	21	**14.1**
(0.36 - 0.40)	179	23	**12.8**
(> 0.40)	11	3	**27.2**
	297	**47**	
28 Week Haematocrit			
(≤ 0.30)	24	3	**12.5**
(0.31 - 0.35)	214	25	**11.7**
(> 0.36)	106	19	**17.9**
	297	**47**	

Table 4.4
Preeclampsia rates (%) by haematocrit value at booking (12-20 weeks) and 28 weeks gestation.

shows that a woman with a booking haematocrit greater than 0.40 has an increased chance for developing preeclampsia. Nevertheless, the numbers in this group were very small. By 28 weeks gestation, a haematocrit greater than 0.35 indicated a slightly increased risk for developing preeclampsia. However, the positive (PPV) and negative (NPV) predictive values for the test were low at 18% and 88% respectively.

DISCUSSION

From this study, measurement of fetal size and maternal haematocrit in the second trimester of pregnancy were not good predictors for the later development of preeclampsia. However, fetal growth impairment associated with preeclampsia is most commonly seen in cases of severe, early onset disease and yet the present sample contained few women with such presentation. Therefore, it is impossible to comment upon the early detection of growth retardation in early disease. Nevertheless, by performing one measurement at 28 weeks gestation, it was not possible to determine a size discrepancy between those women who later developed mild to moderate disease and those who remained normal. Therefore, although preeclampsia is considered a placental disease, disturbed fetal growth does not appear to be an early feature of mild to moderate disease.

It was not possible to confirm the findings of a previous study (Murphy et al, 1986), that second trimester haematocrit levels may predict the later onset of preeclampsia. There was a slight trend towards women with higher haematocrit levels exhibiting later preeclampsia, but a great degree of overlap was present between the measurements of the two outcome groups. In addition, the fall in the haematocrit from early second trimester to 28 weeks gestation was similar between the two groups. This may indicate that a contracted maternal plasma volume does not antedate clinical disease, or alternatively as reported previously, serum parameters do not reflect the plasma volume correctly (Goodlin et al, 1983). However, it has been shown that maternal plasma volume is normal when associated with a normally grown fetus and mild preeclampsia (Cope 1961;

Hays et al, 1985) which may explain the present findings.

Finally, no difference in either measurement (fetal abdominal circumference or maternal haematocrit) was observed between those women who demonstrated a positive or negative response to the AST. This may be inevitable, if in this population, none of the three tests were measuring an early sign of preeclampsia (see Chapter III, Discussion p57).

In conclusion, neither the measurement of the fetal abdominal circumference nor maternal haematocrit in the second trimester of pregnancy appear to have potential as a screening test for preeclampsia.

CHAPTER V

Screening: 24 Hour Ambulatory Blood Pressure

BACKGROUND

Single or duplicate measurements of blood pressure in the second trimester have been shown to distinguish between groups of women who later develop preeclampsia and those who do not (Fallis and Langford, 1973; Page and Christianson, 1976; Gallery et al, 1977; Oney and Kaulhausen, 1983; Moutquin et al, 1985). However these differences are difficult to detect in routine clinical practice (Gallery et al, 1977) and have poor individual predictive value for disease (Villar et al, 1989).

Errors associated with blood pressure measuring technique using the standard sphygmomanometer such as observer bias, digit preference and threshold avoidance may explain some of these findings. In addition, the alerting response (white coat hypertension) [Mancia et al, 1983] and the large fluctuations in an individual's blood pressure over a short period of time compound the problem.

Measurements of blood pressure by ambulatory blood pressure monitoring (ABP) avoid the difficulties associated with subjective measurement, and the repeated readings provide a more reliable estimate of the subject's averaged blood pressure. Theoretically these could have more clinical significance but as yet no long-term follow-up studies of nonpregnant or pregnant subjects are available. However, if it is assumed that if a weak but definite relationship is evident between second trimester blood pressure and the outcome of preeclampsia taken on single and not always standardised measurement, then a much stronger relationship may be found when the blood pressure is measured many times over a 24 hour period. Furthermore, 24-hour ABP will show the circadian pattern of blood pressure variation, a feature particularly relevant to preeclampsia, as alterations in the day/night cycle have been observed in women with established disease (Redman et al, 1976c; Seligman, 1971).

The aim of this study was to determine prospectively whether 24-hour ambulatory blood pressure recordings at 18 and 28 weeks in nulliparous pregnancy can be used to predict reliably and accurately which individual women will develop preeclampsia. In addition, comparison of the 24-hour ABP to the Angiotensin Sensitivity Test (AST) was

performed in a subgroup of these women. The latter was to investigate whether women who demonstrate an increased sensitivity to angiotensin II also exhibit a greater increase in their real blood pressure from 18 to 28 weeks gestation (measured as ABP). If so this may provide a much simpler alternative to the test.

Numbers

To assess 24-hour ABP alone, it was estimated that for a study with a power of 0.9, and 10 controls per case (assuming a preeclampsia rate of 10%), it would be necessary to study approximately 150 women (Kahn and Sempos, 1989). Within the time available, approximately 50 subjects was considered adequate to compare ABP to the AST.

MATERIALS AND METHODS

The ABP Monitor (Fig 5.1)

The TM2420 monitor and TM2020 decoder (A&D Co. Ltd., Tokyo, Japan) uses the Korotkoff method for blood pressure measurement. The instrument was validated for use in pregnancy (according to the recommendations of the Association for the Advancement of Medical Instrumentation for electronic and/or automated sphygmomanometers [AAMI, 1986]) in an earlier study (Clark et al, 1991). Korotkoff Phase 1 and Phase 5 were shown to represent systolic (SBP) and diastolic (DBP) pressures respectively.

The monitor (3 x 5 x 10 cm [500g]) contains internal mechanisms to both inflate the arm cuff and to record the pressure from a microphone sited over the brachial artery. It is connected to the cuff by a 1 cm wide tube containing the air inflation and microphone leads. The monitor is programmed using the decoder to determine the frequency of measurement, duration of monitoring, and display of the measurement or not. Furthermore, the decoder can serve as a printer, providing a simple numerical and graphical record of the measurements. Alternatively the information can be transferred directly to an IBM compatible computer. The reliability of the machine to maintain accurate baseline blood pressure measurement was tested by static accuracy immediately before and following the trial (see results section).

Figure 5.1
The TM2420 monitor and TM2020 decoder (A&D Co., Tokyo, Japan).

Subjects

162 healthy nulliparous women, mean age 28.3 (range 18 -38) were recruited for the 24-hour ABP study and 56 in addition consented to participate in the AST study. Twenty-four hour ABP was measured at 18 and 28 weeks gestation using the TM2420 monitor. The AST was performed as described in Chapter III p37, immediately following the second ABP monitoring.

24-Hour ABP Monitoring

The monitoring was performed in addition to the subject's routine antenatal care, and no person was hospitalised at either stage of gestation. Cuff size was determined by upper arm circumference at the time of each visit. A cuff bladder size of 12 x 22 cm, and 14 x 31 cm was used for arm circumferences of (22-32 cm) and (33-42 cm) respectively. With the patient seated and the cuff on the left arm, three automated readings were taken initially over ten minutes with simultaneous auscultation by the observer to confirm the reading. These readings provided "clinic" measurements and confirmed correct cuff placement; they are not equivalent to a routine clinical measurement because observer bias was eliminated. For the subsequent 24 hours, the subject was encouraged to continue normal activities except to avoid bathing and extreme physical exertion. She was requested to keep the arm still during readings and to switch the monitor off while driving (for insurance purposes, in case of distraction during cuff inflation). Finally, a simple diary was given to document the awake and sleep intervals. Readings were taken every 15 minutes during the awake period and every 30 minutes during sleep. Blood pressure was not displayed, but following the monitoring, the subject was given a printout from the TM2020 decoder and printer. The data were then transferred to an IBM compatible computer and then to an Apple MacIntosh for editing and analysis. Analysis was based on awake / sleep intervals determined by the subject's diary.*

* The editing of the data was performed by Dr S. Clark, Department of Cardiovascular Medicine, John Radcliffe Hospital, Oxford, United Kingdom and the investigator (PMK) performed the overall analysis.

A record was not accepted unless it contained at least 20 and 8 valid readings during the awake and sleep periods respectively. The criteria used to determine a valid reading included a pulse and diastolic pressure greater than 10 and 25 mmHg respectively, and a heart rate less than or equal to 150 beats per minute; these excluded nonphysiological readings only. Outcome was determined by review of the notes post delivery as described in Chapter II p33.

Statistics

Values were expressed as the mean and standard deviation (SD) for the awake and sleep period for each individual, before calculating the mean and variance for each outcome group. The Student's t-test was used to test for between group differences and intragroup serial comparisons.

RESULTS

Static Accuracy

Five machines were used in the study and each was first tested for accuracy against a standard mercury sphygmomanometer.

Method. A 50 ml syringe was attached to a 3-way tap. The second outlet was connected by tubing to a cleaned standard mercury sphygmomanometer and the other to the recording component of the automatic monitor, i.e. the arm cuff was bypassed. To enable manual control of the machine, a circuit card was inserted into the ABP monitor to disable the automatic deflation mechanism. With all taps open, air was injected to increase the mercury column (top of the meniscus) to 50 mmHg, and simultaneously, the pressure reading from the automatic monitor was taken. This procedure was then repeated successively at 100, 150, 200 and 250 mmHg. The monitor readings for each mmHg level are listed in Table 5.1. Static accuracy in the monitors was checked and confirmed before and after the study period.

		Standard Sphygmomanometer (mmHg)				
		50	100	150	200	250
TM2420 Monitor						
1	A	51	101	151	200	250
	B	51	101	152	200	250
2	A	52	102	152	201	251
	B	52	102	151	200	252
3	A	52	102	153	202	252
	B	52	102	152	203	252
4	A	52	102	152	202	252
	B	51	101	152	202	252
5	A	51	102	152	202	252
	B	51	102	152	202	252

Table 5.1
Static Accuracy Results. Simultaneous readings from the TM2420 monitor compared to the standard mercury sphygmomanometer. Testing results obtained before (A) and following (B) the trial.

24 Hour Ambulatory Blood Pressure

161 (99.4%) women provided adequate awake readings at 18 weeks gestation and 145 (89.5%) at both 18 and 28 weeks gestation. The second record was not obtained because the women moved away (2), went into preterm labour requiring medical treatment (3), or declined (11). The analyses of awake and "clinic" readings were based on the smaller group of 145 women. One hundred and twenty-seven (78.4%) women provided adequate data for both the awake and sleep periods at both gestations and these were used for the main analysis of the 24-hour cycle. Nineteen (11.8%) of the 161 women and 17 of the smaller groups of 145 and 127 (11.7% and 13.4% respectively) developed preeclampsia. The five women with proteinuria were included in all three groups. All were normotensive (diastolic < 90 mmHg) at the time of each measurement.

In Table 5.2, the clinical characteristics at the first clinic visit for booking of the two outcome groups are listed; none of these were significantly different.

Awake Readings (Table 5.3)

In those women with a normal outcome, there were significant rises in the systolic, diastolic and mean arterial pressures and heart rate between 18 and 28 weeks ($p < 0.00001$ paired t-test). At 18 weeks gestation, the mean differences (95% CI) between those who did and did not get preeclampsia were 4.7 mmHg (0.2, 9.4) $p < 0.05$, for systolic pressure, 1.1 mmHg (-1.6, 3.8) for the diastolic pressure (nonsignificant), and 2.3 mmHg (0.5, 4.1) $p < 0.02$, for the MAP. These differences were more apparent at 28 weeks [6.9 mmHg (2.3, 11.6), 4.4 mmHg (1.3, 7.5), and 5.2 mmHg (2.4, 8.0) respectively ($p < 0.01$ for all three differences)]. The increases in ABP from 18 to 28 weeks for diastolic and MAP were significantly greater in the women who later developed preeclampsia. The group with incipient preeclampsia had a significantly faster heart rate 6.6 bpm (2.6, 10.5),$p < 0.002$, at 18 weeks as well as at 28 weeks, 7.2 bpm (3.1, 11.4), $p < 0.002$. Because this finding was unexpected, an analysis of an

	NORMAL (N=128)		PREECLAMPSIA (N=17)	
Gestation (weeks)	16.2	(1.3)	16.5	(1.3)
Age (years)	28.3	(4.2)	28.0	(4.5)
Weight (kg)	66.8	(12.5)	66.5	(16.6)
Height (cm)	165.6	(6.2)	162.2	(7.1)
Body Mass Index (kg/m^2)	24.3	(4.2)	25.3	(6.2)
Systolic BP (mmHg)	116.4	(12.8)	116.8	(17.2)
Diastolic BP (mmHg)	69.5	(8.0)	68.2	(9.0)
Mean BP [MAP] (mmHg)	84.3	(8.5)	83.6	(10.1)
Smoking (no.)	6	(5.0%)	2	(12.0%)

Table 5.2
The clinical characteristics at booking represented as mean (SD) of both outcome groups. There were no significant differences between groups (2-sample Student's t-test)

	NORMAL (N=128)		PREECLAMPSIA (N=17)	
18 Weeks Gestation				
		"CLINIC"		
Systolic BP	106.4	(10.0)	111.8	(10.1)*
Diastolic BP	64.2	(6.6)	67.0	(9.2)
Mean BP (MAP)	77.5	(6.5)	81.1	(7.7)
Heart Rate	79.4	(10.8)	85.7	(9.4)**
		AMBULATORY		
Systolic BP	107.7	(8.1)	112.4	(8.6)*
Diastolic BP	66.3	(4.7)	67.4	(5.1)
Mean BP (MAP)	79.3	(4.6)	81.6	(3.2)
Heart Rate	82.4	(7.8)	89.0	(7.2)***
28 Weeks Gestation				
		"CLINIC"		
Systolic BP	109.2	(9.6)	117.4	(11.6)**
Diastolic BP	67.3	(8.2)	70.5	(7.9)
Mean BP (MAP)	80.5	(7.3)	85.3	(7.3)**
Heart Rate	85.9	(11.2)	96.3	(9.5)***
		AMBULATORY		
Systolic BP	110.3	(8.3)	117.2	(8.6)**
Diastolic BP	68.2	(5.4)	72.6	(5.8)**
Mean BP (MAP)	81.4	(5.0)	86.6	(5.3)***
Heart Rate	87.1	(8.3)	94.3	(7.7)***

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.002$

Table 5.3
Comparison of awake clinic and ambulatory systolic, diastolic and MAP (mmHg) and heart rate (bpm) measurements at 18 and 28 weeks for the 2 outcome groups. Values listed are the mean (SD) and p values represent significance from the 2-sample Student's t-test.

independent group of nullipara selected by the same criteria was sought. 437 nulliparous women who underwent the AST but not the ABP study (see Chapter III) had a resting manual pulse taken at 28 weeks gestation. The average heart rate of the 57 women who later developed preeclampsia (by the criteria of this study) was significantly faster, 3.15 bpm [(95% CI: 0.3, 5.98) $p = 0.015$ one-tail 2-sample Student's t-test], than that of the remainder who did not.

Sleep Readings

In the women with a normal outcome who showed a satisfactory sleep recording (N=109), the blood pressure and heart rate during sleep rose significantly between 18 and 28 weeks gestation. The mean changes (95% CI) were 3.4 mmHg (2.2, 4.6) for systolic pressure, 2.6 mmHg (1.8, 3.4) for the diastolic pressure, 2.8 mmHg (2.1, 3.6) for the MAP and 2.8 bpm (1.9, 3.7) for the heart rate ($p < 0.00001$ paired t-test for all four differences). At both stages of gestation the higher blood pressures were sustained during sleep in the incipient preeclamptic group which meant that the awake - sleep differences were similar in relation to each outcome (Fig 5.2). The increase in heart rate was also sustained during sleep.

"Clinic" Readings

The "clinic" blood pressures taken at the beginning of the monitoring as the first or the mean of three automated readings showed a similar increase in the pressure and heart rate to the ABP recordings (Table 5.3). However, the variability was greater, and hence the separation between the two outcome groups were less. Overall, the "clinic" pressures and heart rate were lower than the respective awake ambulatory recording.

A separate analysis of the awake and sleep readings was performed to assess whether the five women who later developed proteinuric preeclampsia showed any significant features in their ABP monitorings compared to those with a normal outcome, but none were found.

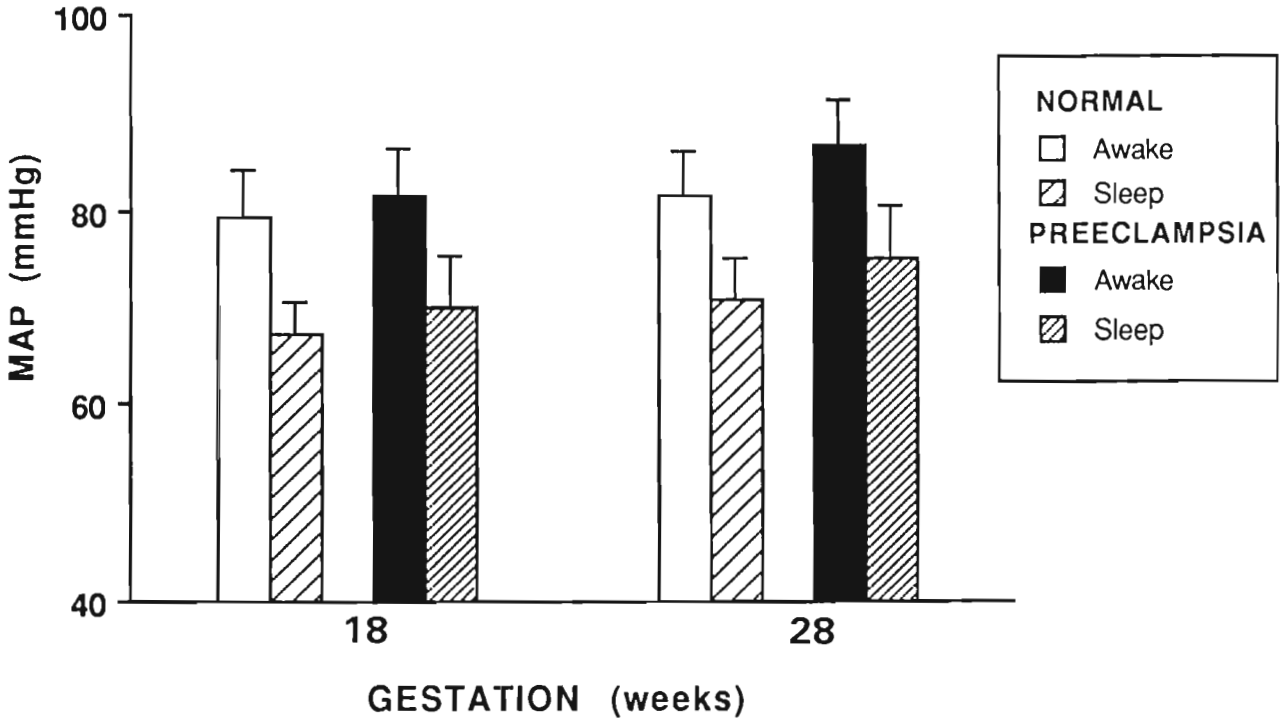

Figure 5.2
The ambulatory awake and sleep mean arterial pressure (MAP) measurements for the two outcome groups at 18 and 28 weeks gestation in 127 nulliparous women. There was no significant difference between the awake - sleep difference in the two outcome groups at either gestation (2-sample Student's t-test).

Value of ABP Recordings for the Prediction of Preeclampsia

The incidence of preeclampsia rose significantly with the rise in blood pressure and heart rate (Table 5.4). The sensitivity, specificity, positive and negative predictive values (PPV and NPV respectively) were calculated for the ABP diastolic, mean arterial pressure and heart rate, to assess the test's value as a screening procedure in this population. As outlined in Table 5.5, the sensitivity and PPV of ABP was greatest for the MAP at 28 weeks, but even these values were low at 65% and 31% respectively. It was a surprise to find that the heart rate showed a similar but independent trend to the MAP. The two variables were not significantly correlated (0.160 at 18 weeks and 0.276 at 28 weeks gestation). By then using the MAP and heart rate in combination as a predictive test, the PPV rose to 45% at 28 weeks gestation.

Comparison ABP to the AST

Of the 56 women who participated in both tests, 53 had suitable 24-hour recordings for awake and sleep analysis; the data from the latter gave the following results. Thirteen (25%) demonstrated a positive AST and the awake ABP was significantly higher (diastolic and mean pressures) at 18 and 28 weeks gestation in this group compared to those with a negative AST, $p < 0.05$ (Table 5.6). A difference between the two groups was also demonstrated in the "clinic" measurements but this was not statistically significant (data not shown). Otherwise, the change in the blood pressure and heart rate between 18 and 28 weeks, the sleep pressures and the difference between sleep and awake pressures at both gestations, were similar between the two AST groups.

DISCUSSION

These results are consistent with previous studies of standard measurments of second trimester blood pressures to predict preeclampsia in which the MAP (Fallis et al, 1973; Page and Christianson, 1976; Moutquin et al, 1985), or diastolic pressure (Gallery et al,

	NUMBER		
	Subjects	Preeclampsia	(%)
MAP (mmHg) 28 weeks			
QUARTILE			
LOWEST			
(69.1 - 78.3 mmHg)	37	2	**5.4**
SECOND			
(78.5 - 81.4 mmHg)	36	0	**0.0**
THIRD			
(81.5 - 84.8 mmHg)	36	4	**11.1**
HIGHEST			
(85.0 - 97.2 mmHg)	36	11	**30.6**
	145	**17**	
Diastolic BP (mmHg) 28 weeks			
LOWEST			
(53.1 - 65.4 mmHg)	37	3	**8.1**
SECOND			
(65.5 - 68.6 mmHg)	36	2	**5.6**
THIRD			
(68.6 - 72.0 mmHg)	36	3	**8.3**
HIGHEST			
(72.2 - 82.9 mmHg)	36	9	**25.0**
	145	**17**	
Heart Rate (bpm) 28 weeks			
LOWEST			
(69.3 - 81.7 bpm)	37	1	**2.7**
SECOND			
(81.8 - 87.8 bpm)	36	2	**5.6**
THIRD			
(87.9 - 93.4 bpm)	36	5	**13.9**
HIGHEST			
(93.8 - 110.5 bpm)	36	9	**25.0**
	145	**17**	

Table 5.4
Preeclampsia rates (%) by diastolic pressure, MAP and heart rate at 28 weeks gestation for each quartile of the study population.

(N = 145)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
18 weeks gestation				
MAP ≥ 80 mmHg	65	51	16	92
MAP ≥ 85 mmHg	24	90	24	90
Heart rate ≥ 85 bpm	59	63	17	92
Heart rate ≥ 90 bpm	47	83	27	92
MAP ≥ 80, HR ≥ 90	29	89	26	91
28 weeks gestation				
Diastolic BP ≥ 75 mmHg*	35	90	32	91
Diastolic BP ≥ 80 mmHg*	12	98	50	89
MAP ≥ 80 mmHg	88	42	17	96
MAP ≥ 85 mmHg	65	81	31	95
Heart rate ≥ 90 bpm	77	69	25	96
Heart rate ≥ 95 bpm	47	83	27	92
MAP ≥ 85, HR ≥ 90	53	94	45	94

Table 5.5
The predictive value of awake ambulatory blood pressure and heart rate in the 2nd trimester in 145 women. * Only the 28 week diastolic BP is included in the calculations because at 18 weeks the difference between the means for the 2 outcome groups was not significant. MAP = mean arterial pressure, PPV = positive predictive value, NPV = negative predictive value

	AST NEGATIVE (N=40)		AST POSITIVE (N=13)	
"Booking"				
Systolic BP	112.5	(12.4)	122.7	(11.1)*
Diastolic BP	69.0	(8.1)	69.6	(8.8)
Mean BP (MAP)8	2.7	(8.5)	86.4	(8.8)
	AMBULATORY BP			
18 weeks gestation	**Awake**			
Systolic BP	106.2	(7.4)	109.2	(8.1)
Diastolic BP	66.2	(4.4)	69.8	(5.4)*
Mean BP (MAP)	78.7	(4.4)	82.1	(5.4)
Heart Rate	83.0	(9.2)	82.9	(5.2)
	Sleep			
Systolic BP	91.0	(7.5)	93.5	(6.6)
Diastolic BP	58.0	(4.3)	60.4	(4.1)
Mean BP (MAP)	68.3	(4.7)	70.7	(4.4)
Heart Rate	72.2	(7.0)	73.1	(5.6)
28 weeks gestation	**Awake**			
Systolic BP	110.4	(8.4)	113.5	(7.9)
Diastolic BP	70.1	(5.3)	73.2	(4.3)*
Mean BP (MAP)	82.7	(5.6)	85.8	(4.8)
Heart Rate	88.9	(8.6)	92.0	(4.8)
	Sleep			
Systolic BP	93.9	(7.5)	94.2	(8.6)
Diastolic BP	60.8	(4.6)	62.0	(5.7)
Mean BP (MAP)	71.1	(5.0)	72.0	(6.2)
Heart Rate	75.9	(6.3)	77.5	(6.1)

$* p < 0.05$

Table 5.6
Comparison of awake and sleep ambulatory blood pressures and heart rate, mean (SD), for those women with a negative and positive Angiotensin Sensitivity Test (AST). The booking measurements are also included. Statistical significance is demonstrated by the 2-sample Student's t-test.

(1977) were significantly greater in those women who later developed preeclampsia than those who did not. In one of these studies, increases in intrauterine growth retardation and perinatal mortality were associated with a MAP $\geq$ 90 mmHg (Page and Christianson, 1976). However, the measurements had limited power to predict which individuals would develop disease, a conclusion confirmed in a recent prospective study of 700 nulliparous women (Villar et al, 1989).

The unreliability of standard indirect blood pressure measurement due to factors such as observer bias, digit preference, and threshold avoidance, heightened in pregnancy by the confusion pertaining to which Korotkoff sound (K4 or K5) best represents diastolic pressure (Perry et al 1990; Perry et al, 1991; de Swiet 1991), may partly explain this weak relationship. ABP corrects some of these factors by avoiding the bias associated with observer measurements, and the TM2420 monitor, has been shown to consistently measure K5 (Clark et al, 1991). In addition, the variability evident with single measurements is less when taking repeated measurements because sampling errors are reduced. If there were a relationship between the variable (blood pressure or heart rate) and the outcome (preeclampsia) it would be stronger using the average of multiple measurements because they are more likely to give an accurate estimate of the real values. Mean awake ABP measurements, particularly the MAP, were significantly higher in those who eventually developed preeclampsia than in those who remained normal. The differences were evident at 18 weeks, and more so at 28 weeks, and were easier to discern than with a carefully measured "clinic" reading.

However, it is clear that the test has limited usefulness for clinical screening because of the overlap between the readings from both groups. The most useful screening measurement was the MAP at 28 weeks and yet although the sensitivity was 65%, the specificity and PPV were low at 81% and 31% respectively.

A decreased fall or even rise in sleep compared to awake blood pressures was not observed, although this has been seen in women with established preeclampsia (Redman et al, 1976c; Seligman 1971). The present results suggest that the circadian rhythm is not

altered before the appearance of clinical disease so that 24-hour measurements cannot provide additional useful information. However, in this study no subject developed preeclampsia before 36 weeks so it is not possible to be precise about the time course of the changes in circadian patterns. Only a large study which included enough subjects who later developed severe preeclampsia over a wider range of gestational ages could answer this question.

The heart rate was increased during both the awake and sleep periods at 18 and 28 weeks in those women who later developed preeclampsia. This unexpected finding was confirmed in a second group of 437 nulliparous women who had a resting manual pulse rate measured at 28 weeks gestation, of whom 57 later developed preeclampsia. It therefore is considered a real finding which does not appear to have been clearly documented before. An equivalent difference has been reported in women with established preeclampsia (Oian et al, 1976; Wallenburg et al, 1988) which persisted for 12 weeks postpartum (Nisell et al, 1985). In contrast, a group of women considered to be at risk for developing preeclampsia by exhibiting increased sensitivity to infused angiotensin II showed a lower resting heart rate (Spitz et al, 1988). An increased heart rate has also been observed in nonpregnant individuals with essential hypertension (Franco-Morselli et al, 1977) and in pregnant women with mild (predominantly essential) hypertension (Lim et al, 1979).

It was considered whether the increased heart rate might arise because the definition for preeclampsia gave a biased selection including women with underlying essential hypertension rather than preeclampsia. However, a subanalysis demonstrated that those women who later developed proteinuria (N=5) had a raised heart rate at 28 weeks similar to those with nonproteinuric preeclampsia (N=12) [mean (SD): 92.6 (6.6) bpm; 95.1 (8.2) bpm respectively], when compared to those who remained normal [87.1 (8.3) bpm]. This was confirmed in the larger study of 437 women with the following results: proteinuric preeclampsia [(N=16) 83.7 (8.1) bpm]; nonproteinuric preeclampsia [(N=41) 83.0 (10.9) bpm] and normal outcome [(N=380) 80.0 (9.0) bpm]. Therefore, a

raised heart rate appears to be a true early feature of preeclampsia. It is not possible to speculate on the underlying cause with the data available.

The "clinic" blood pressure and heart rate readings were lower than the respective awake ambulatory readings. This finding may reflect that the women were resting at the time of the "clinic" measurement whereas ambulatory measurements include periods of activity. In addition, those women with a raised booking blood pressure were excluded from the study. It is most likely that the women who demonstrated an "alerting response" to clinic measurements would have been in this group.

The comparison of ABP to the AST showed that those women with a positive AST had slightly increased diastolic and mean pressures at 18 and 28 weeks gestation. These differences were not evident in either the baseline standard sphygmomanometer readings taken immediately prior to the AST in the main study (see Chapter III p41) or in the "clinic" readings taken immediately before the ABP recording. Therefore, this indicates that those women demonstrating a positive response to angiotensin II may have increased arterial resistance which is only detected by the more sensitive measurement of ABP. However, the differences between the two groups were small, so that even if the AST had been an effective screening test, it would be difficult to replace the test by ABP alone. The rise in blood pressure was similar between the two AST groups, and so a pattern of increasing arterial resistance in those with a positive AST was not confirmed.

In conclusion, an increase in blood pressure and heart rate using ABP has been demonstrated in the second trimester in women who later develop preeclampsia. However, the low predictive values for ABP measurements alone indicate that this is not a useful screening procedure for incipient preeclampsia. The efficiency of the test is increased marginally by combining the heart rate and MAP together at 28 weeks gestation.

CHAPTER VI

Screening: Platelet Intracellular Free Calcium Response to Arginine-Vasopressin Stimulation

BACKGROUND

Preeclampsia is a systemic disease characterised by vasoconstriction and coagulation derangement (Bonnar et al, 1971; Howie et al, 1971; Redman 1990). Platelet function is abnormal in well-established disease evident by decreased platelet number (Bonnar et al, 1971; Howie et al, 1971: Redman et al, 1978; Gibson et al, 1982), increased platelet volume (Giles et al, 1981) and increased levels of the platelet-specific protein ß-thromboglobulin [a product of the platelet release reaction] (Redman et al, 1977b). These alterations in platelet function may be an early feature of the disease (Redman et al, 1978). These changes may be important in the pathogenesis of the disease because antiplatelet therapy, that is the daily intake of small amounts of aspirin (low-dose aspirin, less than 150mg per day), appears to prevent the occurrence of proteinuric preeclampsia and some forms of intrauterine growth retardation [IUGR] (Imperiale and Petrulis, 1991; Collins 1992). This effect may well be mediated by low-dose aspirin's ability to selectively reduce the production from platelets of thromboxane A2 [TXA2] (Weksler et al, 1983), a prostaglandin with potent vasoconstrictor and platelet aggregatory properties, mainly derived from platelets, and whose production may be increased in preeclampsia (Fitzgerald et al, 1990).

Mechanism of Platelet Activation

Platelets are involved in normal haemostasis by adhesion and aggregation. Adhesion is mediated by platelet surface receptors which have high affinity for adhesive glycoproteins in the subendothelium of damaged blood vessels (George and Shattil, 1991). Aggregation is controlled exclusively by the platelet surface receptor GPIIb/IIIa (Coller 1990). The activated receptor can bind several glycoproteins such as von Willebrand factor and fibrinogen and due specifically to this latter compound's dimeric structure, two platelets can interact simultaneously (Gralnick et al, 1984).

Platelet activation is the process leading to the exposure and activation of the GPIIb/IIIa receptor. It involves reorganisation of the structural proteins to produce a

dramatic change in shape and subsequently to release agonists from the internal granules to stimulate aggregation. In vivo activation occurs through exposure to certain circulating agonists such as ADP, adrenalin, serotonin (5HT), thrombin, platelet activation factor (PAF) and vasopressin. All these agonists will release arachidonic acid from the platelet membrane. Arachidonic acid is then converted to the prostaglandin thromboxane A2 (TXA2) by the enzymes cyclo-oxygenase and thromboxane synthetase. TXA2 amplifies the aggregation process by first releasing other agonists from the platelet granules, and second, by diffusing out of the platelet to bind to specific membrane receptors to enhance aggregation. Activation of platelets may also occur by a noncyclo-oxygenase pathway, but this still involves the GIIb/IIIa receptor (Coller 1990).

Certain agonists such as thrombin and TXA2 can also activate phospholipase C (George and Shattil, 1991). This enzyme converts membrane phosphatidylinositol diphosphate into two intermediary messengers: diacylglycerol and inositol triphosphate. The former activates protein kinase C to initiate certain biological steps and the latter induces release of ionised calcium from intracellular stores. The increase in intracellular calcium activates various calcium and calmodulin dependent reactions which are fundamental to platelet activation.

Methods to Detect Platelet Activation

Measurement of platelet activation may be a method for detecting certain diseases early. Techniques for detection have included measurement of secretory substances or their metabolites in serum or urine [fibrinogen, ß-thromboglobulin, Platelet Factor 4 (PF4)]; platelet aggregometry to detect hyperaggregable platelets; flow cytometric measurement of surface antigens using monoclonal antibodies on resting and activated platelets; and resting and stimulated levels of platelet intracellular calcium.

The clinical application of some of these methods has some limitations. Secretory products can be difficult to measure and interpret accurately because they may be released during collection and processing of the platelets. A technique described to

improve the method is to use the "release reaction", quantified by the amount of radio-labelled compound released from the granules in platelets which have been preincubated with the label and then exposed to an aggregating agent (Louden et al, 1990).

Another problem general to all methods is that platelets which have been activated and have undergone granule release *in vivo* may then become hypo-aggregable when examined *in vitro*; these "exhausted" platelets are a sign of more established disease (Howie et al, 1971; Whigham et al, 1978; Ahmed et al, 1991). This should be recognised in any functional system of measurement which relies on in vitro stimulation to assess platelet activation, such as aggregometry and measurements of intracellular calcium in stimulated platelets.

Intracellular calcium and monoclonal antibody binding levels are new methods, still under investigation, which may provide an earlier and more sensitive method to detect changes in platelet physiology that underlie activation.

Intracellular Free Calcium

Intracellular free calcium plays a crucial role in a second messenger system that activates platelets and other cell types (Rasmussen et al, 1986). The system relies on a balance between free ionised calcium in the cell cytosol, bound calcium in the intracellular stores, and transport into the cell of extracellular calcium through membrane calcium channels. Intracellular free calcium concentration increases rapidly following agonist stimulation which leads to a cascade of enzyme activation. A rise in cytoplasmic free calcium is fundamental in the mechanisms of platelet shape change, the release reaction and aggregation (Rink et al, 1982). Therefore increased intracellular free calcium levels in resting platelets, or in those immediately following stimulation, measures early platelet activation.

Intracellular free calcium measurements are now possible using fluorescent dyes which can be loaded into intact cells as an ester derivative and retained therein because

the action of intracellular esterases renders them membrane impermeable. Either their fluorescence intensity or wavelength changes when they chelate cytoplasmic ionised calcium (Tsien, 1980; Tsien, 1981; Tsien et al, 1982). Hence the fluorescence signal can reflect the calcium concentration, measured either quantitatively or qualitatively. First, second and third generation dyes include quin-2 (Tsien et al, 1982); fura-2 and indo-1 (Grynkiewiez et al, 1985); and fluo-3 (Minta et al, 1989) respectively.

Intracellular free calcium can be measured by spectrofluorimetry in resting or activated platelets (Hallam and Rink, 1987) or by flow cytometry (Davies et al, 1988; Jennings et al, 1989). The advantages of the latter method are that individual platelets are assessed and a smaller number of cells is required. In addition, it is possible to identify different cell populations within a sample (Davies et al, 1988).

Fluo-3

Fluo-3 is a recently developed indicator which increases its fluorescence intensity on binding to calcium by 40-fold (Minta et al, 1989). It has been used for the measurement of intracellular calcium in human T lymphocytes (Vandenberghe and Ceuppens, 1990) and platelets (Merritt et al, 1990). The absorption and emission spectra is similar to fluoroscein; this makes it suitable for use with most commonly available flow cytometers which have a 488 nm argon laser excitation source. The earlier dyes are stimulated by light in the ultraviolet (UV) spectrum using spectrofluorimetry (quin-2, fura-2) or by flow cytometry with a UV argon or krypton laser excitation source (indo-1). Fluo-3 has a low affinity for ionised calcium which makes it more suitable to measure transient peaks and high levels of calcium (Minta et al, 1989). The dye's main disadvantage is that it does not display significant wavelength shifts following calcium binding, and therefore the method for calculating the free calcium level relies on a comparison to the baseline level of fluorescence.

Arginine-Vasopressin

Arginine-vasopressin (AVP), otherwise known as antidiuretic hormone (ADH), is a pituitary neuropeptide predominantly concerned with the control of water conservation. It does however, have other functions as a vasoconstrictor and also as a stimulus to platelet aggregation. The hormone interacts with two types of membrane receptors. The pressor response and platelet activation are mediated by V1 receptors (to induce an increase in intracellular calcium) whereas the antidiuretic effects are mediated by V2 receptors (Michell et al, 1979). The latter are unlikely to be present on platelets (Hallam et al, 1984a).

In platelets, vasopressin causes a rapid but transient increase in intracellular calcium (Pollock and MacIntyre, 1986). In the presence of 1 mM external calcium, a saturating dose of vasopressin of 1 μM, increases intracellular calcium by 700% (Hallam et al, 1984a). With low levels of external calcium, the response is dramatically reduced which implies that it is dependent on the presence of extracellular bivalent cations (Haslam and Rosson, 1972; Pletscher et al, 1985). With the addition of small quantities of aspirin to irreversibly inhibit cyclo-oxygenase, the rise in calcium following vasopressin stimulation is reduced slightly, a reflection that TXA2 is only minimally involved in the mechanism (Hallam and Rink, 1987).

The transient nature of the calcium response may reflect rapid desensitisation of the receptor or alternatively, the calcium channels may only open briefly; the increased free calcium ion would rapidly reduce due to processes of cellular homeostasis (Hallam et al, 1984b).

Depending on the platelet donor, maximum calcium responses have been documented using 100 - 1000 nM vasopressin and the threshold to obtain a response is approximately 10 nM (Pollock and McIntyre, 1986). Intracellular calcium increases from basal levels (around 100 nM), to levels of 500-700 nM at maximal stimulation. This is an interesting finding, because the levels of intracellular calcium required to elicit the platelet changes of shape change, secretion and aggregation are in the order of 500, 700, 2000 nM

respectively (Rink et al, 1982), and yet even in situations of increased vasopressin secretion, the circulating levels are too low to induce such calcium levels (Pollock and MacIntyre, 1986). Therefore, the physiological function of vasopressin alone and the V1 receptors on platelets remains unknown. However it is possible that potentiation of the vasopressin effect may occur with other circulating hormones such as ADP and adrenalin (Launay et al, 1987).

Pregnancy Studies of Platelet Intracellular Calcium

Platelet intracellular free calcium has been assessed in normotensive and hypertensive pregnancies at different gestational ages with measurements of basal levels and of those following stimulation with serotonin (5HT), thrombin, angiotensin II (AII) and arginine-vasopressin (Haller et al, 1989; Barr et al, 1989; Kilby et al, 1990; Zemel et al, 1990). Intracellular free calcium was measured by spectrofluorimetry using quin-2, or fura-2. Basal levels were similar in preeclamptic compared to control pregnancies in some studies (Barr et al, 1989; Zemel et al, 1990) but not in others (Haller et al, 1989; Kilby et al, 1990). Significant differences in intracellular calcium mobilisation following stimulation with 5HT, AII, and arginine-vasopressin have been found: a reduction following stimulation with 5HT (Barr et al, 1989); an increase (Haller et al, 1989) or no response following AII (Zemel et al, 1990); and an increase with arginine-vasopressin (Zemel et al, 1990). In the original description of the latter study, an increased response to arginine-vasopressin was evident at 10 weeks gestation in urban black women with incipient preeclampsia but not in those who remained normal. The authors proposed that the test would be eminently suitable as a screening test for preeclampsia (Zemel et al, 1990). There is only one other report of the platelet response to arginine-vasopressin in severe preeclampsia in which reduced aggregation was found (Whigham et al, 1978). Therefore, because the underlying mechanism is unknown and also the changes observed in the Zemel Study could be potentially an intrinsic feature of the black population studied, this platelet response to arginine-vasopressin needs to be confirmed to show that it is a real change associated with preeclampsia.

In this study, the levels of resting and arginine-vasopressin stimulated intracellular free calcium were evaluated in the platelets of women with established preeclampsia and normal pregnancy, and the response was assessed as a screening test for preeclampsia alone and in parallel to the Angiotensin Sensitivity Test (AST). Flow cytometry, rather than spectrofluorimetry, was chosen as a potentially more sensitive method to detect the changes in intracellular calcium levels, and fluo-3 was the most suitable calcium indicator for use with the 488 nm laser excitation source available.

MATERIALS

Fluo-3-acetoxymethylester (fluo-3/AM) was obtained from Molecular Probes Inc, Eugene, Oregon, USA; [Arg^8]-vasopressin-acetate, 4-bromo-A23187 calcium ionophore, probenecid (p-[Dipropylsulphamoyl] benzoic acid) were obtained from the Sigma Chemical Company, Poole, Dorset; and anhydrous dimethyl sulphoxide (DMSO) from Aldrich, Gillingham, Dorset. Fluo-3/AM was prepared as a 1 mM stock solution in anhydrous DMSO each week. [Arg^8]-vasopressin was prepared as 1 mM stock solution in phosphate buffered saline (PBS) and stored as aliquots at -20°C and thawed just before the experiment. 4-bromo-A23187 was prepared as a stock solution of 1 mM in DMSO and stored at 4°C. The buffer for these experiments contained 145 mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, 2.5 mM probenecid. Probenecid was added to the buffer just before the experiment (2.5 mM final concentration, pH adjusted to 7.4).

SUBJECTS

The study involved 94 women, age range 18-39, with no history of hypertension or renal disease, and who had taken no antiplatelet medication for the preceding two weeks. The nonpregnant women were healthy volunteers from the hospital staff. The pregnant women were all nulliparous; those who were normotensive at the time of testing were outpatients recruited at antenatal clinic whereas the women with established proteinuric preeclampsia were inpatients.

The following groups were studied:

1. Ten healthy normotensive nonpregnant women

2. Ten women with proteinuric preeclampsia and their normotensive pregnant controls, matched for age +/- 4 years and gestational maturity +/- 13 days. The proteinuric preeclamptic subjects had greater than 0.5 g protein per 24 hour urinary collection. The matched controls remained normotensive throughout the remainder of the pregnancy. Five of the preeclamptic women were taking α-methyldopa for treatment of hypertension at the time of testing, a drug considered not to interfere with platelet function.

3. Seventy-four normotensive nulliparous women at 28 weeks gestation who in addition participated in the AST study (see Chapter III).

METHOD

Platelet Intracellular Free Calcium Measurement

Venous blood was collected by venipuncture using a 21 gauge butterfly needle, or an 18 gauge venflon (28 week gestation subjects). Ten mls were taken for full blood and platelet counts (STKR T890, Coulter Electronics, Luton, Bedfordshire) and serum urate level (oxidation method, Parallel Discrete Analyser, Monitor Bioscience, West Sussex) followed by a specimen of 8.6 mls taken in a separate syringe (to be less disturbed) for platelet calcium analysis. Blood was anticoagulated in acid-citrate-dextrose (1.3g trisodium citrate, 0.5g citric acid and 1.5g dextrose in 100 ml water) in a ratio of 6:1, and spun (200 **g**, 21°C for 10 min) to obtain platelet-rich plasma. This was aspirated and incubated for 30 minutes at 37°C in the dark with 5 μM fluo-3-acetoxymethylester (final concentration). In order to prevent dye leakage, probenecid (2.5 mM final concentration) was added to the platelet-rich plasma, and to all buffers throughout the experiment (Merritt et al, 1990). The platelets were pelleted by centrifugation for 10 mins at 200**g**, at 21°C.

The supernatant plasma was discarded, and the platelets were resuspended in 1 ml buffer at a concentration of approximately 3 x 10^7 /ml cells. The samples were stored at room temperature between measurements.

Angiotensin Sensitivity Test

The AST was performed in those subjects at 28 weeks gestation as described in Chapter III p37. The blood was withdrawn for platelet analysis at the time of venflon insertion immediately prior to the screening test.

Flow Cytometry Measurements

Fluorescence measurements were made using an EPICS 541 Flow Cytometer (Coulter Electronics, Luton, Bedfordshire) equipped with a 5W Argon Laser. The excitation wavelength of the laser was 488 nM at 300 mW. The fluorescence detector was standardised before each experiment using Immuno-Brite beads (Coulter Electronics). For all measurements, the mean fluorescence was calculated from a linear scale of 1024 channels using 10,000 platelets.

A prestimulation value was initially obtained by averaging the mean fluorescence from three consecutive measurements. The sample was then removed, the agonist added and then the sample was reanalysed in the flow cytometer. The first time-point for measurement post agonist addition was approximately 10 seconds. Subsequent measurements were made until the maximum response had been obtained (Fig 6.1). The concentrations of arginine-vasopressin used were 0.1, 1.0, and 10 μM. In another sample, 5 μM of 4-bromo-A23187 was added [a calcium ionophore which opens calcium channels to induce maximal intracellular calcium levels and hence maximal fluorescence (Deber et al, 1985) [Fig 6.2]. The concentration of 5 μM was shown to have a consistent saturating effect on calcium flux (Fig 6.3).

The mean platelet intracellular calcium was not calculated as described by Tsien et al, 1982, because when using fluo-3 there is no shift in the wavelength following calcium

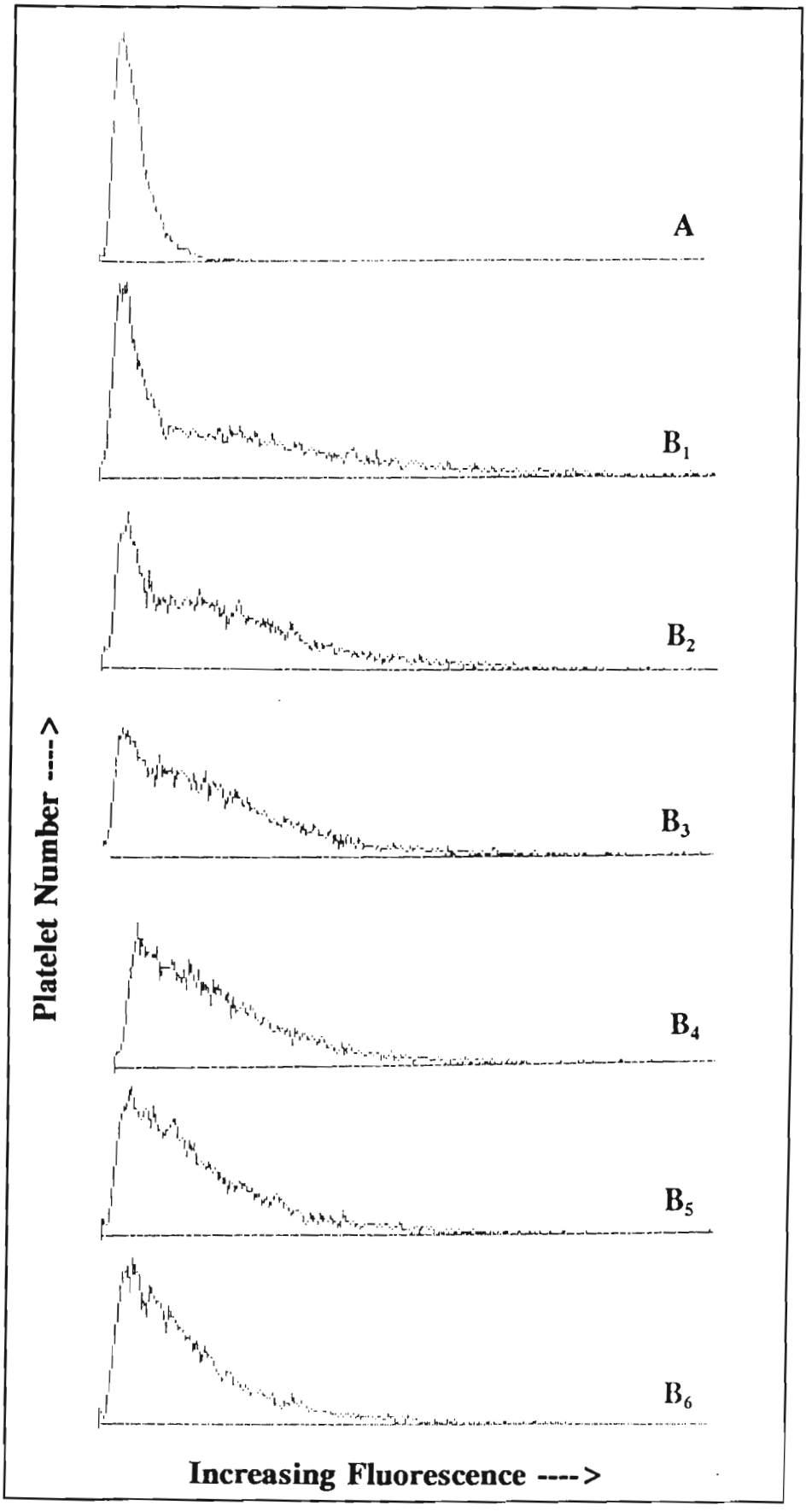

Figure 6.1
Flow cytometry printouts representing the fluorescence emitted from fluo-3 platelets as a baseline (A) and post μM arginine-vasopressin stimulation (B1-6). The interval between the readings was 10 seconds. The mean fluorescence was taken as the fluorescence measurement.

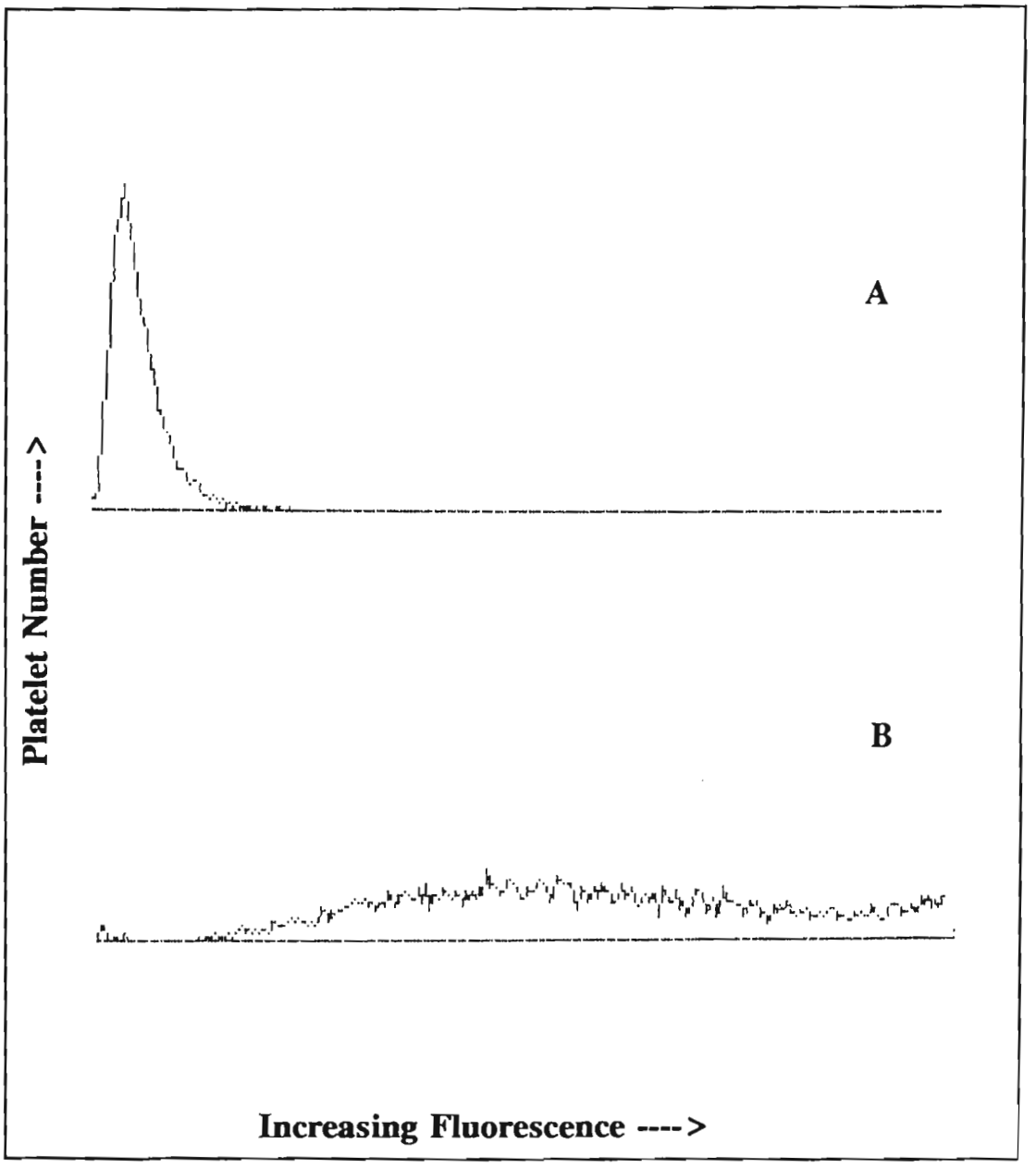

Figure 6.2
Flow cytometry computer printout of the fluorescence emitted from fluo-3 loaded platelets as baseline (A) and post 5 μM 4-bromo-A23187 calcium ionophore stimulation (B).

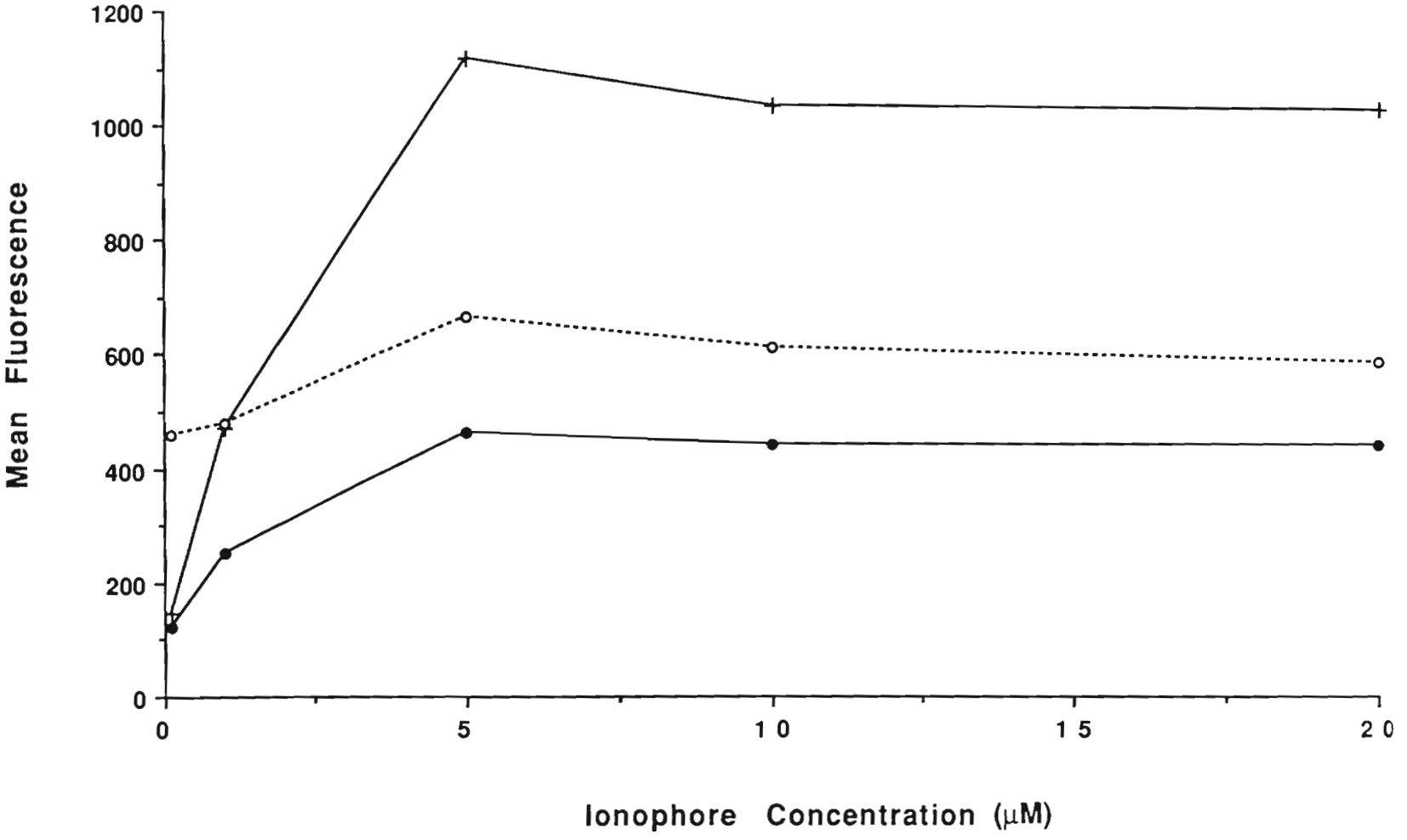

Figure 6.3
Graph showing titration curves for 4-bromo-A23187 calcium ionophore stimulation of fluo-3 loaded platelets in three different subjects. Five μM ionophore was shown to maximise the calcium flux consistently.

binding and therefore the ratio between wavelengths cannot be used. Instead the ratios of the stimulated platelet fluorescence to the baseline fluorescence were determined, or maximal fluorescence following stimulation with 4-bromo-A23187 (see results section).

Statistics

Because of the small numbers involved and the non-normal distribution of the data, the values were expressed as medians (range) and nonparametric tests were used for statistical comparisons. The Wilcoxon one-sample test was used for comparison between proteinuric preeclamptics and matched controls; the Kruskal-Wallis test for multiple intergroup comparisons and the Mann-Whitney-U test for two intergroup comparisons. Statistical significance was taken at $p < 0.05$.

RESULTS

Calculation of Results

To determine the influence of variations in dye loading on the measurement of the platelet response to stimulation, fluo-3 loading concentrations (1.0, 2.5, 5.0, 7.5 μM) were titrated using two incubation periods (15 and 30 minutes). The intensities of both the baseline and maximal fluorescence (following 4-bromo-A23187 stimulation) increased with increasing dye concentrations and incubation times, but the ratio between baseline and maximal fluorescence was unaffected by variations in dye loading (Table 6.1). With all subsequent measurements the platelets were loaded with 5 μM fluo-3 for 30 minutes. Variations caused by small random fluctuations in dye loading were corrected by calculating the ratio of fluorescence intensity following arginine-vasopressin stimulation to that following maximal stimulation with 4-bromo-A23187. The ratio with maximal fluorescence was chosen to avoid the potential problem of increased baseline fluorescence induced by uncontrolled spontaneous platelet activation in vitro. The group means of the platelet fluorescence at maximal stimulation were similar for all the four groups of

	Fluo-3 Loading Concentration (μM)			
	1.0	2.5	5.0	7.5
Incubation Time 15 minutes				
Baseline	12	25	36	50
Stimulated	127	292	361	443
Baseline/Stimulated				
% Maximal Stimulation	**9.4**	**8.6**	**10.8**	**11.3**
Incubation Time 30 minutes				
Baseline	22	50	77	94
Stimulated	220	472	830	962
Baseline/Stimulated				
% Maximal Stimulation	**10.0**	**10.6**	**9.3**	**9.7**

Table 6.1
Table showing platelet fluorescence for a titration of fluo-3 loading concentrations over two incubation periods. Baseline and stimulated (4-bromo-A23187 calcium ionophore) fluorescence increased with increasing fluo-3 concentration and incubation time. The % maximal stimulation [baseline / stimulated (%)] corrected for the variation in dye loading.

women so that the differences between the ratios reflected the intensity of this platelet response to the agonist. Therefore, the response to arginine-vasopressin stimulation was calculated as the % maximal response, namely:

$$\frac{\text{arginine-vasopressin stimulated fluorescence}}{\text{4-bromo-A23187 stimulated fluorescence}} \%$$

Arginine-Vasopressin Concentration

An increasing titration response was shown for the three concentrations of arginine-vasopressin, with a saturating effect at 10 μM (Fig 6.4). Therefore, unless otherwise stated the results are presented using the latter concentration.

Validation

The inter-assay coefficient of variations (CV) of the fluorescence response to arginine-vasopressin in the platelets of two nonpregnant healthy female volunteers were calculated from measurements taken on six separate occasions over six weeks (Fig 6.5). The values were 7.0 and 10.8%. In one of these volunteers, the CV, in addition, determined on four separate days over one week to represent 'day-to-day' variation; this was 3.5%. The repeated readings for each individual were of similar magnitude even when tested many months apart, but were consistently different between the two individuals.

Proteinuric Preeclampsia and Matched Controls

The median gestational ages at measurement for both groups were 33 weeks (range 28 - 37). The booking clinical features in both groups were similar, but the final maximum blood pressures were significantly different ($p < 0.006$ Wilcoxon one-sample test), Table 6.2. Serum urate, but not platelet count was significantly different between the two groups. There were wide intersubject variations in the platelet fluorescence

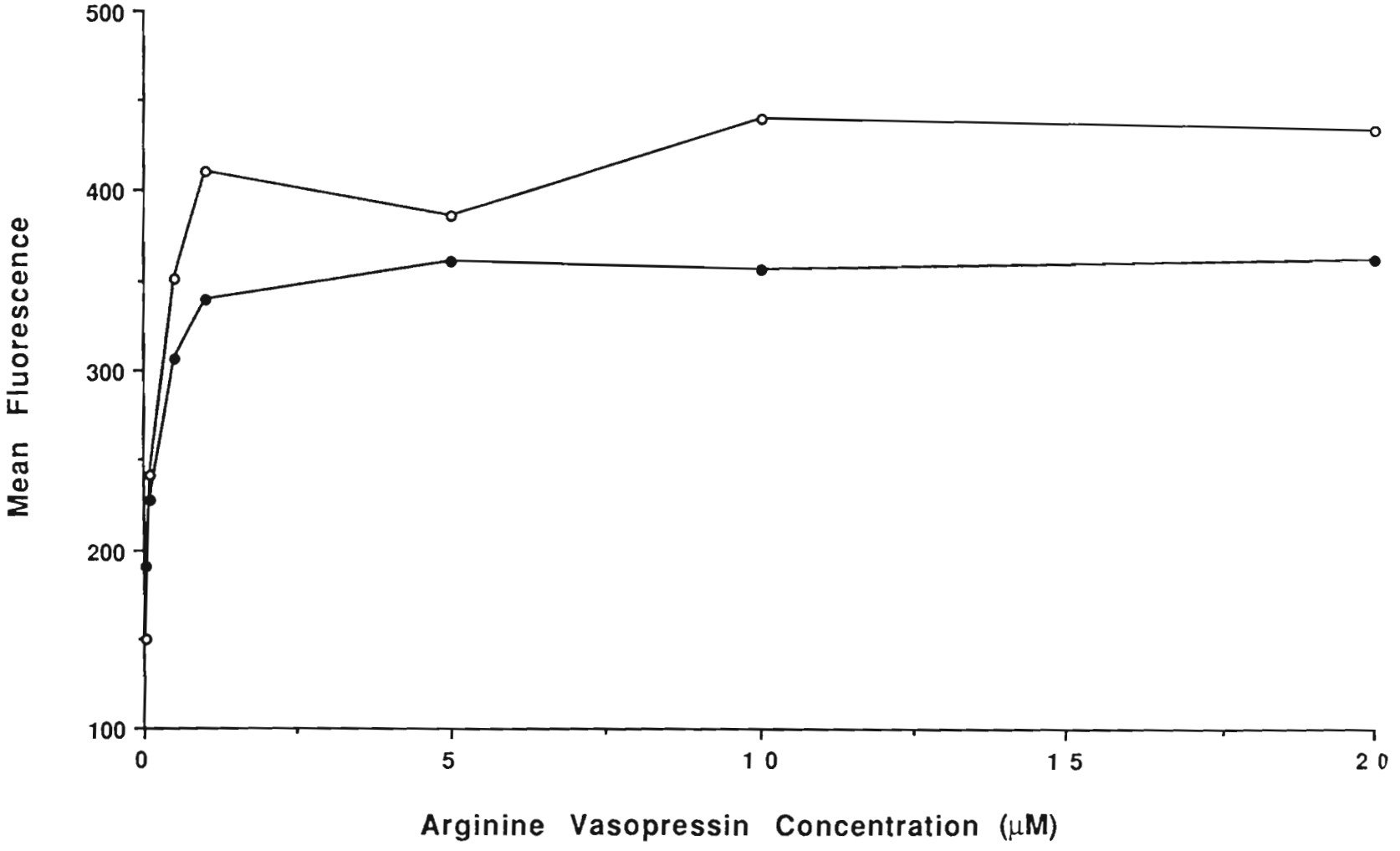

Figure 6.4
Graph showing titration curves of arginine-vasopressin stimulation of fluo-3 loaded platelets in two different subjects. Ten μM was taken as the saturating dose.

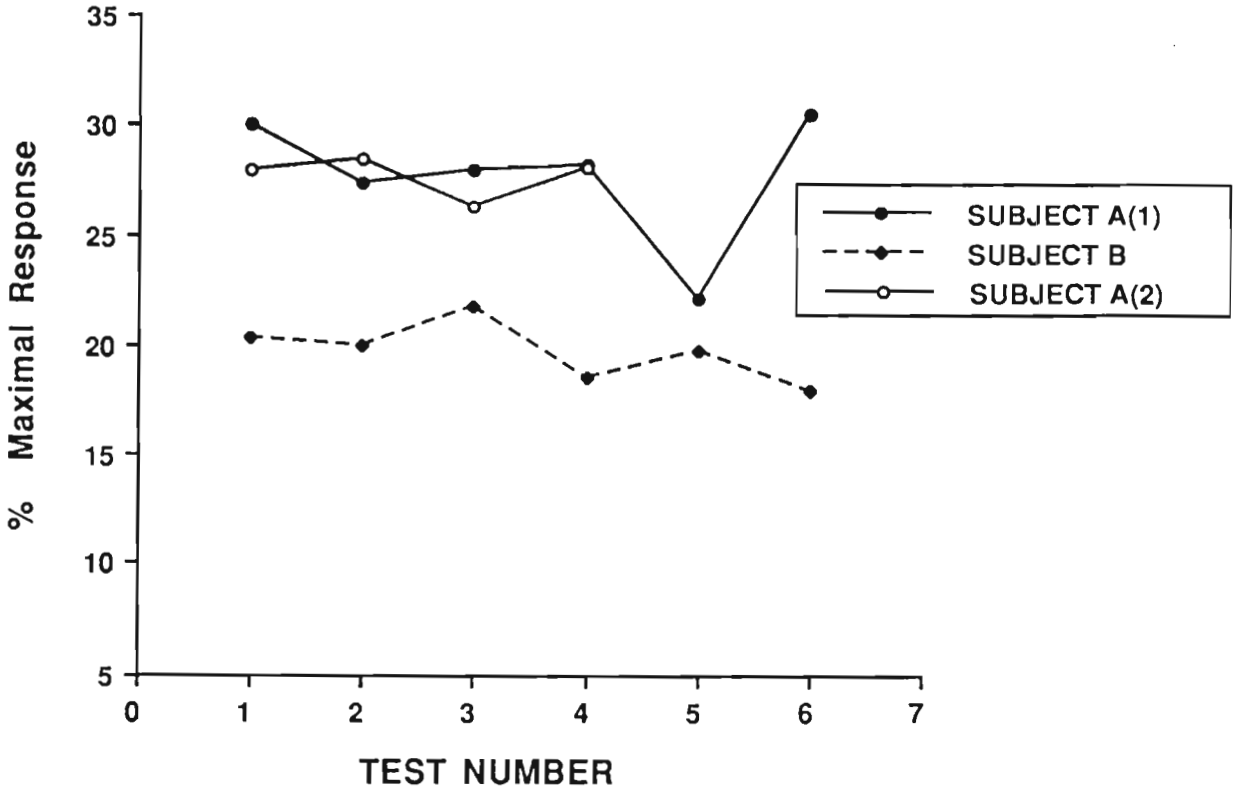

Figure 6.5
Weekly testing of the intracellular free calcium response to 10 μM arginine-vasopressin on two subjects to assess reproducibility [coefficient of variation (CV)]. The continuous lines relate to subject A tested over six weeks on the first occasion (●) and over one week four months later (○). The dotted line relates to subject B tested over six weeks. The CV for the groups were: 10.8% (subject A1); 7.0% (subject B) and 3.5% (subject A2).

	ESTABLISHED PREECLAMPSIA (N=10)		CONTROL (N=10)	
Booking				
Age (years)	24.5	(18.0 - 34.0)	25.5	(18.0 - 34.0)
Weight (kg)	67.5	(53.0 - 100.0)	68.5	(53.0 - 80.0)
Height (cm)	163.0	(158.0 - 172.0)	167.0	(152.0 - 175.0)
Systolic BP (mmHg)	120.0	(100.0 - 160.0)	120.0	(95.0 - 130.0)
Diastolic BP (mmHg)	70.0	(50.0 - 80.0)	70.0	(60.0 - 80.0)
Time of Testing				
Gestation (weeks)	33.5	(28.0 - 37.0)	33.0	(29.0 - 34.0)
Max Systolic BP (mmHg)	170.0	(150.0 - 210.0)	127.5	(95.0 - 150.0)*
Max Diastolic BP (mmHg)	107.5	(98.0 - 130.0)	77.5	(70.0 - 90.0)*
Urate (mmol/l)	406.0	(304.0 - 526.0)	237.0	(143.0 - 318.0)$\doteqdot$
Platelet Count (x 10^9/l)	255.0	(146.0 - 253.0)	253.0	(172.0 - 273.0)
Infant Birth Weight (g)	1931	(1460 - 3101)	3350	(2845 - 4128)**

$\doteqdot$ $p = 0.006$ * $p < 0.002$ ** $p < 0.0001$

Table 6.2

The clinical characteristics of the ten proteinuric preeclamptic subjects and their matched controls represented as median (range). Significant differences (1-sample Wilcoxon test) were found at the time of testing and at final outcome between the two groups.

measurements but no significant difference was observed between the groups (Fig 6.6).

Normotensive Nulliparous Women at 28 weeks Gestation

Of the 74 women involved in the screening study, 17 (23%) demonstrated a positive response to the AST. The platelet intracellular free calcium levels were similar between those women who exhibited a positive or negative response to the AST (Table 6.3). All of the women with a positive AST accepted randomisation into the CLASP trial and at uncoding of the CLASP allocation, it was determined that 10 had been randomised to low-dose aspirin and seven to placebo.

The comparison of platelet intracellular free calcium in relation to pregnancy outcome was performed twice, to both include and exclude those randomised to aspirin. Ten (13.5%) and nine (14.1%) [including or excluding aspirin cases respectively] developed preeclampsia (3 with proteinuria in both analyses). The clinical characteristics at the booking clinic visit and at 28 weeks gestation were similar between both outcome groups (preeclampsia and normal), independent of whether aspirin takers or not were included (Table 6.4). There were no significant differences in the platelet count, serum urate, or platelet calcium fluorescence measurements (before or following arginine-vasopressin stimulation) between the two outcome groups (Fig 6.7), again uninfluenced by whether those allocated to aspirin were or were not included in the analysis.

Comparison Between Nonpregnant and Pregnant Women

The nonpregnant women were of similar age to the proteinuric preeclamptic women (Group 2) and those pregnant women screened at 28 weeks gestation (Group 3). The platelet count was higher in the nonpregnant women compared to those with proteinuric preeclampsia or those at 28 weeks, either normal or with incipient preeclampsia ($p = 0.019$ Kruskal-Wallis test). The median (range) platelet counts (x 10^9/l) were 312 (233 - 375), 201 (141 - 428), 255 (146 - 339) and 307 (182 - 395) for nonpregnant women, women with proteinuric preeclampsia, and women at 28 weeks gestation with subsequent normal or preeclampsia outcomes respectively. Baseline fluorescence measurements were

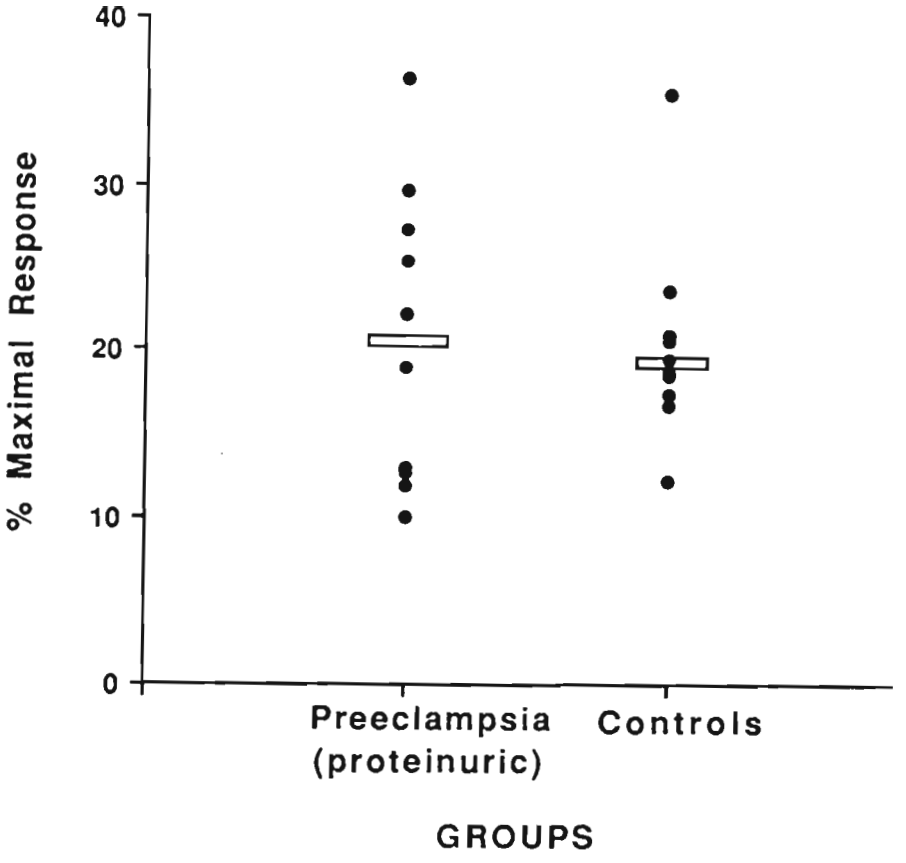

Figure 6.6
Comparison of the intracellular free calcium response to 10 μM arginine-vasopressin between 10 proteinuric preeclamptic patients and their matched controls. No significant difference was observed between the medians (1-sample Wilcoxon test).

	AST NEGATIVE (N=57)		AST POSITIVE (N=17)	
MAXIMUM % response				
10 μM arg-vasopressin	22.1	(10.3 - 36.6)	20.2	(11.9 - 37.9)
28 wk platelet count (10^9/l)	264.0	(143.0 - 422.0)	246.0	(179.0 - 338.0)
28 wk urate (mmol/l)	235.0	(146.0 - 339.0)	238.0	(184.0 - 322.0)

Table 6.3
The platelet intracellular free calcium response to 10 μM arginine vasopressin [median (range)] performed at 28 weeks gestation in those with a negative and positive Angiotensin Sensitivity Test (AST). The platelet and urate measurements at this time are also listed. No significant differences were evident between the two groups (Mann-Whitney-U test).

	Normal		"Incipient" Preeclampsia	
A: Aspirin cases included	(N=64)		(N=10)	
Booking				
Age (years)	27.3	(18.6 - 40.5)	26.4	(19.0 -34.9)
Weight (kg)	63.0	(47.0 - 103.0)	60.5	(52.0 - 89.0)
Height (cm)	163.0	(150.0 - 180.0)	164.0	(155.0 - 173.0)
Systolic BP (mmHg)	110.0	(90.0 - 140.0)	110.0	(100.0 - 140.0)
Diastolic BP (mmHg)	70.0	(50.0 - 80.0)	65.0	(55.0 - 85.0)
28 weeks gestation				
Weight (kg)	70.0	(53.0 - 105.0)	69.5	(57.0 - 98.0)
Systolic BP (mmHg)	108.5	(91.0 - 126.0)	109.0	(105.0 - 129.0)
Diastolic BP (mmHg)	69.0	(56.0 - 82.0)	69.5	(64.0 - 78.0)
Platelet Count (x 10^9/l)	258.0	(143.0 - 422.0)	295.5	(182.0 - 395.0)
Urate (mmol/l)	235.5	(146.0 - 339.0)	224.5	(172.0 - 273.0)
B: Aspirin cases excluded	(N=55)		(N=9)	
Booking				
Age (years)	27.6	(18.6 - 39.2)	25.5	(18.0 - 34.0)
Weight (kg)	62.0	(47.0 - 84.0)	68.5	(53.0 - 80.0)
Height (cm)	163.0	(150.0 - 179.0)	167.0	(152.0 - 175.0)
Systolic BP (mmHg)	110.0	(90.0 - 140.0)	120.0	(95.0 - 130.0)
Diastolic BP (mmHg)	70.0	(0.0 - 80.0)	70.0	(60.0 - 80.0)
28 weeks gestation				
Weight (kg)	70.0	(53.0 - 90.0)	71.0	(62.0 - 98.0)
Systolic BP (mmHg)	108.0	(91.0 - 126.0)	110.0	(105.0 - 129.0)
Diastolic BP (mmHg)	69.0	(56.0 - 82.0)	70.0	(65.0 - 78.0)
Platelet Count (x 10^9/l)	255.0	(143.0 - 422.0)	307.0	(182.0 - 395.0)
Urate (mmol/l)	235.0	(146.0 - 253.0)	253.0	(172.0 - 273.0)

Table 6.4
The clinical characteristics of the nulliparous women tested at 28 weeks gestation grouped according to their outcome. Part A and B delineates whether the aspirin cases were or were not included in the analysis. Results presented as median (range). No statistically significant differences were observed between the two outcome groups (Mann-Whitney-U test).

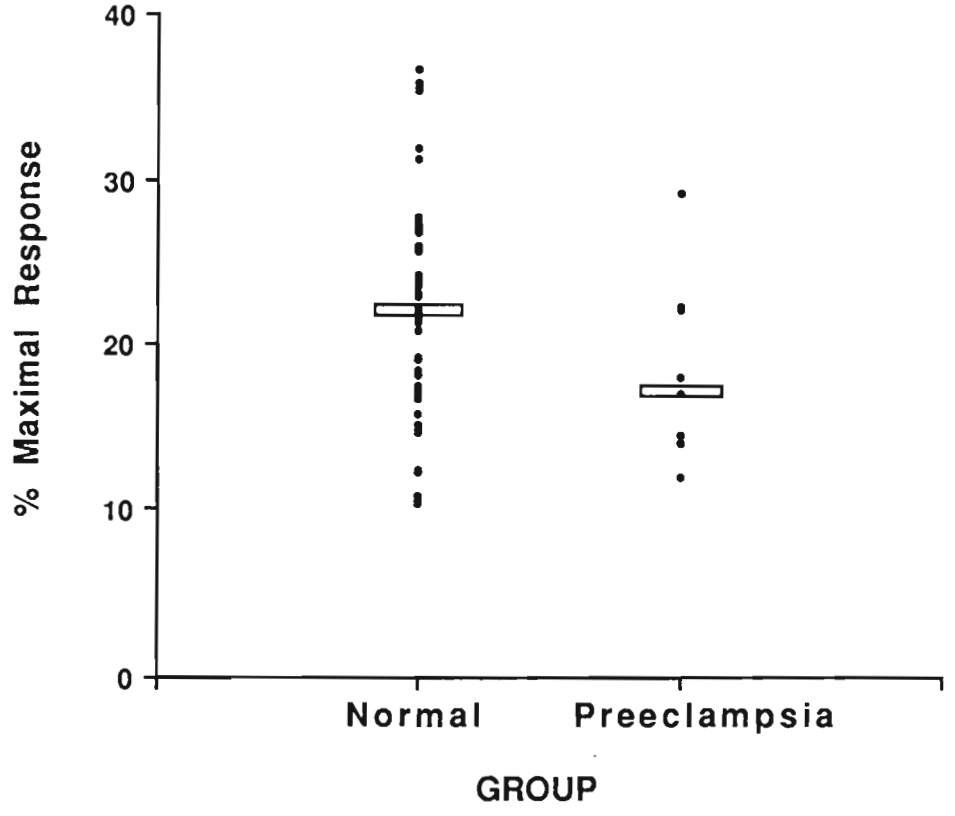

Figure 6.7
Comparison of the intracellular free calcium response to 10 μM arginine-vasopressin between the two outcome groups (preeclampsia or normal) from nulliparous pregnant women tested at 28 weeks gestation. No significant difference was observed between the medians (Mann-Whitney-U test).

similar in all groups. When stimulated by arginine-vasopressin there were no significant differences between the three groups of pregnant women (Mann-Whitney-U test $p > 0.05$). However, the response of stimulated platelets from nonpregnant women was significantly greater than those of the three pregnant groups at all concentrations of arginine-vasopressin used [$p < 0.001$ Kruskal-Wallis test] (Fig 6.8).

DISCUSSION

In this study, there was no difference in the response of platelet intracellular calcium to arginine-vasopressin between women at 28 weeks gestation who later did or did not develop preeclampsia. Furthermore, no differences in this response were detected between women with established proteinuric preeclampsia and their matched normotensive pregnant controls. Therefore, these results do not confirm the findings of Zemel et al (1990), who demonstrated an increased sensitivity to arginine-vasopressin in the platelets of women destined to develop preeclampsia, which was apparent in the first and second trimesters, antedating the clinical signs of disease.

The disparity in the results may have been influenced by several factors. The study populations were different. Zemel et al (1990) investigated urban black women whereas caucasian women were studied in the present trial. Differences in gestational age between the times of measurement cannot explain the discrepancy because the increased sensitivity documented by Zemel et al (1990) was consistently present until the third trimester. Second, a different definition for preeclampsia was used in each study; the present definition was chosen in an attempt to exclude those with underlying chronic hypertension, but nevertheless, a reasonable degree of overlap in the women diagnosed to have preeclampsia by either definition should have been expected. A final point is that the platelet calcium responses to arginine-vasopressin stimulation were measured differently in the two studies. Spectrofluorimetry and the calcium indicator fura-2 were used by Zemel et al (1990), whereas flow cytometry and the indicator fluo-3 were used in this study. This in turn led to different methods for analysis, calculation and

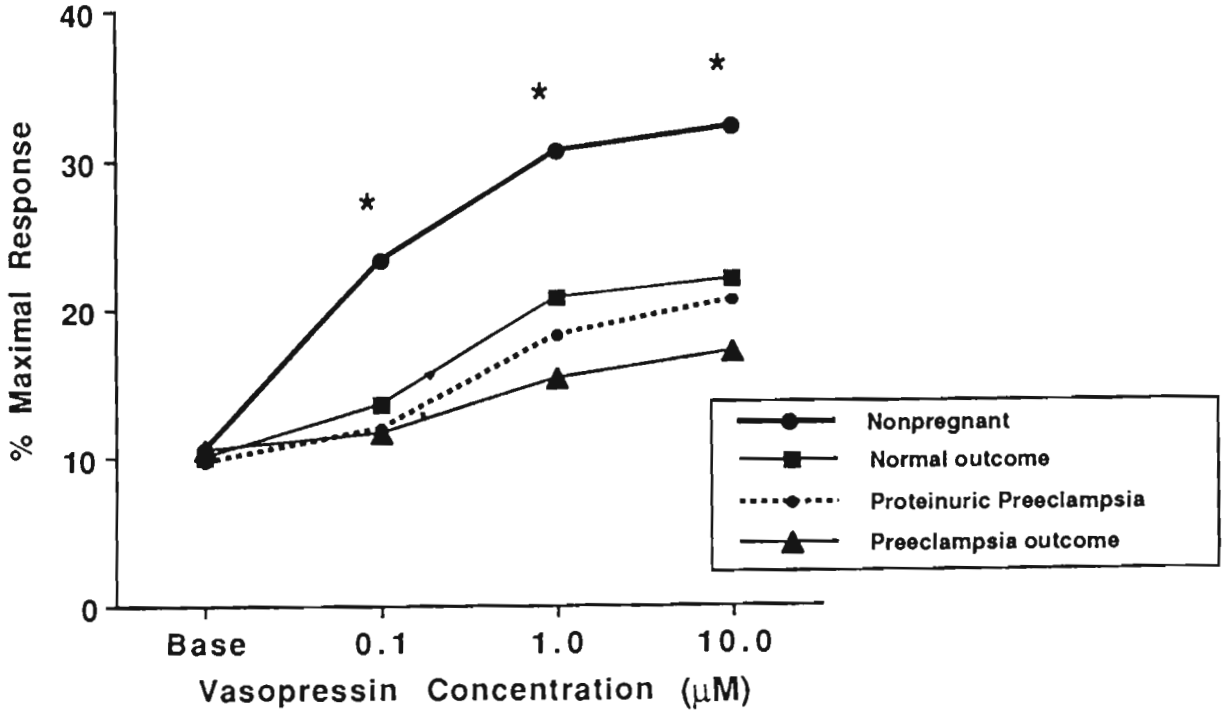

Figure 6.8

The baseline intracellular free calcium level and the response to 0.1, 1.0, and 10.0 μM arginine-vasopressin for the four groups of women: nonpregnant (N=10), proteinuric preeclampsia (N=10), preeclampsia outcome (N=9) and normal outcome (N=55). The results are represented as the % maximal response (median). * $p<0.001$ (Kruskal-Wallis test) comparing nonpregnant and pregnant groups.

presentation of the results. However, these should not have interfered with the final result if a fundamental platelet response was being determined.

Furthermore, the results of Zemel and his associates now need to be interpreted with caution following the later correction to their paper (Zemel et al, 1992). The number of women who developed proteinuric preeclampsia was reduced from 14 to 10, with only 31 instead of 48 women being sampled early in pregnancy. Because the gestational ages at first sampling were originally miscalculated they were corrected from 10 weeks to 18 weeks. The smaller numbers available for final analysis make their findings less conclusive.

Although it was not demonstrated, it might have been expected to find a decreased response to arginine-vasopressin in the platelets of women with established proteinuric preeclampsia. Diminished aggregation has been reported (Whigham et al, 1978), and second, there is evidence for decreased responses to various agonists *in vitro* interpreted as "platelet exhaustion" (Ahmed et al, 1991; Louden et al, 1991). The significantly higher sensitivity to arginine-vasopressin in nonpregnant than in pregnant subjects is very similar to that reported by Whigham and her colleagues (1978). This may arise because platelets from nonpregnant women have an increased density of arginine-vasopressin receptors or alternatively a greater responsiveness to receptor stimulation. The effect cannot be explained by the higher platelet count in nonpregnant women because measurements were made in a standardised number of 10,000 cells.

Five of the patients with proteinuric preeclampsia were on α-methyldopa for control of blood pressure at the time of testing. They were included in the study because the drug is not known to interfere with platelet function, nor were we attempting to assess the relationship between blood pressure and platelet intracellular calcium levels. A correlation between these two variables has not been shown to exist in pregnancy (Barr et al, 1989; Kilby et al, 1990; Zemel et al, 1990).

The methods used in this study had several limitations. The rapid and transient response to arginine-vasopressin, which was unanticipated, is more suited to a continuous

recording not available on the flow cytometer used in this study. Secondly, variations in cellular dye loading could not be measured because the emission wavelength of fluo-3 does not shift when it binds to calcium; therefore, absolute intracellular calcium concentrations were not measured. However, using the index (% maximal response) described in this study it was possible to measure a titrated response to different concentrations of arginine-vasopressin and demonstrate consistent differences between individuals and groups (nonpregnant and pregnant). It was therefore considered that a valid measurement reflecting the intracellular free calcium response to arginine-vasopressin was being made.

There were no significant differences between the platelet intracellular calcium responses of those women who demonstrated a positive or negative AST. This is not surprising as neither of the tests were predictive for later preeclampsia and therefore may not have been measuring any common feature.

Finally, as shown in Chapter III, the introduction of low-dose aspirin did not appear to alter the pregnancy outcome and therefore the assessment of the screening test in this population.

In conclusion, an increase in the platelet free calcium response to arginine-vasopressin stimulation was not demonstrated in women with incipient preeclampsia at 28 weeks gestation or established preeclampsia in the third gestation. This does not provide evidence for an early disturbance of platelet function antedating clinical signs nor the basis for an effective screening test for preeclampsia.

CHAPTER VII

Screening: Inactive Urinary Kallikrein / Creatinine Ratio

BACKGROUND

Normal pregnancy is characterised by vasodilation, reduced blood pressure and expanded extracellular fluid volume, whereas the characteristic signs of preeclampsia are secondary to vasoconstriction and increased peripheral resistance. This has led to the detailed investigation of some of the vasoconstrictor hormone systems in the disease, while the involvement of those that are vasodilatory has been relatively ignored. The kallikrein-kinin system is one such example.

The Kallikrein-Kinin System

The kallikrein-kinin system is a vasoactive circulatory hormonal system with predominantly vasodilatory functions. Although it has many similarities to the renin-angiotensin system, it is less well known, primarily because characterisation of the different components and their respective functions has not been easy.

Similarities to the Renin-Angiotensin-Aldosterone System

The kidney has several hormonal systems which serve a self-regulatory function. These include the prostaglandin, kallikrein-kinin and renin-angiotensin systems.

It is most likely that the kallikrein-kinin system is a parallel but opposing hormonal system to the renin-angiotensin system (Fig 7.1). They have similarities because both involve an enzyme which acts on a substrate (α-2 globulin) to produce a vasoactive compound; lysyl-bradykinin, bradykinin (kallikrein-kinin system) and angiotensin II (renin-angiotensin-aldosterone system). The kinins have a vasodilatory function whereas angiotensin II is one of the most potent vasoconstrictors known. In addition, there appears to be multiple points of interaction between these two systems. Aldosterone stimulates the release of tissue kallikrein (Margolius et al 1974); kallikrein *in vitro* activates the conversion of pro-renin to active renin (Sealey et al, 1978); and kininase II, the enzyme which inactivates bradykinin, is identical to angiotensin converting enzyme (ACE) which converts angiotensin I to angiotensin II. Finally, both systems are involved

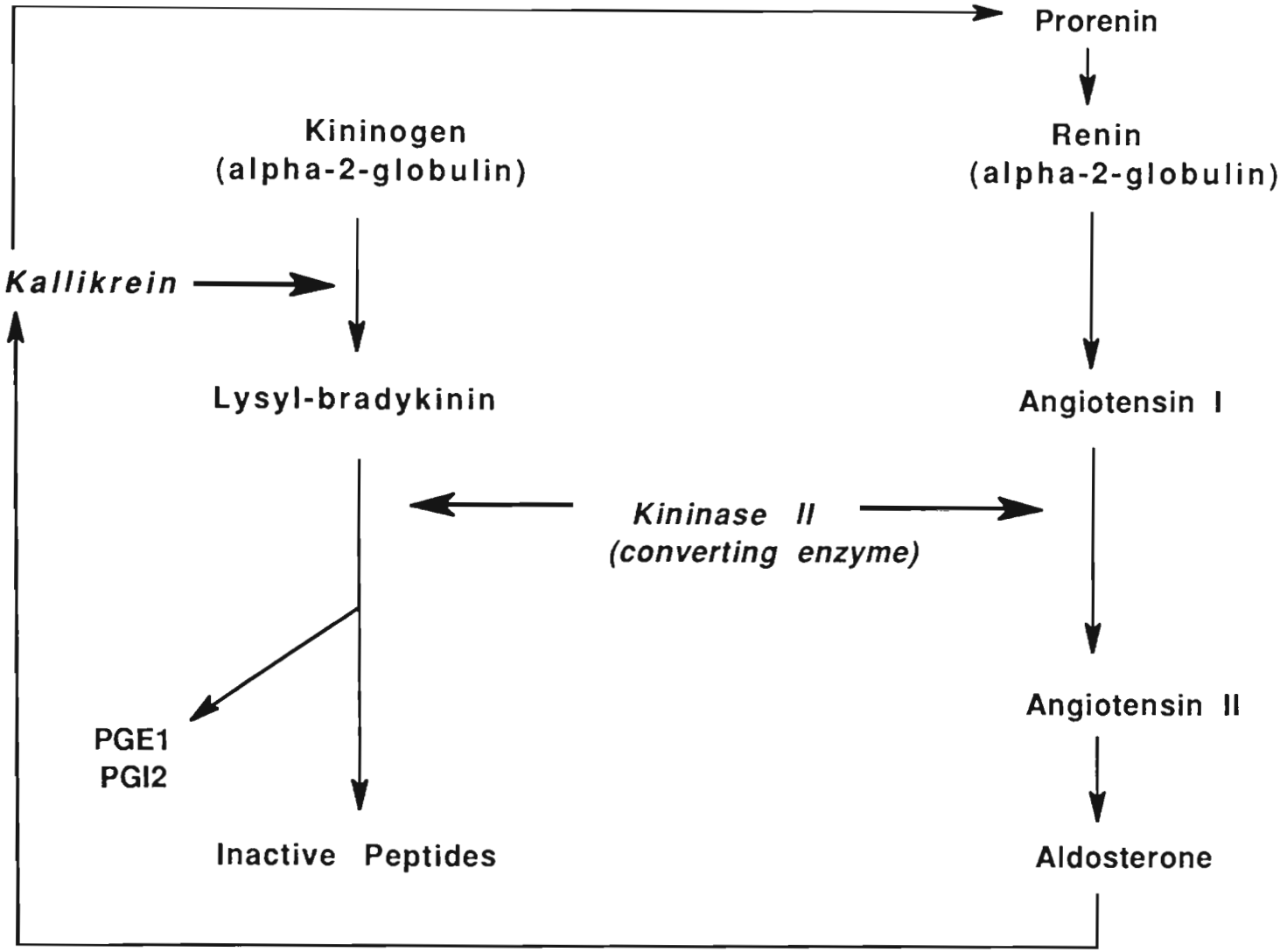

Figure 7.1
Flow diagram showing the relationship between the renin-angiotensin-aldosterone and kallikrein-kinin systems.

in activating certain prostaglandins to enhance their effect.

Many of the renal components of all three systems are produced and released in close proximity within the renal collecting duct system, and therefore it is likely that they interact as a paracrine system to regulate renal blood flow and possibly salt and water transport (Carreter and Scicli, 1983).

Kallikrein

Kallikrein is the major enzyme involved in this vasodilatory system, converting kininogen to the active components, lysyl-bradykinin (kallidin) and bradykinin. The effect of these two compounds is then amplified by release of PGE2 and PGI2 (Terragno et al, 1972).

There are two types of kallikrein - tissue (or glandular) and plasma kallikrein. They differ in their site of origin, molecular weight, immunoreactivity and substrate specificity and furthermore, they appear to have different functions (Carretero and Scicli, 1983).

Plasma Kallikrein

Plasma kallikrein is an intrinsic component of the coagulation system. Prekallikrein (the inactive form) interacts with the Hageman factor and high molecular weight kininogen (HMWK) to initiate the clotting cascade. To what extent plasma kallikrein is involved in blood pressure regulation is unknown. Bradykinin, produced from the interaction of plasma kallikrein with HMWK, is a vasodilator, causes increased vessel permeability and may induce bronchoconstriction. However, it is rapidly destroyed by kininase I and II, peptidases which circulate in the plasma. Therefore it is more likely that these transient effects are involved in local circulatory responses such as inflammation rather than in blood pressure homeostasis (Carretero and Scicli, 1983).

Tissue Kallikrein

Tissue kallikrein is produced in the kidney, and in other glandular tissues such as the

pancreas, gut and pituitary gland. Its function in some of these tissues has not been determined. Low molecular weight kininogen (LMWK) is the preferred substrate for tissue kallikrein to produce lysyl-bradykinin, and later bradykinin as the vasoactive hormones.

The kallikrein excreted in urine is considered to be renal in origin (Scicli et al, 1976) and the probable site of synthesis is the cells of the macula densa within the distal nephron (Levinsky 1979). Although by using immunogenic methods (Rabito et al, 1982), renal tissue kallikrein has been detected in the circulation, present information suggests that its vasoactive effects are exerted indirectly via a paracrine mechanism within the kidney.

Inactive and Active Kallikrein

Kallikrein is excreted in urine as both active urinary kallikrein (AUK) and inactive urinary kallikrein (IUK) with the latter predominating (Pisano et al, 1978). It is undetermined whether IUK is a reservoir for subsequent activation, or is produced by a different pathway of synthesis, but certainly, the behaviour of these two compounds does not always follow in parallel. For example, the excretion of IUK depends on the urinary flow rate, whereas the excretion of AUK does not and therefore the former must be expressed as a concentration rather than excretion rate (Waller et al, 1985).

Methods for Measurement

Measurement of urinary kallikrein has not been without difficulties. At present the enzyme activity is measured using a specific synthetic substrate to produce a radiolabelled by-product or colourimetric change, or alternatively, kallikrein activity can be measured by the rate at which kinin is generated once the substrate is added to the sample. The problem with the former methods is that they lack specificity because there are other esterases in the urine which also can act on the synthetic substrates (Carretero et al, 1983). In addition, these methods will only measure AUK. To overcome this

problem, IUK can be converted to AUK by addition of trypsin. The AUK measurement prior to trypsin addition is then subtracted from total urinary kallikrein to give the IUK measurement. The measurement of kinin generation is more specific but involves time-consuming radioimmunoassays which make it unsuitable for large-scale population studies such as screening (Levinsky 1979). Kallikrein can be measured directly by radio-immunoassay, but these will not differentiate between inactive and active kallikrein (Carretero et al, 1978).

Urinary Kallikrein in Essential Hypertension

It has been proposed that the kallikrein-kinin system may be involved in the pathogenesis of essential hypertension. In 1934 it was first shown that urinary kallikrein excretion is lower in subjects with essential hypertension (Elliot and Nuzum, 1934), and this was confirmed in 1971 (Margolius et al, 1971).

However, there has been criticism of these earlier studies as the subjects were not matched for age, race or sex. Black subjects, whether hypertensive or not, excrete lower levels of urinary kallikrein (Levy et al, 1977), and black children have significantly lower urinary kallikrein levels than white children (Zinner et al, 1976). In addition, children with lower levels of urinary kallikrein tend to have parents with higher blood pressures (Zinner et al, 1978). With consideration of such epidemiological data, subsequent studies have used matched controls for these variables. Some have shown decreased levels in essential hypertension (Lechi et al, 1978), whereas others have not (Lawton et al, 1977; Holland et al, 1980). The reason for these disparate findings is not clear and lends doubt to whether urinary kallikrein is lower in essential hypertension. It may be that the deficiency in urinary kallikrein is limited to some subclasses of essential hypertension and even in these situations it is unknown whether it is a primary or secondary change.

Pregnancy Studies

Longitudinal and cross-sectional studies have shown that AUK excretion over 24 hours is raised over nonpregnant levels during the first trimester of pregnancy returning to non-pregnant levels by term. The stage of pregnancy at which these higher levels are no longer sustained varies between studies. Some have shown that the higher levels are only present during the first trimester (Campbell et al, 1987) whereas others have demonstrated increased levels to at least the beginning of the third trimester (Elebute et al, 1976). By contrast, IUK is increased dramatically in pregnancy, and these levels are sustained until term (Campbell et al, 1987).

AUK and IUK are reduced in women with established preeclampsia (Karlberg et al, 1984; Kovatz et al, 1985; Campbell et al, 1987), and furthermore, the reduced levels may be detected prior to the development of clinical signs. Two-hour, carefully timed urine samples collected at 16-20 weeks, demonstrated lower levels in women who later developed preeclampsia (Campbell et al, 1987). IUK was the chosen index to measure when using the reduced collection period because the calculation of the ratio between IUK and urinary creatinine, provided a more discriminatory index than AUK (Campbell et al, personal communication). Subsequently, single, untimed mid-stream urine (MSU) samples showed a similar relationship (Campbell et al, 1990). The clinical endpoint for these two latter studies was the development of hypertension (diastolic BP $\geq$ 90 mmHg).

Measurement of the IUK/creatinine ratio (using the colourimetric assay) in an untimed mid-stream urine sample may provide a simple and inexpensive screening test for preeclampsia. However, it requires confirmation.

The aim of the present study was to measure the IUK/Creatinine ratio at 28 weeks gestation in a population of nulliparous women who were currently undergoing the Angiotensin Sensitivity Test (AST). This would enable two assessments to be made. First, the efficiency of each as a screening procedure for preeclampsia, using the endpoints described previously, and second, a comparison of the relationship between the two hormonal systems. The purpose of the latter is that with recognition of the

similarities between the two hormone systems, it was proposed that women responsive to angiotensin II may have lower urinary kallikrein levels.

METHOD

The analysis of samples for this study was performed by S. Campbell, Department of Renal and Endocrine Medicine, University of Southampton, St Mary's Hospital, Portsmouth, United Kingdom.

A midstream urine sample was collected from 459 normotensive nulliparous women prior to the AST (see Chapter III p38). The samples were sent immediately to Portsmouth by overnight mail. They were stored at 4°C until analysis was performed (within five days of sample collection).

Materials

Substrate S-2266 [H-D-Val-Leu-Arg-pNA . 2HCl] was obtained from AB Kabi Diagnostica, Stockholm, Sweden; Trasylol [lyphilized aprotinin] was obtained from Bayer, Leverkusen, Germany; Trypsin and Paranitroaniline were obtained from Sigma Chemical Company, Poole, Dorset; Freeze-dried Human Urinary Kallikrein from Channel Diagnostics, Walmer, Kent, U.K. The buffer (pH 8.2, 25°C) was made up from Tris (Sigma Chemical Company) and distilled water.

Determination of IUK

Kallikrein-like activity was measured using the chromogenic substrate S-2266 by a modification of the method of Amunsden et al (1979). Kallikrein in urine splits the substrate H-D-Val-Leu-Arg-pNA and the rate of paranitroaniline (pNA) formation, measured at OD 405 nM, increases linearly with increasing concentration of kallikrein. The assay is performed using microtubules from which samples can be aliquoted directly onto a microplate. Two mls of sample are incubated with trypsin (15 μl, 5 mg/ml) to convert

the inactive kallikrein to the active form. This sample then gives a measure of the total kallikrein present in the sample. The reaction is stopped using SBTI (soya bean trypsin inhibitor, 15 μl, 10 mg/ml). The active and the total kallikrein samples are the aliquoted into duplicate 1 ml samples in microtubules. Using a multichannel pipette 200 μl are dispensed into microtubules where:

a = sample blank

b = active kallikrein

c = total kallikrein

inactive kallikrein fraction = b-c

These are incubated for one minute in a 37°C waterbath and to the "a" tubes, 250 μl of tris/tras (0.05M tris : 20 kIU/ml trasylol) buffer is added. Trasylol is a potent and specific inhibitor of urinary kallikrein and therefore any production of the chromogen measured at OD 405 nM in the blank would represent protease activities other than kallikrein or the colour of the urine sample itself. The tubes are incubated for a further 3 minutes and then thoroughly mixed following addition of 50 μl of substrate (S2266) 1.5 mmol/l. They are then incubated for 30 minutes in a 37°C waterbath and the reaction is terminated with the addition of 50 μl of 50% acetic acid.

Using a multichannel pipette, 200 μl from each tube is transferred to a microplate and read at 405 nM. Standardised measurements are made using pNA and freeze dried human urinary kallikrein, and a quality control is performed in every assay.

For the purposes of this study, the inactive urinary kallikrein (IUK) activity was used for the analysis. IUK was represented as a ratio to urinary creatinine: IUK [mU/l] / Cr [mmol/l]. Urinary creatinine was measured by an enzymatic method (creatinine PAP, Boehringer) using a microplate reader. The intra and interassay coefficients of variation were 2.3% (n=12) and 3.1% (n=10) respectively (Waller et al, 1988).

Statistics

Intergroup comparisons were performed using the Student's 2-sample t-test and Oneway ANOVA after a Log_{10} transformation was performed owing to positive skewed distributions. The data was then reconverted and presented as raw numbers. Statistical significance was taken at $p < 0.05$.

RESULTS

459 IUK/Cr results were available for comparison to the AST outcome and due to loss of follow-up of one subject (see Chapter III p46), 458 were available for comparison to the pregnancy outcome.

Comparison to the AST

By dividing the AST results into Positive and Negative outcome (EPD = 10 ng/kg/min), the IUK/Cr mean was significantly lower in the AST positive group ($p < 0.0001$ 2-sample Student's t-test) [Table 7.1(A)]. This trend was observed further when we divided the AST results into Positive (EPD $\leq$ 10 ng/kg/min), Borderline (EPD $>$ 10, $\leq$ 14 ng/kg/min) and Nonresponsive (EPD $>$ 14 ng/kg/min), $p < 0.0001$ One-way ANOVA [Table 7.1(B)]. To examine this association further, the maximum angiotensin concentration achieved during the infusion was plotted against the IUK/Cr value for all the subjects (Fig 7.2). Although there was a positive trend between the two parameters, the correlation coefficient (Pearson) was nonsignificant ($r = 0.151$).

Comparison to Pregnancy Outcome

63 (13.8%) women developed preeclampsia, 20 (4.4%) with proteinuria. The IUK/Cr level [geometric mean (95% confidence interval)] was lower in those who developed preeclampsia compared to those who remained normal [239.3 (223.9, 257.0) vs 161.4 (138.0, 190.6) (mU/l)/(mmol/l)], $p < 0.0001$ 2-sample Student's t-test]. In the preeclampsia group, those who developed proteinuria demonstrated a similar result to those

	N	IUK/CR [mU/l]/[mmol/l] Geometric MEAN	95% C.I.	
(A)				*
AST POSITIVE *EPD ≤ 10ng/kg/min*	75	151.4	(130.3, 174.2)	
AST NEGATIVE *EPD > 10ng/kg/min*	384	245.5	(231.7, 260.0)	
(B)				*
AST POSITIVE *EPD ≤ 10ng/kg/min*	75	150.6	(132.0, 171.9)	
AST BORDERLINE *EPD > 10, ≤ 14ng/kg/min*	58	195.9	(168.5, 227.7)	
AST NONRESPONSIVE *EPD > 14 ng/kg/min*	326	256.5	(240.8, 273.2)	

* $p < 0.00001$

Table 7.1
The IUK/Cr levels, geometric mean and 95% confidence interval, for those women with a positive and negative Angiotensin Sensitivity Test (AST) [Part A] and a positive, borderline and nonresponsive AST [Part B]. A $\log_{10}$ transformation was performed (with back transformation) to correct for the skewed distributions. A highly significant difference between each of the groups was evident ($p < 0.00001$) 2-sample Student's t-test (Part A) and oneway ANOVA (Part B).

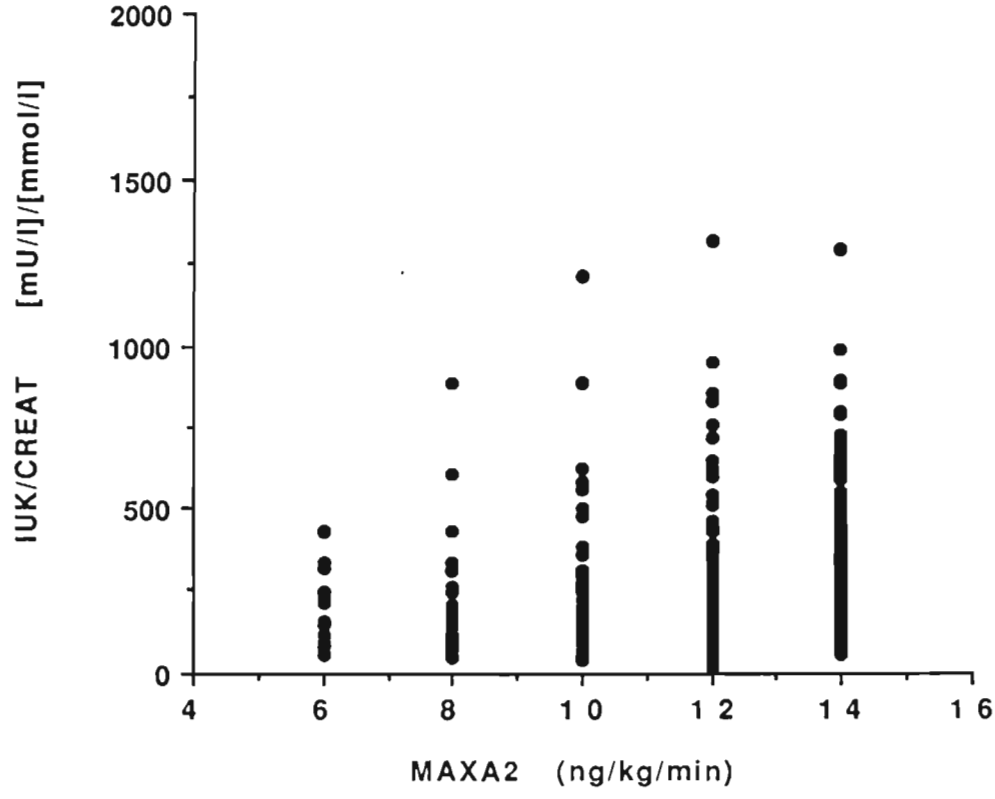

Figure 7.2
The maximum dose of angiotensin II (MAXA2) infused versus the IUK/Cr result for the 459 women. No significant correlation was found (r = 0.15).

who did not [156.3 (117.5, 208.9) vs 163.7 (134.9, 204.2), $p > 0.05$]. The addition or removal of those women randomised to aspirin did not alter the results of the analysis; therefore they were retained to enable calculation of the screening efficiencies of both the tests. The PPV and NPV were respectively 19% and 87% for the AST, and 30% and 93% for the IUK/Cr (Table 7.2).

The thresholds taken to calculate these results were $\leq$ 10 ng/kg/min for the AST and $<$ 170 (mU/ml)/(mmol/l) for the IUK/Cr; the latter was derived from previous studies of Campbell et al (unpublished). To verify this level, a comparison of different thresholds was performed on the present data (Table 7.2). 170 (mU/ml)/(mmol/l) was chosen as the middle of the optimal range.

DISCUSSION

The IUK/Cr analysis is a simple and inexpensive test to perform with potential as a screening test for preeclampsia (Campbell et al, 1990). In this study, the analysis was performed in parallel to the Angiotensin Sensitivity Test (AST) to assess if a relationship between the two hormonal systems was discernible in pregnancy and prior to the development of preeclampsia, and second, to compare the IUK/Cr test to the reference Angiotensin Sensitivity Test.

Some relationship between the two tests was shown as those who demonstrated a lower effective pressure dose (EPD) to angiotensin II (i.e. a more positive response), also demonstrated lower IUK/Cr levels and vice versa. However, this trend was not always present and so the correlation between the two tests was low. Many factors, such as the variability inherent in each of the tests, may have blurred this relationship, but it was interesting to see that a trend was present, even if neither of the tests predicted preeclampsia accurately.

The AST did not perform well as a screening test in this population as described in Chapter III. The screening results presented above represent the smaller number of AST studies performed concurrently with the IUK/Cr test. The IUK/Cr test, performed

Threshold	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
IUK/Cr [mU/l]/[mmol/l]				
130	38	88	34	90
150	56	81	32	92
170	67	75	30	93
190	68	69	30	93
210	70	61	22	93
Angiotensin Sensitivity Test (AST) ng/kg/min				
≤ 10	22	85	19	87

Table 7.2
The efficiency of the IUK/Cr and AST tests for predicting preeclampsia when performed concurrently at 28 weeks gestation in 458 women.

better than the AST as a screening test, but still only demonstrated a sensitivity and PPV of 67% and 30% respectively. This screening efficacy was much lower than reported in the original study (Campbell et al, 1990). Several factors may have contributed to this difference. First, the IUK/Cr samples were collected and analysed at different gestations. In the original study, the analysis was performed at 16-20 weeks gestation, compared to 28 weeks gestation in the present study. Nevertheless, this difference would seem unlikely to alter the results greatly because a screening test would normally be expected to have more predictive accuracy (although possibly less practical application) closer to the onset of clinical symptoms, that is, closer to term for preeclampsia. Second, the samples were posted overnight to Portsmouth, and therefore were not maintained at 4°C for this period of time. However, an analysis of duplicate samples, one kept at room temperature and the other at 4°C for 18-24 hours showed no difference in the measured result (Campbell S, personal communication). Finally, the original study used a threshold level of hypertension to define disease, while the definition used in this study, required an increment of 25 mmHg diastolic pressure in addition to the final threshold level (see Chapter II p35). However, analysis of the group of women who showed a diastolic pressure rise to > 90 mmHg but an increment less than 25 mmHg had similar IUK/Cr levels to those whose blood pressures remained normal (Table 7.3).

In conclusion, in the present study population, the IUK/Cr test was only moderately efficient as a screening test for preeclampsia, although it was much superior to the AST. With the high negative predictive value of the IUK/Cr test (93%), it would seem worthwhile to assess the test again under the original study protocol (presently in progress) and second, to investigate the efficacy of the test in predicting preeclampsia in a high-risk population where the prevalence of the disease will be higher.

	IUK/Cr [mU/l]/[mmol/l]		
	N	Median	Range
Final maximum diastolic BP < 90 mmHg	352	241.0	(20.0 - 1316.0)
Rise in diastolic BP ≥ 90 mmHg but increment < 25 mmHg	43	265.0	(45.0 - 886.0)
Rise in diastolic BP ≥ 90 mmHg plus an increment ≥ 25 mmHg	63	144.0	(41.0 - 988.0)

Table 7.3
The IUK/creatinine levels, median (range) measured at 28 weeks gestation compared to the change in the diastolic blood pressure (BP) during the pregnancy and the final maximum diastolic pressure measured before the onset of labour.

CHAPTER VIII

The Maternal Cerebral Response to an Acute Blood Pressure Rise: Transcranial Doppler Ultrasound (TCD) of the Middle Cerebral Artery During Angiotensin II Infusion

BACKGROUND

The cerebral complications of hypertension in pregnancy remain a significant cause of morbidity and mortality. The pathophysiology is poorly understood, but may involve a breakdown in cerebral autoregulation, the homeostatic mechanism that maintains constant cerebral perfusion over a wide range of arterial pressure. This leads to abnormally increased cerebral blood flow, and vascular damage (Donaldson 1989). Until recently, cerebral blood flow and perfusion could be assessed only by invasive methods requiring X-ray imaging or radiolabelled isotopes, that cannot be used routinely in pregnancy.

Transcranial Doppler ultrasound (TCD), is a noninvasive technique for measuring blood flow velocities in the basal cerebral arteries. It was first described by Aaslid et al in 1982, and subsequently has been validated by angiography in neurological studies (Aaslid et al, 1984), vascular measurements during surgery (Lindegaard et al, 1987) and anatomical cadaver studies (Gillard et al, 1986). It is used for the clinical assessment of intracranial vascular disease such as stenosis (Lindegaard et al, 1985; Weschler et al, 1986) and post subarachnoid vasospasm (Seiler et al, 1986). New applications are being explored such as the assessment of migrainous disorders (Dahl et al, 1990; Friberg et al, 1991; Thomas et al, 1990) and the physiological mechanism of autoregulation (Aaslid et al, 1989). The technique is ideally suited for use in obstetrics, but it appears not to have been described in pregnancy before. Internal carotid artery flow velocities have been measured in pregnancy via an extracranial approach (Ikeda et al, 1990), but because other factors are more likely to influence these recordings, transcranial Doppler should be more informative about intracerebral events.

The middle cerebral artery (MCA) [Fig 8.1] is the most accessible of the basal cranial arteries for flow velocity measurement (Aaslid et al, 1982). This is performed through the thin squamous temporal bone above the zygomatic arch (temporal window). The relationship between the observed and actual velocities of the arterial blood depends on the angle of insonation. This angle is unknown but, for the MCA, is limited to +/-

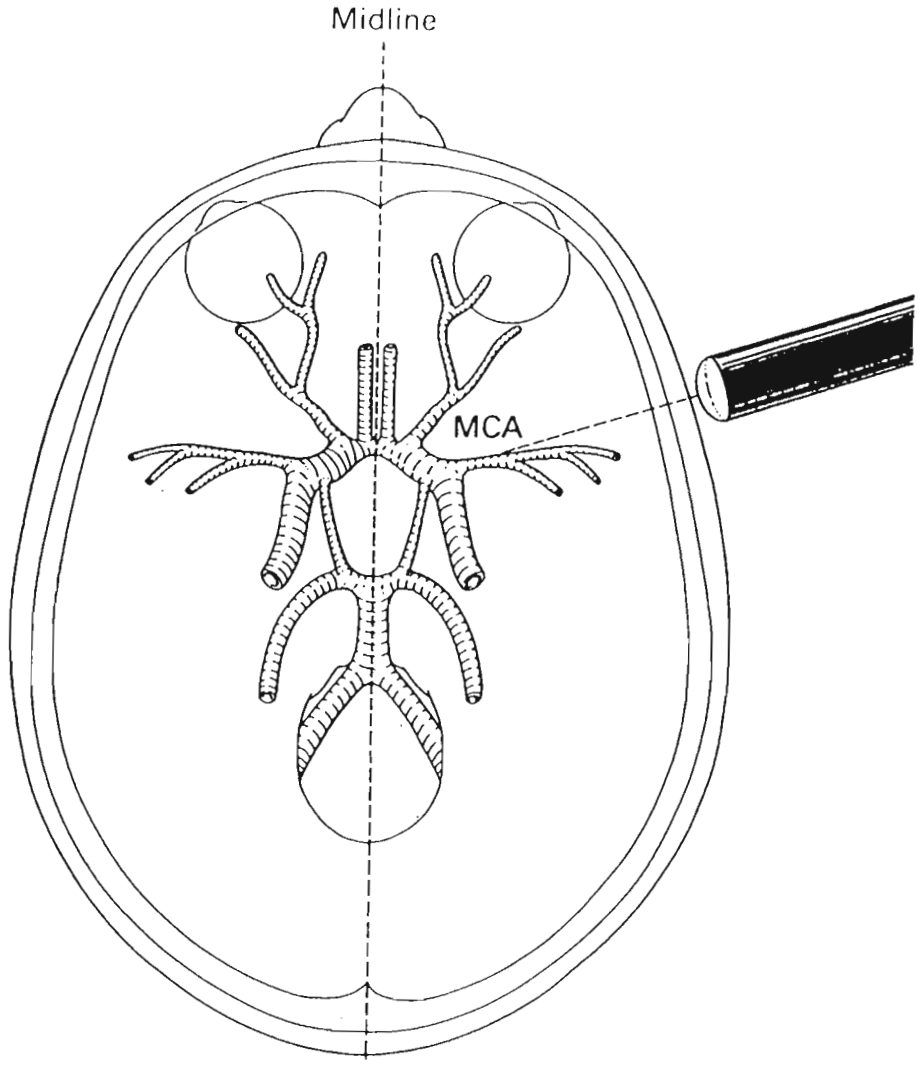

Figure 8.1
Schematic diagram of the cerebral arteries and the temporal approach used to obtain middle cerebral artery (MCA) flow velocity waveforms.

30° (Aaslid et al, 1986a; Kirkham et al, 1986; Padayachee et al, 1986).

If v = observed velocity , | v | = actual velocity, and Θ = the angle of insonation, then $V = |V| \cos \Theta$.

If $\Theta \leq 30°$, then cosine Θ lies between 0.86 and unity and therefore the measurements taken relate closely to the actual flow velocity. For this reason MCA Doppler measurements are given as velocities (cm/s) rather than the dimensionless indices used for other Doppler ultrasound measurements in obstetric practice. These include the resistance index (RI) (Pourcelot 1974), pulsatility index (PI) (Gosling et al, 1975), and systolic/diastolic frequency ratio (S/D) (Stuart et al, 1980) and were developed to circumvent the problem of an unknown and potentially wide angle of insonation. However, the PI calculated as *(systolic velocity - diastolic velocity)/ mean velocity* provides some additional information about the shape of the waveform within the recording (Aaslid et al, 1986b) and so has been included for description during the present study. Flow velocity is an essential component for the calculation of the blood flow through a vessel, which is the product of the instantaneous mean velocity and the cross-sectional area of the vessel. It is assumed that the flow velocities, without actual calculation of blood flow, provide a useful representation of the dynamic events occurring in the cerebral circulation (Lindegaard et al, 1987; Report 1990).

The angiotensin II infusion test provides an experimental situation of an induced blood pressure rise which rapidly returns to baseline levels when the infusion is stopped. In conjunction with the AST screening study (see Chapter III) TCD ultrasound was performed in a cohort of nulliparous women to determine whether changes in the MCA flow velocity could be detected in response to the induced rise in blood pressure associated with the hormone infusion. If so, this would provide a preliminary step in the assessment of Doppler ultrasound to measure the cerebral vascular response to physiological alterations in blood pressure, and ultimately, the pathological disturbance of this response that may occur in preeclampsia.

SUBJECTS AND METHODS

The subjects were either healthy nonpregnant female volunteers (age 25-35) or nulliparous women with singleton pregnancies. The latter were recruited from antenatal clinic prior to 28 weeks gestation as described in Chapter II.

Angiotensin Sensitivity Test (AST)

The blood pressure response to intravenously infused angiotensin II was performed at 28 weeks gestation as described in Chapter III p37.

MCA Doppler Recordings

MCA flow velocity measurements were made using 2 MHz pulsed range-gated Doppler ultrasound (Appleton Floscan Plus, Chilworth, UK) through the temporal skull window as described previously (Aaslid et al, 1982). Subjects in the left semirecumbent position maintained 15° head elevation. This position enabled bilateral examination to be performed. The handheld transcranial probe was focused at a depth of 50.2 mm which occasionally required minor adjustments to obtain maximum flow velocity waveforms. The high pass filter was set at 150 Hz and low pass at 10 kHz. The output was read through a fast Fourier transform spectral analyser and displayed on a screen as a plot of time versus Doppler shift.

The MCA measurements were taken bilaterally, at three stages of the infusion: baseline (preinfusion), at the time when the maximum dose of angiotensin II had been achieved and 10 minutes following discontinuation of the infusion. Systolic, diastolic and mean flow velocities, as well as the PI were calculated automatically by the spectral analyser for the recordings taken over periods of 4 seconds.

All angiotensin II tests and TCD studies were performed by the investigator.

Statistical Analysis

Correlations were analysed using the Pearson correlation coefficient. Conditions of measurement were compared using the paired Student's t-test. The angiotensin II MCA

recordings were analysed at different stages of the infusion using the paired Student's t-test and multiple regression. Statistical significance was taken as $p < 0.05$.

RESULTS

Measurements taken from the right and left MCA did not differ significantly. This is consistent with other studies (Aaslid et al, 1982; Grolimund et al, 1988; Hennerici et al, 1987; Sorteberg et al, 1990). Therefore, when recordings from both MCA were obtained (82%), the average was taken as the measurement for that subject otherwise the single recording was used in the analysis.

Intra and Inter-observer Variation

MCA flow velocity recordings were obtained from the 10 nonpregnant subjects bilaterally, and repeated at a mean interval of 1 (1-3) days by the same investigator (PMK). The mean and SD of the differences between the observations are presented in Table 8.1. Using the Bland Altman Plot to assess agreement between measurements (Bland et al, 1986), the width of the limits of agreement ($\pm$ 2 SD), was $\pm$ 9.3 cm/s (Fig 8.2). As the mean interval between observations was at least 24 hours, this value was considered to be clinically acceptable. In 10 nonpregnant subjects, recordings were obtained bilaterally by investigator A (PMK) and then repeated by investigator B (VSS)* within a mean interval of 4 (1-14) days. The results were not disclosed to the other investigator before both recordings were obtained. The Bland Altman plot showed that the width of the limits of agreement between observers was $\pm$ 7.7 cm/s which again was considered to be clinically acceptable (Fig 8.2).

* Dr V. Serra Serra, Research Fellow, John Radcliffe Maternity Hospital participated in the validation study.

	INTRAOBSERVER		INTEROBSERVER	
Systolic velocity (cm/s)	0.45	(5.2)	-2.75	(4.3)
Diastolic velocity (cm/s)	-0.15	(4.0)	0.75	(4.1)
Mean velocity (cm/s)	0.25	(4.6)	-1.35	(3.9)
Pulsatility Index	-0.003	(0.07)	-0.01	(0.10)

Table 8.1
Mean (SD) of the differences between observations for the assessment of intra and interobserver agreement in 10 nonpregnant women. The average of the left and right sides for each Doppler index was used for comparison.

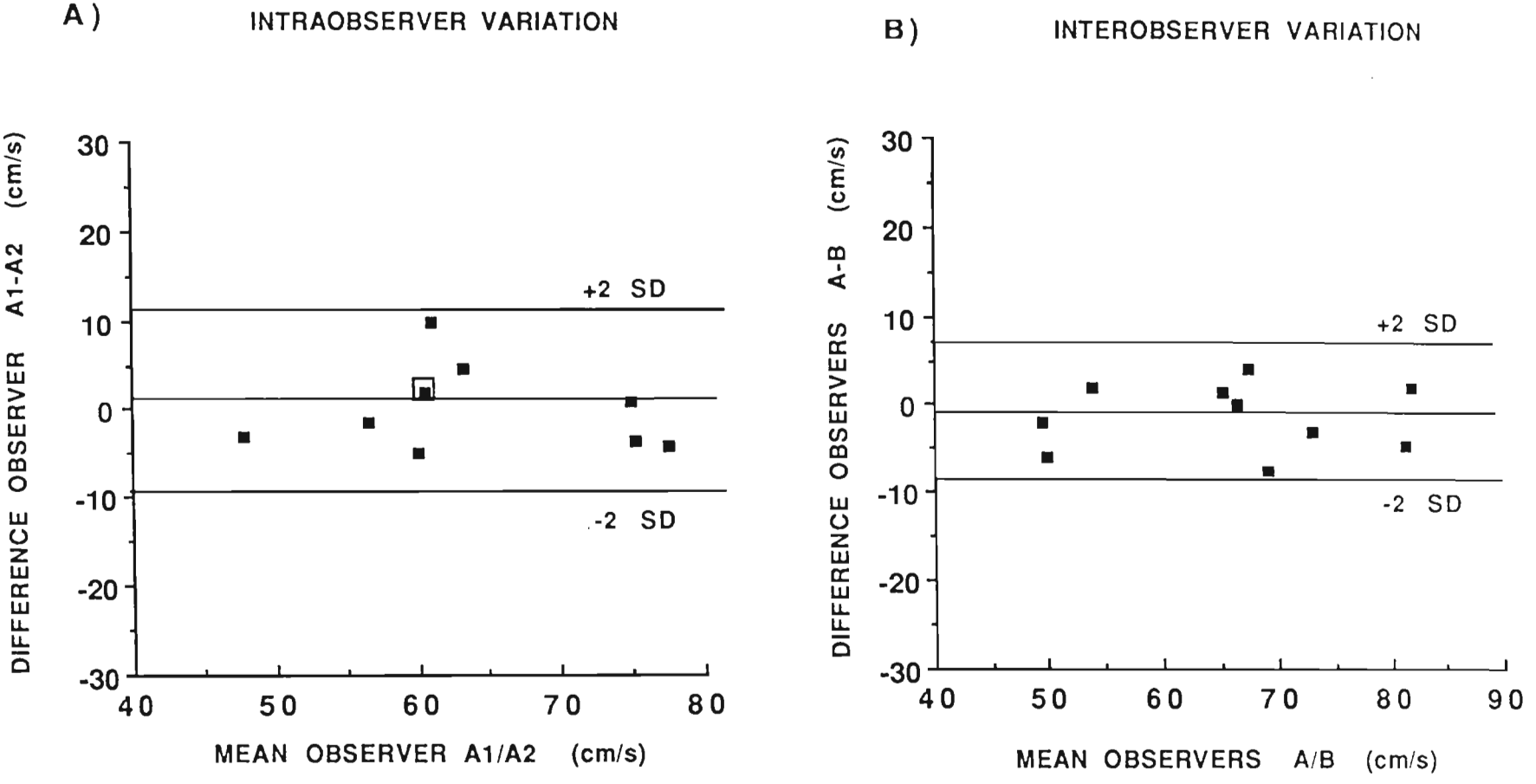

Figure 8.2
Comparison of the MCA flow velocity measurements in 10 nonpregnant women, estimating intra (A) and interobserver (B) variation for mean velocity using the Bland Altman plot. The mean of the two observations are plotted against the differences between the two observations. □ Only 9 points are plotted in the intraobserver variation graph because 2 observations were identical.

Conditions of Measurement (Table 8.2)

Bilateral recordings were obtained from ten nonpregnant subjects after resting supine for five minutes and in the sitting position for two minutes. No significant differences were found between the two sets of recordings for BP, pulse, flow velocity and pulsatility index.

Bilateral measurements were recorded from 10 nonpregnant subjects in the morning after an overnight fast, and then an hour after breakfast. There was a significant rise in pulse, systolic velocity and pulsatility index.

MCA measurements were taken from 10 women who regularly smoked 1-10 cigarettes per day. No subject had smoked for at least four hours before the first measurement. When this was complete she smoked the cigarette after which the measurement was immediately repeated. Bilateral recordings were obtained from eight women and right sided recordings from two. Although the heart rate and diastolic pressure increased after smoking this was not statistically significant. However, the systolic, diastolic and mean MCA velocities increased, and PI fell significantly ($p < 0.05$).

Angiotensin II Infusion Study

MCA flow velocities were measured in 101 primigravid women at 28 weeks, 83 bilaterally and 18 on one side only. The results are summarised in Table 8.3. The mean change (95% CI) between baseline and maximum angiotensin readings were 9.1 mmHg (8.0, 10.3) for the systolic pressure, 15.8 mmHg (14.7, 16.9) for the diastolic pressure, and -5.0 bpm (-6.6, -3.4) for the heart rate; all these changes were significant ($p < 0.0001$). There was no correlation between baseline systolic and diastolic pressures and initial Doppler values. During the infusion the systolic velocity in the MCA decreased -3.3 cm/s (-4.6, -2.0) and the diastolic and mean velocities increased 7.1 cm/s (6.3, 7.9), 5.7 cm/s (4.8, 6.6) respectively, $p < 0.0001$. These indices returned to baseline levels 15 minutes postinfusion, as did the blood pressure and heart rate ($p < 0.0001$). The PI also

	POSTURE		EATING		SMOKING	
	Mean Difference (95% CI) between 2 states:					
Systolic BP (mmHg)	2.0	(-3.0,7.0)	-1.8	(-8.6,5.0)	0.9	(-2.0,3.8)
Diastolic BP (mmHg)	0.7	(-3.5,4.9)	-3.3	(-8.0,1.4)	3.0	(-0.6,6.6)
Heart Rate (beats/min)	-0.2	(-5.1,4.7)	7.9	(4.1,11.7)**	6.6	(-0.2,13.4)
Systolic Velocity (cm/s)	-1.0	(-3.4,1.4)	4.3	(2.0,6.5)**	3.4	(0.3,6.5)*
Diastolic Velocity (cm/s)	-0.8	(-2.7,1.2)	-2.0	(-4.1,0.2)	5.9	(2.1,9.6)**
Mean Velocity (cm/s)	-0.9	(-2.5,0.7)	-0.5	(-2.8,1.9)	4.7	(1.4,8.0)*
Pulsatility Index	0.01	(-0.03,0.06)	0.09	(0.04,0.13)	0.05	(-0.09,-0.01)*

* $p < 0.05$ ** $p < 0.01$

Table 8.2
Conditions of measurement in 10 nonpregnant women. Values listed are the differences (SD) in BP and flow velocities between the 2 states for each condition tested: lying - sitting; fasting - 1 hour postprandial; baseline - 5 mins post cigarette. p-values calculated from the paired Student t-test.

	BASELINE		MAXIMUM		POST	
BP Systolic	109	(8)	119	(8)	111	(8)
BP Diastolic	67	(6)	83	(7)	66	(6)
Heart Rate	80	(11)	75	(11)	79	(12)
Systolic Velocity	101	(12)	98	(12)	101	(13)
Diastolic Velocity	42	(7)	49	(7)	41	(6)
Mean Velocity	65	(9)	70	(9)	65	(9)
Pulsatility Index	0.9	(0.14)	0.70	(0.09)	0.94	(0.12)

Table 8.3
Blood Pressure (mmHg), heart rate (beats/min) and averaged flow velocity recordings (cm/s) recordings taken at pre, maximum and post angiotensin II infusion. Values listed as mean (SD). Changes between baseline and maximum, and maximum and post infusion recordings were all highly significant ($p < 0.0001$).

declined -0.22 (-0.24, -0.20), $p < 0.0001$] during the angiotensin II infusion and returned to normal afterwards. An example of the MCA flow velocity waveform before, during and after the infusion is shown in Figure 8.3.

The correlation between the increase in BP [systolic, diastolic, mean arterial pressure (MAP)], angiotensin II dose, and the change in Doppler indices were analysed. A correlation (95% CI) was present between the increase in diastolic BP and mean velocity increment [r = 0.23 (0.03, 0.40)] and between the increase in MAP and mean velocity increment [r = 0.22 (0.02, 0.40)] $p < 0.05$. One outlying result affected this correlation markedly (Fig 8.4). If this is removed the correlation coefficients are considerably greater [r = 0.32 (0.13, 0.48), r = 0.30 (0.11, 0.47), $p < 0.01$]. There was no correlation between angiotensin II dose and the change in MCA flow velocities or PI.

It was recognised that both the rise in the BP and the increase in angiotensin II dose could have affected the flow velocity recordings. To examine this problem 2 subgroups were selected who were standardised for either angiotensin II dose (6 ng/kg/min, N = 16), or rise in diastolic BP ($\geq$ 20, $\leq$ 25 mmHg, N = 18). A further fourteen women were studied to expand the data necessary for this analysis. In these women, the angiotensin II infusion was performed as described earlier, but Doppler recordings were repeated every 5 minutes just before each increment of the infusion. Correlations were then performed between the change in the variable factor and the change in flow velocity.

At a constant rate of infusion of angiotensin (6 ng/kg/min), the systolic and diastolic pressures increased by an average of 4 mmHg (range -2 to 11 mmHg) and 10 mmHg (range 5 to 21 mmHg) respectively. Both the change in mean MCA velocity and PI were significantly correlated ($p < 0.05$) with the increase in systolic pressure [r = 0.51 (0.05, 0.81) and -0.55 (-0.82, -0.08) respectively]. The increases in diastolic and MAP were similarly correlated but to a lesser and nonsignificant degree. However, when the diastolic BP increment was standardised to 20-25 mmHg, with the dose of angiotensin II ranging from 6-14 ng/kg/min, none of the changes in the Doppler

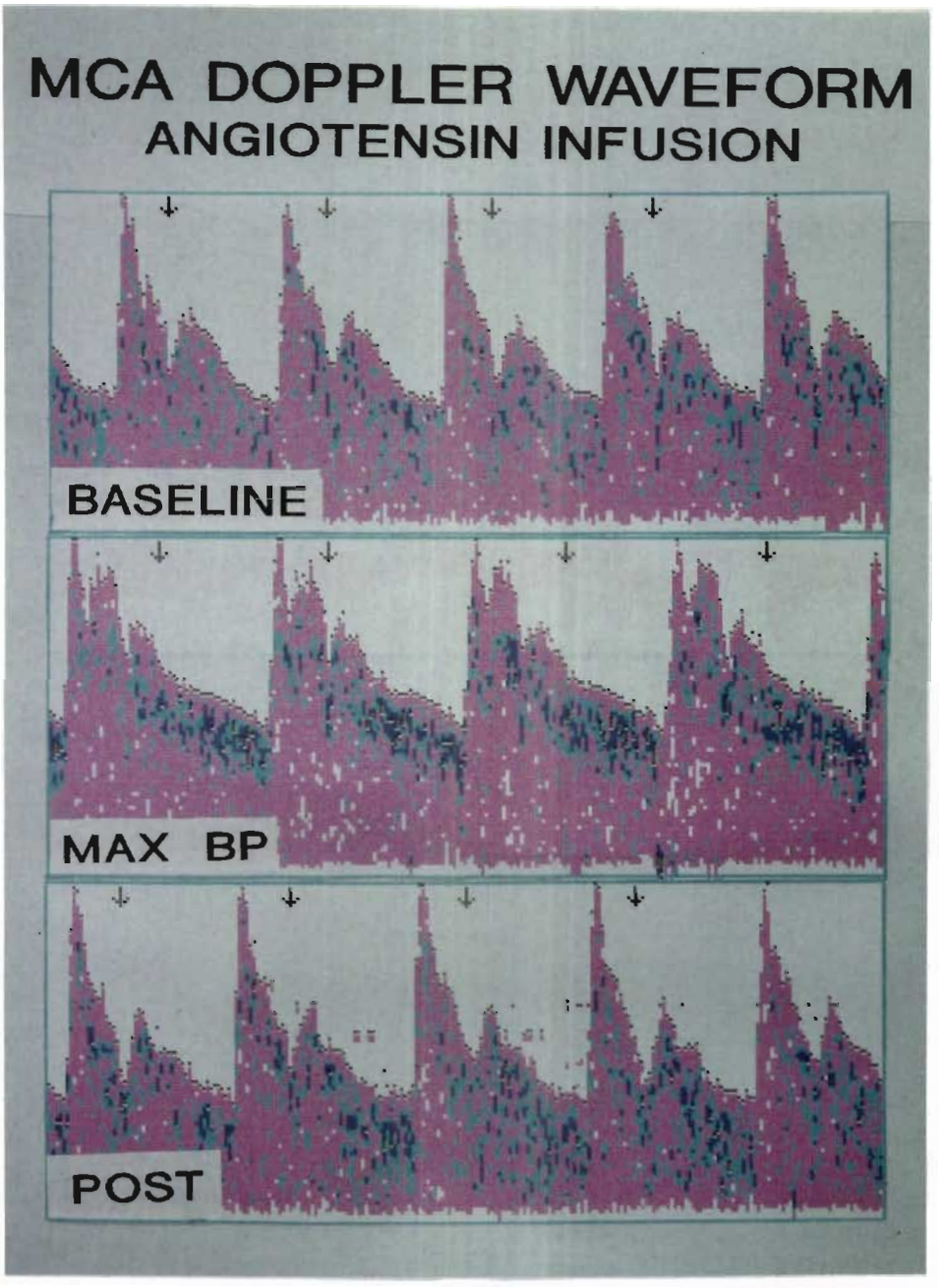

Figure 8.3
The middle cerebral artery (MCA) flow velocity waveform recorded before, at maximum, and 15 minutes post angiotensin II infusion.

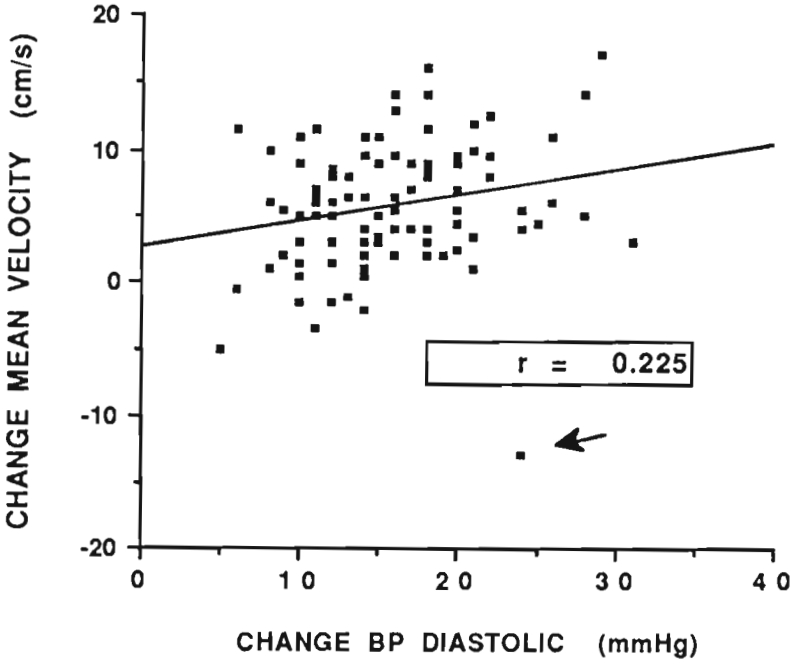

Figure 8.4
Graph showing the relationship between the increment in diastolic BP plotted against the change in mean velocity (pre to maximum angiotensin II infusion) in 101 subjects. The arrow indicates the one outlying result.

measurements correlated with dose of infused angiotensin.

Finally, a multiple regression analysis was performed with the change in mean flow velocity as dependent (δMV), and the change in diastolic BP (δDBP) and angiotensin II dose (MAX A2) as independent variables (the one outlying result was excluded for this analysis). This showed that the increase in the mean flow velocity associated with the rise in diastolic BP could not be explained by the simultaneous increase in angiotensin II dosage. The equation (regression coefficients with their SE in brackets) is $\delta MV = 1.72\ (4.19) + 0.26\ (0.08)\ \delta DBP + 0.01\ (0.30)\ MAXA2$. The increase in velocity associated with an increase of 1 mmHg diastolic pressure is expected to be 0.26 cm/s (95% CI 0.1, 0.42).

DISCUSSION

This study has demonstrated that maternal MCA flow velocity measurements can be obtained easily by TCD to provide information about the cerebral circulation in pregnancy. The MCA was chosen as the most accessible cerebral artery to insonate in order to maximise the quality and reproducibility of the recordings. The reproducibility of the technique was shown to be acceptable.

A wide variation in baseline recordings of flow velocity between individuals was noted, as have others (Aaslid et al, 1982; Hennerici et al, 1987; Lindegaard et al, 1986), and these showed no correlation with the BP or heart rate. This is considered to reflect variation in the MCA diameter (Hennerici et al, 1987). The wide range of normality may indicate that the technique will be more useful for monitoring individual changes, i.e. serial measurements, rather than defining abnormal versus normal values based on an isolated reading (Hennerici et al, 1987). However, other studies have shown that absolute mean velocities above 200 cm/s indicate a potentially dangerous situation associated with a critical degree of vasospasm (Aaslid et al, 1984; Caplan et al, 1990; Seiler et al, 1986).

The time-mean velocity has been recommended as the most useful index of flow velocity, because it is the most reproducible, least variable within the population, and the

least dependent on central cardiovascular factors such as contractility, aortic compliance and heart rate (Aaslid et al, 1986b, Hennerici et al, 1987). In addition, blood flow can be calculated from the product of the "time-mean" velocity and the cross-sectional area of the vessel measured radiographically. The present measurements of mean velocity in both nonpregnant and pregnant women are consistent with reports on nonpregnant individuals (Aaslid et al, 1982; Lindegaard et al, 1985; Maeda et al, 1990). The results were therefore primarily interpreted based on the mean velocities, although the other indices of systolic, diastolic velocities and PI have been included. The study could be criticised because although it is known that pCO2 affects cerebral blood flow, pCO2 was not measured by end-tidal expirometry. However, as angiotensin II was infused while the subjects were resting in the semirecumbent position, it was assumed that pCO2 would remain constant as has been already reported (Sorteberg et al, 1990).

Posture and eating did not induce significant changes in the mean velocities although the systolic velocity and PI declined after eating. Variation in the flow velocity has been reported when subjects move from lying to standing (Brunholzl et al, 1986). The lying and sitting positions were only investigated as these are the most practical for cerebral Doppler examination. The results show that such differences in the position of the subject are not critical for the Doppler measurement. A significant rise in the pulse rate was observed after eating, but when examined by multiple regression this did not account for the observed fall in systolic velocity and PI (results not included). The mean velocity rose significantly after smoking one cigarette, as has been observed by others. This effect has been attributed to cerebral vasoconstriction induced directly by nicotine (Cruickshank et al, 1989; Rundek et al, 1990). The magnitude of the change might have been greater if more cigarettes had been smoked as the effect is dose-dependent and in addition, is subject to individual tolerance in habitually heavy smokers. Therefore, it is considered that subjects should not smoke immediately before TCD examination; in the angiotensin study no women smoked for at least 8 hours prior to the infusion.

During the angiotensin II infusion, MCA flow velocities increased in association with

a rise in arterial pressure but the changes were only weakly correlated. This could have reflected real variation in individual response in the cerebral circulation to the increase in systemic blood pressure, or alternatively, angiotensin II may have contributed directly to vasoconstriction of the cerebral blood vessels. In experimental studies intravenously administered angiotensin II does not induce cerebral vasoconstriction (Kontos et al, 1978; Olesen et al, 1972). Nevertheless, a cerebral vascular response during the angiotensin II infusion could be detected by TCD. In various subanalyses it was attempted to separate the effects of changes in blood pressure from those of angiotensin II. The results were consistent with the conclusion that angiotensin II has a negligible direct effect on flow through the MCA. In addition, the same analyses were compatible with an indirect response of MCA flow waveforms to changes which might be ascribed to cerebral autoregulation.

Cerebral autoregulation occurs in the resistance vessels, predominantly the precapillary arterioles (Donaldson 1989). Blood flow is maintained constant by reflex vasoconstriction or vasodilation as perfusing pressure rises or falls. The finding of increased flow velocity in the MCA when the blood pressure rose during the angiotensin infusion is consistent with autoregulatory vasoconstriction. Others have observed a similar autoregulatory effect but in the opposite direction following peripheral infusion of the hypotensive, vasodilator agent nitroglycerine in nonpregnant subjects (Dahl et al, 1989). The possibility that the increased flow velocities reflect increases in cerebral blood flow cannot be excluded without simultaneously measuring the vessel diameter. However, when regional cerebral blood flow has been measured, vasoactive agents have had no effect despite changes in flow velocity (Dahl et al, 1989; Friberg et al, 1991). Therefore, it is considered that the increased MCA flow velocities observed reflect cerebral vasoconstriction.

Chapter IX

Conclusions

Screening Tests

For many years an effective and practical screening test for preeclampsia has been sought to enable rationalisation of antenatal care and to prevent sudden emergence of fulminating disease. Various tests had been promoted and later discredited, until in 1973, the Angiotensin Sensitivity Test (AST) was described as a highly effective screening test (Gant et al, 1973). The test demonstrated positive and negative predictive values of greater than 90%, although the limitations of using this highly impractical procedure at 28 weeks gestation were recognised. Subsequently, the AST has continued to receive credit as the "gold standard" screening test for preeclampsia, although its validity has been tested in only five small studies. None of these reproduced the screening efficiency of the original study, although the incidence of preeclampsia was much lower in the later studies.

In the present study, the AST was performed on 495 women, but it was not found to be an efficient screening test for preeclampsia. Some of the most severe cases of preeclampsia developed in women who demonstrated a negative response to the test, and overall the positive and negative predictive values for the test were low at 19% and 87% respectively. These results suggest that the screening abilities of the test are not transferable to other populations, or alternatively, the test itself is not reproducible. The latter point cannot be addressed from this study alone.

It has always been recognised that even if the AST was an efficient screening test for preeclampsia, it was too complicated for routine pregnancy use. Several more acceptable and practical tests have been evaluated in this study. These included **(1)** maternal haematocrit **(2)** fetal abdominal circumference **(3)** ambulatory blood pressure **(4)** platelet intracellular calcium response to arginine-vasopressin stimulation and **(5)** inactive urinary kallikrein/creatinine ratio; all of which showed a range of efficiency to predict later preeclampsia. Several had no value as a screening test **(1) (2) (4)**, but daytime ambulatory blood pressure (including heart rate) and the IUK/Cr ratio showed some efficacy; the positive predictive values were 45% and 30% respectively. Nevertheless,

these predictive values indicate that a high degree of overlap exists between the screening results of the two outcome groups (normal and preeclampsia), and therefore, a more effective screening test is still required.

The inability to find an efficient screening test for preeclampsia is not surprising since the cause and pathogenesis of the syndrome is undetermined. There is no test available to define those women who actually have the disease with 100% sensitivity and specificity. Furthermore, because the disease presents at a wide range of gestations, a predictive test performed at one stage of pregnancy is unlikely to be effective. Until the cause of preeclampsia is known, and a marker for the disease is identified, all women at risk will not be identified.

Low-Dose Aspirin

In this study the efficacy of low-dose aspirin as a preventive agent for preeclampsia was poor; a finding that is in direct contrast to that of a comparable study performed previously (Wallenburg et al, 1986). Although both of the studies were small and therefore exhibited limited power, the efficacy of low-dose aspirin to prevent preeclampsia when commenced at this late gestation must now be in doubt. If increased placentation is the beneficial effect attributed to low-dose aspirin, then the potential for this action when the drug is commenced as late as 28 weeks gestation must be limited. However, low-dose aspirin may also suppress the development and extension of "acute atherosis" by preventing excessive platelet deposition in the uteroplacental arteries. If this latter mechanism is real, low-dose aspirin may have more potential to prevent preeclampsia in the second half of pregnancy. Only an increased understanding of the pathogenesis of the disease combined with the results of subsequent clinical trials will answer these questions.

Furthermore, the efficacy of low-dose aspirin to prevent preeclampsia has not been confirmed irrefutably. Although the initial studies and their meta-analysis (conducted in organised trials involving high-risk populations) showed positive results, two recent studies (in moderate to low-risk populations) have not (Italian Study of Aspirin in

Pregnancy, 1993; Sibai et al, 1993). Low-dose aspirin may only be effective when used selectively and commenced within a certain gestation. It is hoped that these questions will be answered at publication of the CLASP trial results.

From the present study, it was not possible to comment upon the potential risks associated with aspirin use in pregnancy. Only one of the previously published studies have reported an obvious side-effect (Sibai et al, 1993) and therefore only a trial involving large numbers and paediatric follow-up will be able to confirm or refute this, in addition to, estimating the risks of those side-effects that are rare. Again, the CLASP trial will address this issue.

Transcranial Doppler Ultrasound

Just as there is a need to predict the onset of preeclampsia with a screening test, there is also a need to predict the onset of cerebrovascular complications in women with established preeclampsia. Most cases of maternal mortality associated with hypertension in pregnancy involve eclampsia.

A unique experimental situation of a series of pregnant women with a controlled blood pressure rise was provided by performing this large number of angiotensin II infusions. This, together with the availability of a new method for studying the hemodynamics of the intracerebral circulation noninvasively, enabled the evaluation of the maternal middle cerebral artery (MCA) by transcranial Doppler ultrasound during changes in blood pressure to be commenced.

The conditions which affected the readings were assessed in addition to the effect of the blood pressure rise. Changes in the MCA flow velocities were observed with the rise and fall of the blood pressure. These were considered to reflect an autoregulatory feature (vasoconstriction or vasodilation) to protect cerebral blood flow. This preliminary investigation, now provides the basis for ongoing studies to evaluate the maternal cerebral circulation in relation to eclampsia.

Conclusion

The ideal management of preeclampsia is to identify all pregnant women at risk so that preventive therapy can be introduced early. This thesis has shown that although our understanding of the pathogenesis of the syndrome has increased considerably over the past decade, we are still far from that goal. The results of the CLASP trial, when released, will increase our understanding of the efficacy of low-dose aspirin to prevent preeclampsia, but still we are unable to screen for preeclampsia and its complications adequately. A measurable marker for preeclampsia needs to be identified, that not only will define the disease accurately, but will also enable early identification of the disease process. Until that time, the study and investigation of preeclampsia will continue.

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