Inflammatory modifications in human pregnancy

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Faculty of Pharmacy
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Modificações inflamatórias na gravidez humana

Dissertation submitted to the Faculty of Pharmacy, University of Porto, for the degree of Doctor of Philosophy

Dissertação de candidatura ao grau de Doutor apresentada à Faculdade de Farmácia da Universidade do Porto

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Esta dissertação é dedicada,
com todo o meu amor,
aos meus PAIS,
à minha irmã SARA
e à minha fonte de encanto,
MÁRCIA,
por
constante incentivo,
inesgotável paciência e
carinho.

Luís Belo

Declaração

Ao abrigo do artigo 8º do Decreto-Lei nº 388/70, declara-se que fazem parte integrante desta dissertação os seguintes trabalhos já publicados ou em publicação:

Artigos em revistas de circulação internacional com arbitragem científica

I. Belo L, Caslake M, Gaffney D, Santos-Silva A, Pereira-Leite L, Quintanilha A, and Rebelo I. (2002). "Changes in LDL size and HDL concentration in normal and preeclamptic pregnancies". *Atherosclerosis* 162: 425–432.

II. Belo L, Rebelo I, Castro EMB, Catarino C, Pereira-Leite L, Quintanilha A, and Santos-Silva A. (2002). "Band 3 as a marker of erythrocyte changes in pregnancy". *European Journal of Haematology* 69: 145–151.

III. Belo L, Caslake M, Santos-Silva A, Pereira-Leite L, Quintanilha A, and Rebelo I. (2002). "Lipoprotein(a): a longitudinal versus a cross-sectional study in normal pregnancy and its levels in preeclampsia". *Atherosclerosis* 165: 393–395.

IV. Belo L, Santos-Silva A, Rumley A, Lowe G, Pereira-Leite L, Quintanilha A, and Rebelo I. (2002). "Elevated tissue plasminogen activator as a potential marker of endothelial dysfunction in pre-eclampsia: correlation with proteinuria". *British Journal of Obstetrics & Gynaecology* 109: 1250–1255.

V. Belo L, Santos-Silva A, Caslake M, Cooney J, Pereira-Leite L, Quintanilha A, and Rebelo I. (2003). "Neutrophil activation and C-reactive protein concentration in preeclampsia". *Hypertension in Pregnancy* 22: 129–141.

VI. Belo L, Gaffney D, Caslake M, Santos-Silva A, Pereira-Leite L, Quintanilha A, and Rebelo I. "Apo E and CETP polymorphisms in normal and preeclamptic pregnancies". Aceite para publicação na revista *European Journal of Obstetrics & Gynecology and Reproductive Biology*, a 27 de Março de 2003.

Comunicações em actas de encontros científicos com arbitragem científica

I. Belo L, Santos-Silva A, Castro E, Caslake M, Pereira-Leite L, Quintanilha A, and Rebelo I. (2000). "Lipid modifications in normal pregnancy and preeclampsia". *Prenatal and Neonatal Medicine* 5 (2): 3.

II. Belo L, Santos-Silva A, Rumley A, Lowe GDO, Pereira-Leite L, Quintanilha A, and Rebelo I. (2000). "Hemostatic variables in normal pregnancy and preeclampsia". *Prenatal and Neonatal Medicine* 5 (2): 4.

III. Belo L, Rebelo I, Santos-Silva A, Castro E, Pereira L, Pereira-Leite L, and Quintanilha A. (2000). "Antioxidants and oxidative stress in normal and preeclamptic portuguese pregnant women". *Atherosclerosis* 151 (1): 308.

IV. Belo L, Santos-Silva A, Pereira-Leite L, Quintanilha A, and Rebelo I. (2001). "Leukocyte activation in the pathophysiology of preeclampsia". *European Journal of Biochemistry* 268 (Supplement 1): 114.

V. Belo L, Santos-Silva A, Caslake MJ, Castro E, Pereira L, Pereira-Leite L, Quintanilha A, and Rebelo I. (2001). "LDL peak particle diameter: a longitudinal study in normal portuguese pregnant women". *Atherosclerosis* 2 (2): 76.

VI. Belo L, Santos-Silva A, Pereira-Leite L, Cooney J, Caslake MJ, Quintanilha A, and Rebelo I. (2001). "C-Reactive protein and its potential involvement in the pathophysiology of preeclampsia". *Journal of Perinatal Medicine* 29 (1): 494.

VII. Belo L, Santos-Silva A, Pereira-Leite L, Rumley A, Lowe G, Quintanilha A, and Rebelo I. (2002). "Lipoprotein(a), endothelial cell function and hemostasis in preeclampsia". *Hypertension in Pregnancy* 21 (Supplement 1): 157.

VIII. Belo L, Santos-Silva A, Rumley A, Lowe G, Pereira-Leite L, Quintanilha A, and Rebelo I. (2002). "Tissue plasminogen activator and platelet count in the clinical evaluation of preeclampsia". *Journal of Hypertension* 20 (Supplement 4): S121.

Em cumprimento do disposto no referido Decreto-Lei, o autor declara que participou activamente na recolha e estudo do material incluído em todos os trabalhos, tendo redigido os textos com a activa colaboração dos outros autores.

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Acknowledgements/Agradecimentos

The present work was carried out at the Department of Biochemistry of the Faculty of Pharmacy and at the Department of Obstetrics and Gynaecology of the Porto Medical School, University of Porto, and in some extent at the Clinical Analysis Laboratory of the Faculty of Pharmacy, at the Institute for Molecular and Cell Biology (IBMC), University of Porto, and at the Department of Pathological Biochemistry and the Department of Medicine of the Glasgow Royal Infirmary (GRI), University of Glasgow.

I would like to express my most deep gratitude to a number of people for their contribution to my work:

- Ao Professor Francisco Carvalho Guerra, pela oportunidade de integração no Laboratório de Bioquímica como bolseiro de doutoramento da Fundação para a Ciência e a Tecnologia (FCT).
- Ao Professor Fernando Sena Esteves, por todo o apoio, considerando-me um privilegiado por ter tido a oportunidade de trabalhar de uma forma tão directa com o Professor.
- Ao Professor Alexandre Quintanilha, pela orientação e apoio científico e por ter disponibilizado no IBMC alguns meios necessários à concretização deste projecto. Marcar-me-ão para a vida, o espírito crítico com que sempre analisou os resultados laboratoriais obtidos e a disponibilidade que sempre demonstrou.
- À Professora Irene Rebelo, pela orientação dada e a amizade demonstrada, bem como o entusiasmo que me transmitiu pela investigação na área da Obstetrícia. Guardarei para sempre os seus ensinamentos e a forma positiva de encarar a vida.
- À Professora Alice Santos Silva, pela orientação e ainda pelos ensinamentos, disponibilidade e apoio constantemente demonstrados. A sua calma, o seu optimismo e dedicação ao trabalho laboratorial muito me influenciaram.
- Ao Professor Luís Pereira Leite, pela coordenação do estudo clínico das grávidas estudadas. Ficam gravadas na minha memória, a forma experiente e sábia como me transmitiu conhecimentos e a calma que sempre revelou nas mais diversas situações que se depararam no decorrer deste trabalho.
- À Professora Elisabeth Castro, pelo seu apoio e por ter tornado possível a minha enriquecedora experiência no GRI.

- To Professor Christopher Packard, for receiving me at the Department of Pathological Biochemistry of the GRI, and for his valuable scientific advices.
- To Dr Muriel Caslake, for her precious contribution to my work in the field of lipids and inflammation. Her advices and friendship will be kept in my heart for all my life.
- To Dr Dairena Gaffney, for her support in the genetic part of this work, and also for her smile, dedication and friendship.
- To Professor Gordon Lowe and to Dr Ann Rumley, for their important collaboration in the haemostatic part of this work.
- À Dra. Laura Pereira e ao Professor Franklim Marques, pelo contributo na determinação e interpretação de parâmetros de bioquímica clínica.
- À Professora Denisa Mendonça, ao Professor Paulo Costa e ao Dr. Filipe Azevedo, pelos pareceres sobre estatística, e também pela disponibilidade e paciência demonstradas.
- Às Professoras Natércia Teixeira, Georgina Correia da Silva, Elsa Bronze da Rocha e Anabela Cordeiro, pelos ensinamentos e pelo constante incentivo.
- A todos os colegas, colaboradores e funcionários do Laboratório de Bioquímica, especialmente aos que de perto me acompanharam e que de forma directa ou indirecta, frequentemente anónima, contribuíram para tornar possível a presente dissertação. Não posso, todavia, deixar de fazer um agradecimento especial à Dra. Cristina Catarino e à Dra. Susana Rocha, pelo apoio no trabalho laboratorial e pelos bons momentos de convívio.
- To Dorothy Bedford, Grace Stewart, and Josephine Cooney, whom I worked with at the GRI, for the expert technical assistance and friendship.
- Aos meus pais, à Sara e à Márcia, pela compreensão e apoio ao longo destes anos de trabalho.

Agradeço ainda o importante apoio financeiro da FCT e do Fundo Social Europeu (FSE) no âmbito do III Quadro Comunitário de Apoio. De facto, a realização do trabalho apresentado nesta dissertação só foi possível mediante a atribuição de uma Bolsa de Investigação por intermédio do Programa PRAXIS XXI (referência da Bolsa: PRAXIS XXI / BD / 16061 / 98).

Abstract

Normal human pregnancy leads to an inflammatory response, involving several organs and systems, usually well tolerated by the maternal body. However, in complicated pregnancies, such as in pre-eclampsia (PE), a typical hypertensive disorder of human pregnancy, the mechanisms regulating physiologic inflammation may malfunction.

Despite intensive research in normal human pregnancy, some modifications in maternal blood are still controversial and/or unexplored. Moreover, there is also no agreement whether PE represents an exacerbated form of inflammation compared with normal pregnancy.

This work investigated, in a cross-sectional and longitudinal manner, systemic changes occurring with pregnancy and throughout the three trimesters of gestation, with a particular interest on biochemical variables that might be altered as a consequence of the inflammatory process. The same variables were also used to compare normal pregnant and pre-eclamptic women in the third trimester of gestation. In addition, genetic variants were investigated in order to assess their potential involvement in PE.

The laboratory analysis included the evaluation of acute phase proteins [C-reactive protein (CRP) and haptoglobin], liver function (aspartate aminotransferase and alanine aminotransferase), the traditional lipid and lipoprotein profile together with the quantification of LDL size (peak and mean particle diameters) and oxidised LDL, the total antioxidant status, and variables related to coagulation (fibrinogen and platelets) and to fibrinolysis [tissue type plasminogen activator (t-PA), plasminogen activator inhibitor type 1, and fibrin fragment D-dimer]. Leukocytes were also quantified (total and differential count) and neutrophil activation was assessed by evaluating the plasma levels of elastase and lactoferrin. The erythrocyte was used as an *in vivo* model of cumulative damage imposed by the inflammatory process. Its study included the determination of the erythrocyte band 3 profile, an indicator of oxidative and proteolytic stress. In the genetic study, two polymorphic regions were evaluated: one associated with the CRP gene and the other with the apolipopotein (apo) E gene.

Normal human pregnancy was associated with increments in acute phase proteins and in leukocytes but no evidence was found that neutrophils are activated during gestation. The inflammatory response in pregnancy was accompanied not only by an increase in younger erythrocytes, but also by an increase in damaged erythrocytes in the maternal circulation.

Human pregnancy was also associated with pronounced changes in the lipid and lipoprotein profile with an apparent "atherogenic-like" feature. Moreover, changes in lipids and lipoproteins occurred with concomitant coordinated changes in haemostasis, namely the development of a procoagulant state.

In PE, there was evidence of an enhanced systemic inflammatory response to pregnancy. Components of the inflammatory response in PE include: an increased concentration of some acute phase proteins; the existence of endothelial dysfunction and neutrophil activation; correlation of neutrophil activation with the severity of this disorder; and the existence of a lipid profile with enhanced atherogenic and/or pro-inflammatory potential. None of the candidate polymorphisms showed any association with PE. However, in pregnant women, apo E genotype was associated with changes in lipid and lipoprotein profiles similar to that seen in the general population.

In conclusion, this work describes an extensive number of changes occurring in the systemic maternal circulation during normal pregnancy and in PE. In addition, it also demonstrates an inflammatory response in normal pregnancy that seems to be enhanced in PE. As this work was undertaken in women with already established PE, it is uncertain whether this inflammation is a cause or a consequence of the pathologic process. However, there is evidence to suggest that triglycerides, t-PA, and elastase, may work as risk factors in PE.

Resumo

A gravidez humana normal desencadeia uma resposta inflamatória, que envolve vários órgãos e sistemas, e que é normalmente bem tolerada pelo organismo materno. Contudo, em gravidezes complicadas, tal como na pre-eclâmpsia (PE), uma perturbação hipertensiva característica da gravidez humana, os mecanismos reguladores da inflamação fisiológica poderão não funcionar correctamente.

Apesar de intensa investigação na área da gravidez humana, algumas das modificações que ocorrem no sangue materno são ainda controversas e/ou não foram exploradas. Além disso, não existe também conformidade sobre se a PE representa uma forma exacerbada da inflamação que ocorre na gravidez normal.

Este trabalho investigou, de uma forma transversal e longitudinal, alterações sistémicas que ocorrem com a gravidez e durante os três trimestres de gestação, com um interesse particular em variáveis bioquímicas que poderão estar alteradas como uma consequência do processo inflamatório. Essas mesmas variáveis foram também usadas para comparar mulheres grávidas normais e pre-eclâmpticas no terceiro trimestre de gestação. Mais ainda, variantes genéticas foram investigadas no sentido de avaliar o seu potencial envolvimento na PE.

O estudo laboratorial englobou a avaliação de proteínas de fase aguda [proteína C reactiva (CRP) e haptoglobina], da função hepática (aminotransférase do aspartato e aminotransférase da alanina), do perfil lipídico e lipoproteico tradicional, juntamente com a quantificação do tamanho das LDL (tamanho predominante e médio das partículas) e das LDL oxidadas, do estado antioxidante total, e de variáveis relacionadas com a coagulação (fibrinogénio e plaquetas) e com a fibrinólise [activador do plasminogénio tecidular (t-PA), inibidor do activador do plasminogénio tipo 1, e D-dímero]. Foram também quantificados os leucócitos (contagem total e diferencial) e a activação dos neutrófilos, sendo esta determinada pela avaliação dos níveis plasmáticos de elástase e lactoferrina. O eritrócito foi utilizado como um modelo *in vivo* de lesões cumulativas impostas pelo processo inflamatório. O seu estudo incluiu a determinação do perfil da banda 3 do eritrócito, um indicador de stresse oxidativo e proteolítico. No estudo genético, duas regiões polimórficas foram avaliadas: uma associada com o gene da CRP, e a outra com o gene da apolipoproteína (apo) E.

Na gravidez humana normal verificaram-se aumentos nas proteínas de fase aguda e nos leucócitos, mas não foi encontrada evidência de os neutrófilos estarem activados durante

a gestação. A resposta inflamatória da gravidez acompanhou-se não só por um aumento de eritrócitos mais novos na circulação materna, mas também por um aumento de eritrócitos lesados. Na gravidez humana, verificaram-se ainda alterações profundas no perfil lipídico e lipoproteico, com desenvolvimento de um quadro "tipo-aterogénico". Mais ainda, as alterações nos lípidos e lipoproteínas ocorreram paralela e coordenadamente com alterações na hemostase, nomeadamente com o desenvolvimento de um estado pro-coagulante.

Na PE, encontrou-se evidência de uma resposta inflamatória sistémica mais marcada à gravidez. Componentes da resposta inflamatória na PE incluem: um aumento na concentração de algumas proteínas de fase aguda; a existência de disfunção endotelial e de activação do neutrófilo; correlação da activação do neutrófilo com a gravidade da PE; e a existência de um perfil lipídico com um potencial aterogénico e/ou pro-inflamatório aumentado. Nenhum dos polimorfismos estudados apresentou alguma associação com a PE. Contudo, nas mulheres grávidas, o genótipo da apo E mostrou-se associado a alterações no perfil lipídico e lipoproteico semelhantes às observadas na população em geral.

Em conclusão, este trabalho descreve um extenso número de alterações que ocorrem na circulação materna sistémica durante a gravidez normal e na PE. Este trabalho evidencia também uma resposta inflamatória na gravidez normal, que parece estar aumentada na PE. Como este trabalho foi realizado em mulheres com PE já diagnosticada, não é possível esclarecer se esta inflamação é uma causa ou uma consequência do processo patológico. Contudo, os resultados obtidos sugerem que os triglicéridos, o t-PA e a elástase deverão ser considerados como potenciais factores de risco da PE.

Résumé

La grossesse chez la femme s'accompagne normalement d'une réponse inflammatoire, impliquant plusieurs organes et systèmes et habituellement bien tolérée par l'organisme maternel. Cependant, lors des grossesses compliquées comme la prééclampsie (PE), une atteinte spécifique de la grossesse chez la femme caractérisée par une hypertension artérielle, les mécanismes de régulation de l'inflammation physiologique peuvent être défaillants.

Malgré de nombreux travaux sur la grossesse chez la femme, certaines modifications dans le sang maternel sont encore controversées et/ou non explorées. De plus, l'idée selon laquelle la PE représente une forme exacerbée de l'inflammation normalement rencontrée au cours de la grossesse ne fait pas l'unanimité.

Au cours de ce travail, nous avons recherché par une étude cas-témoin et longitudinale, des modifications systémiques survenant avec la grossesse et tout au long du troisième trimestre de gestation, avec un intérêt particulier pour les paramètres biochimiques susceptibles d'être modifiés par un processus inflammatoire. Les mêmes paramètres ont également été utilisés pour comparer les femmes avec une grossesse normale et les femmes avec PE lors du troisième trimestre de grossesse. De plus, des polymorphismes génétiques ont été recherchés de manière à établir leur rôle éventuel dans la PE.

Nous avons analysés les paramètres biologiques suivants : protéine de la phase aiguë de l'inflammation [Protéine C Réactive (CRP), haptoglobine], fonction hépatique (aspartate aminotransférase et alanine aminotransférase), bilan lipidique classique, taille des LDL (pic et taille moyenne), LDL oxydées, pouvoir antioxydant total, paramètres de la coagulation (fibrinogène et plaquettes) et de la fibrinolyse [activateur tissulaire du plasminogène (t-PA), inibiteur de l'activateur du plasminogène de type 1 et D-dimères]. Nous avons également réalisé une numération formule sanguine et l'activation des polynucléaires neutrophiles a été évaluée par la concentration plasmatique d'élastase et de lactoferrine. L'ensemble des dommages en relation avec l'inflammation ont été déterminés sur les érythrocytes. Nous avons déterminé le profil de la bande 3 des érythrocytes, un indicateur du stress oxydatif et de la protéolyse. En ce qui concerne l'étude génétique, deux régions de polymorphisme ont été étudiés : l'un associé avec le gène de la CRP, l'autre avec le gène de l'apo E.

La grossesse normale chez la femme s'accompagne d'une augmentation des protéines de la phase aiguë de l'inflammation ainsi que des leucocytes, mais nous n'avons pas retrouvé d'activation des polynucléaires neutrophiles. La réponse inflammatoire au cours de la

grossesse est associée à une augmentation dans la circulation maternelle, des jeunes érythrocytes mais aussi des érythrocytes altérés. La grossesse chez la femme est par ailleurs associée à d'importantes modifications des lipides et des lipoprotéines, avec un profil similaire à un profil athérogène. Parallèlement aux modifications des lipides et des lipoprotéines, surviennent des modifications de l'hémostase, avec notamment l'apparition d'un état procoagulant.

Au cours de la PE, la réponse inflammatoire à la grossesse est accrue. Les composants de la réponse inflammatoire de la PE comprennent une augmentation de la concentration de certaines protéines de l'inflammation, une dysfonction endothéliale, une activation des polynucléaires neutrophiles corrélée avec la gravité du désordre, un profil lipidique plus athérogène et un potentiel proinfammatoire plus important. Aucun des polymorphismes recherché n'était associé à la PE. Cependant, chez la femme enceinte, le génotype de l'apo E était associé avec les mêmes modifications des lipides et des lipoprotéines que celles observées dans la population générale.

En conclusion, ce travail décrit de multiples modifications survenant dans la circulation sanguine maternelle au cours de la grossesse normale et de la PE. De plus, il met en évidence une réponse inflammatoire au cours de la grossesse normale, réponse qui semble accrue lors de la PE. Dans la mesure où nous avons travaillé sur des femmes avec une PE déjà installée, nous ne pouvons affirmer si l'inflammation est une cause ou une conséquence de ce processus. Cependant, les triglycérides, le t-PA et l'élastase apparaissent comme des facteurs de risque probables de la PE.

PART I

INTRODUCTION

Section One: Inflammation

Inflammation is the body's response to a variety of noxious stimuli such as tissue injury caused by infection or physical damage. It is a complex process that involves the participation of several cells and molecules and may present different intensities and durations.

Inflammation is normally a localised, protective response that destroys, dilutes, or walls-off the injurious agent and the injured tissue. In the acute form, it is characterised by the classic signs of pain (*dolor*), heat (*calor*), redness (*rubor*), swelling (*tumor*), and loss of function (*functio laesa*). Microscopically, the inflammatory response involves a series of events, which includes dilation of the microvasculature, with increased blood flow to the site of inflammation; permeabilisation of capillaries with exudation of fluids; migration and accumulation of leukocytes into the inflammatory focus. On completion of the repair process, the inflammatory mechanisms are normally turned down to a low level. However, inflammation disturbances, including excessive or prolonged inflammation, can compromise the body and may lead to inflammatory diseases such as atherosclerosis, Alzheimer's disease or cancer. Therefore, inflammation may play an important role in both health and disease.

Given the complexity of the inflammatory process, this section will address just some of the components of inflammation and will express some considerations on atherosclerosis, as it is the major cause of mortality and morbidity in the western world and also presents some similarities with pre-eclampsia (PE), a characteristic multisystem disorder of human pregnancy.

1. The Acute Phase Proteins

Inflammation usually refers to a localised process; however, if the noxious stimulus is severe enough, distant systemic changes may also occur. These changes are referred to collectively as the acute phase response (APR). The APR is not limited to acute inflammatory states but is also found in chronic inflammation and includes a large number of changes (Table 1–1). Despite the fact that many of these changes commonly occur together, not all occur in all situations, suggesting that they must be individually regulated. Acute phase phenomena are primarily induced by inflammation-associated cytokines, including tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and interferon-γ (IFN-γ), which may be produced by many

different cells, including macrophages, fibroblasts and endothelial cells (Kushner and Rzewnicki, 1999). The cytokine effects are influenced by a variety of circulating molecules, namely cytokine modulators and some endocrine hormones (Kushner and Rzewnicki, 1999).

Table 1–1. Acute Phase Phenomena

Neuroendocrine changes	Fever, poor appetite and somnolence Increased secretion of adrenocorticotropic hormone (ACTH) Increased secretion of cortisol and catecholamines
Metabolic changes	Increased protein catabolism Hepatic production of acute phase proteins (APPs) Increased hepatic lipogenesis Increased adipose tissue lipolysis Decrease in bone mass Increased gluconeogenesis
Hematopoietic changes	Anaemia (in chronic disease) Leukocytosis Thrombocytosis
Changes in non-protein plasma constituents	Hypozincaemia, hypoferraemia, and hypercupraemia Increased plasma retinol and glutathione concentrations

(Adapted from Ceciliani *et al.*, 2002)

The influence on concentration of a group of plasma proteins, known as the acute phase proteins (APPs), is one of the most interesting features of the APR. Although these proteins are electrophoretically heterogeneous and have diverse functions, their levels increase (positive APP) or decrease (negative APP) in association with the inflammatory stimulus. Their changes may vary from 25 percent to more than 1,000 fold, as in the case of C-reactive protein (CRP) and serum amyloid A (SAA), the two major human APPs (Kushner and Rzewnicki, 1999). Table 1–2 describes some of the positive APPs. Negative human APPs include: albumin, transferrin, transthyretin, α_2-HS glycoprotein, α-fetoprotein, T4-binding globulin, and insulin-like growth factor-I (IGF-I) (Ceciliani *et al.*, 2002). The liver is not the only organ capable of producing APPs: some can also be produced at extrahepatic sites.

Table 1–2. Human positive acute phase proteins

Major APR	C-reactive protein (CRP), serum amyloid A (SAA)
Complement system	C3, C4, C9, factor B, C1 inhibitor, C4b-binding protein, mannose-binding protein
Coagulation and fibrinolytic system	Factor VIII, fibrinogen, fibronectin, plasminogen, plasminogen activator inhibitor (PAI)-1, prothrombin, von Willebrand factor (vWf), tissue plasminogen activator (t-PA)
Transport/Scavenger proteins	Caeruloplasmin, haptoglobin, haemopexin, lipopolysaccharide (LPS)-binding protein, ferritin
Participants in inflammatory response	Secreted phospholipase A2, granulocyte-macrophage colony-stimulating factor (GM-CSF), kallikreins
Protease inhibitors	α1-protease inhibitor, α1-antichymotrypsin, pancreatic secretory trypsin inhibitor, inter-α-trypsin inhibitors

(Adapted from Ceciliani *et al.*, 2002)

The increase in APPs modulates the inflammatory response, but if present for prolonged periods it can lead to detrimental consequences in the host. Moreover, while the changes in some APPs present evident beneficial effects, for others the biological advantage is less clear. In the coagulation and fibrinolytic systems, the overall improvement of defence and restoration of tissue integrity is evident. The up-regulation of several APPs can satisfy the requirement for wound healing. Moreover, the whole coagulatory and wound-healing system is overexpressed to avoid the unbalancing of the repair process (Ceciliani *et al.*, 2002). The increase in plasma proteins involved in coagulation and fibrinolytic systems is one of the interesting links between inflammation and haemostasis. Moreover, the interaction between inflammation and coagulation involves interesting cross talk, in which the endothelium seems to play a major role (Cicala and Cirino, 1998; Esmon *et al.*, 1999; Esmon, 2000).

Haptoglobin is one of the APPs that may play a multifunctional role. This APP is capable of binding haemoglobin, thus preventing iron loss and renal damage; haptoglobin also acts as an antioxidant (Halliwell and Gutteridge, 1990), has antibacterial activity and is considered an angiogenic factor (Kushner and Rzewnicki, 1999; Raijmakers *et al.*, 2003). Changes in the measured concentrations of haptoglobin in serum may help to assess the disease status of patients with inflammations (increases) as well as in haemolytic conditions (decreases).

Another interesting feature of some APPs is that they can play a dual role in inflammation and therefore, their precise effect is somewhat unclear. For instance, caeruloplasmin is an APP that has long been recognised as an antioxidant, by its ferroxidase activity and its copper-binding property (Halliwell and Gutteridge, 1990). However, recent studies have shown that carefully isolated caeruloplasmin is prooxidant and can oxidise LDL (Khovidhunkit *et al.*, 2000). Thus, whether caeruloplasmin is prooxidant or antioxidant *in vivo* is uncertain. CRP is another interesting APP since it may play both pro-inflammatory and anti-inflammatory roles (Kushner and Rzewnicki, 1999). CRP is one of the APPs that has gained considerable attention in clinical research. The CRP response has no clinical specificity and CRP has been used as a traditional acute phase marker of systemic inflammation. CRP is also used to monitor disease activity and response to treatment in patients with a variety of inflammatory diseases (Pepys and Berger, 2001). The development of high sensitivity assays has recently allowed clinicians to better explore the role of CRP in inflammatory diseases. Subtle differences in CRP levels, even within the reported normal range, can be quantified accurately and used to detect inflammation before clinical consequences become evident (Pepys and Berger, 2001).

In conclusion, one of the most explored features in APR is the change in APPs level. Newly recognised APPs are constantly being reported and, moreover, a lot of their protective and/or deleterious effects remain to be established or clarified.

2. Inflammation-Induced Changes in Lipids, LDL and HDL

Lipids and lipoproteins are strongly associated with inflammation, not only because changes in their concentrations and/or compositions are observed in inflammatory processes but also because some can mediate inflammatory responses.

During the APR, lipid metabolism is markedly altered. Hepatic production of triglycerides (TG) and very low-density lipoprotein (VLDL) secretion are increased (resulting from increased adipocyte lipolysis, *de novo* fatty acid synthesis, and decreased fatty acid oxidation in the liver) but do not necessarily result in high concentration of plasma TG (Carpentier and Scruel, 2002). Plasma TG level may increase, remain unchanged, or decrease in different types of acute conditions, depending on the efficacy of the mechanisms removing VLDL-TG (TG catabolism and tissue uptake of remnant particles). In contrast, acute conditions lower plasma cholesterol (Chol) by decreasing its content in both low and high-density lipoproteins (LDL and HDL). Chol lowering does not result from a decrease in Chol

production (which in fact increases) but from an increased catabolism of Chol-rich lipoproteins, their margination outside the plasma compartment, or both (Carpentier and Scruel, 2002).

Furthermore, major changes in the composition of lipoproteins (namely in LDL and HDL) occur, which may increase their atherogenic and inflammatory properties (Khovidhunkit *et al.*, 2000; Navab *et al.*, 2001; Carpentier and Scruel, 2002).

LDL

In normal conditions, LDL particles transport Chol, phospolipids, and lipid-soluble vitamins from the liver to extra-hepatic tissues. Under certain conditions, some of the phospholipids contained in LDL can yield oxidised phospholipids that induce an inflammatory response (Navab *et al.*, 2001). Platelet-activating factor (PAF) is a pro-inflammatory phospholipid and oxidised LDL (Ox-LDL) contains oxidised phospholipids with PAF-like bioactivity (Marathe *et al.*, 2001).

The oxidation of LDL depends on several factors. Polyunsaturated fatty acids (PUFAs) in LDL are the primary target of oxidising compounds and therefore the LDL content in PUFAs is a major determinant of oxidative susceptibility (Witztum, 1993). These PUFAs are protected by antioxidants and thus the oxidation of LDL will only occur after consumption of its endogenous antioxidants. The primarily protective component is likely to be vitamin E, as it is the most abundant antioxidant in LDL, with minor contributions given by other antioxidants present in smaller amounts, such as β-carotene, γ-tocopherol, ubiquinol, and others (Witztum, 1993). The distribution of the PUFAs within LDL, or the content of free Chol or TG may also play a role. Finally, different LDL particles may exhibit differences as to the content of intrinsic phospholipase A2 activity, which is necessary for oxidative modification to occur (Witztum, 1993).

LDL itself is not a homogeneous entity; it contains discrete particles varying in size, density, composition and function. Although some workers have divided LDL into multiple fractions, it is clear that by density gradient centrifugation, three distinct fractions are usually seen in the range of 1.019–1.063 g/ml (LDL-I, *d* 1.025–1.034 g/ml, LDL-II, *d* 1.034–1.044 g/ml and LDL-III, *d* 1.044–1.060 g/ml) (Packard and Shepherd, 1997). Larger and more buoyant LDL particles (LDL I and II) are distinct from small, dense LDL (LDL III). Smaller, denser LDL particles are more susceptible to *in vitro* oxidation than the larger and more buoyant subfractions (Graaf *et al.*, 1991). It seems that the most dense LDL subfraction is

relatively enriched in PUFAs and depleted in vitamin E. The presence of small, dense LDL particles is determined by genetic as well as environmental factors, such as elevated plasma TG. The most widely accepted mechanism of LDL subclass formation and modulation is that of neutral lipid exchange (Packard and Shepherd, 1997) and a metabolic model for the formation of small, dense LDL is described in Figure 2–1. Thus, increases in plasma TG concentration generally result in the formation of smaller LDL particles.

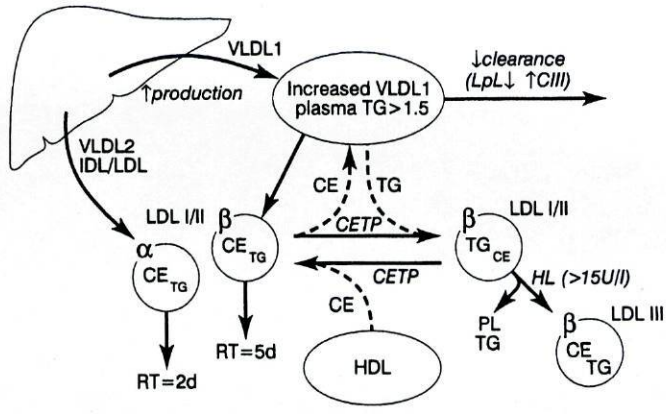


Figure 2–1. Model integrating LDL structural and metabolic heterogeneity. Kinetic evidence suggests that the two metabolically distinct pools in LDL (α and β) arise from different sources. Pool α is the major species detected by multicompartmental modelling of subjects with low normal plasma triglyceride (TG) levels. It is postulated to arise when apo B is secreted into the S_f 0–60 density range; LDL with the kinetic properties of pool β has been shown to be the product of $VLDL_1$ delipidation. The two LDL species have substantially differing residence times (RT) in the circulation. LDL-III generation is favoured when plasma TG exceeds 133 mg/dl (1.5 mmol/l) and hepatic lipase (HL) is in the male range (i.e. >15 u/l). $VLDL_1$ is the principal species that accumulates as plasma TG levels rise in the population as a result of overproduction of the lipoprotein or its defective removal (LpL or elevated apo CIII levels). It is envisaged that above the 133 mg/dl (1.5 mmol/l) threshold enough $VLDL_1$ is present to both produce long-lived pool β LDL and to cause TG enrichment of the particle to a level that makes LDL-III formation possible. The action of HL removes lipid from the LDL-II to form LDL-III. (From Packard and Shepherd, 1997)

As referred, some inflammatory conditions can be associated with an increase in TG levels and patients with AIDS frequently present a high proportion of small, dense LDL particles (subclass pattern B) (Khovidhunkit *et al.*, 2000). Moreover, inflammation is associated with increased levels of caeruloplasmin, secretory phospholipase A2 (sPLA2) and PAF-acetylhydrolase, all of which can be involved in LDL oxidation (Khovidhunkit *et al.*, 2000). Consistent with these findings, is infection and inflammation in animals inducing LDL oxidation (Memon *et al.*, 2000).

HDL

In normal conditions, high-density lipoprotein (HDL) particles play a major role in the reverse transport of Chol from peripheral tissues (including foam cells in the artery wall) to the liver. In addition, HDL may also have other important functions, namely anti-inflammatory functions. HDL can prevent the formation of or inactivate the inflammatory LDL-derived

oxidised phospholipids and this is attributed to HDL associated enzymes [such as paraoxonase and lecithin:cholesterol acyltransferase (LCAT)] and apolipoproteins [namely apolipoprotein (apo) A-I, the major protein component of HDL] (Mertens and Holvoet, 2001). Moreover, recent studies have shown that HDL also plays an anti-inflammatory role by interacting with leukocytes and by inhibiting endothelial cell activation. HDL-associated apo A-I inhibits contacted-mediated activation of monocytes by T lymphocytes, thus inhibiting TNF-α and IL-1β production (Hyka *et al.*, 2001). HDL also inhibits endothelial cell expression of cytokine-induced leukocyte adhesion molecules (Cockerill *et al.*, 2001; Nofer *et al.*, 2002).

During acute inflammation, HDL particles are altered in composition and become pro-inflammatory (Navab *et al.*, 2001; Van Lenten *et al.*, 2001). Major modifications in HDL protein pattern are an abrupt rise in SAA, apo J, sPLA2, and caeruloplasmin, together with a decrease in apo A-I, paraoxonase, LCAT, and phospholipid transfer protein (Navab *et al.*, 2001; Van Lenten *et al.*, 2001; Carpentier and Scruel, 2002). "Acute-phase" HDL particles seem to loose their protective effect against the formation of inflammatory LDL oxidised phospholipids (Carpentier and Scruel, 2002). Thus, HDL has a "chameleon-like" nature, being anti-inflammatory in the basal state and pro-inflammatory during an APR (Navab *et al.*, 2001; Van Lenten *et al.*, 2001).

3. Neutrophil Activation and Endothelial Cell Injury

Leukocytosis and recruitment of circulating leukocytes into the affected areas are hallmarks of inflammation. Leukocytes are attracted to inflammed regions by leukotactic chemicals and their migration from blood to the injured tissue is initially mediated by the expression of cell-adhesion molecules in the endothelium, which interact with surface receptors on leukocytes (Clark *et al.*, 1998; Muller, 1999; Sullivan *et al.*, 2000). When the leukocyte adhesion cascade is triggered, a sequence of adhesion and activation events results in extravasation of the leukocyte, whereby the cell exerts its effect on the inflammed site (Clark *et al.*, 1998; Muller, 1999; Sullivan *et al.*, 2000). In acute inflammation, the leukocyte infiltration is predominantly of neutrophils, whereas in chronic inflammation a mononuclear cell infiltration (predominantly macrophages and lymphocytes) is observed. Although leukocyte-endothelial cell interactions (mediated by various cell adhesion molecules) are important for leukocyte extravasation and trafficking, there is increasing evidence that altered leukocyte-endothelial

interactions are implicated in the pathogenesis of diseases associated with inflammation, possibly by damaging the endothelium or altering endothelial function (Harlan, 1985).

Leukocytosis is essential in the primary host defence against infections, and neutrophils, the major population of blood leukocytes, have a primordial role in this feature. It is well known that neutrophils possess mechanisms that are used to destroy invading microorganisms. Neutrophils recruited to the site of injury undergo a respiratory burst, i.e. they consume large quantities of oxygen, which is transformed almost quantitatively into the superoxide anion radical (Southorn and Powis, 1988). This radical is subsequently converted into hydrogen peroxide, hydroxil radical, and possibly singlet oxygen. Activated neutrophils also release proteases (namely elastase) with microbicidal activity. Lactoferrin is released from activated neutrophils and there is evidence that it may participate in granulocyte aggregation, adhesion to endothelial cells (Harlan, 1985) and antioxidant defences (Halliwell and Gutteridge, 1990). Activated neutrophils are therefore important sources of reactive oxygen species and proteases that exert antimicrobial activity. However, the release of these substances into the environment may impose oxidative and proteolytic damage on neighbouring cells, such as endothelial cells (Harlan, 1985) and circulating erythrocytes (Santos-Silva *et al.*, 1998). Thus, activated neutrophils may exhibit both protective and harmful effects, which express the utility of assessing neutrophil function.

4. The Erythrocyte Band 3 Profile

The red blood cell (RBC), an anucleated cell, presents a very limited biosynthesis capacity and poor repair mechanisms. Thus, whenever exposed to physical and/or chemical stress, RBC suffers and accumulates physical and/or molecular damage. The ageing of the cell is associated with a decrease in the activity of several enzymes [such as glucose-6-phosphate dehydrogenase (Imanishi *et al.*, 1986; Santos-Silva *et al.*, 1998) and glutathione peroxidase (GPX) (Imanishi *et al.*, 1986)] and with modifications in membrane proteins (Brovelli *et al.*, 1991), which ultimately may lead to its destruction.

Band 3, a 911 amino acid glycoprotein, is the most abundant protein in the erythrocyte membrane (Wang, 1994). Band 3 has diverse biological functions: it serves as an anion exchanger, playing a crucial role in the CO_2 transport system and also serves as a protein anchor, connecting components of the cytoskeleton to the membrane (spectrin binds to band 3 via ankyrin, band 4.1 and band 4.2; Figure 4–1) and providing bindings sites for haemoglobin and glycolitic enzymes. In addition to these functions, band 3 also acts as a senescense

neoantigen for aged and damaged RBCs (Wang, 1994). Modifications in the erythrocyte membrane band 3 protein, by proteolytic cleavage, clustering or exposure of unusual epitopes, trigger the binding of specific anti-band 3 autoantibodies and complement activation, marking the cell for death (Kay *et al.*, 1983; Kay, 1984; Lutz *et al.*, 1987; Kay *et al.*, 1991).

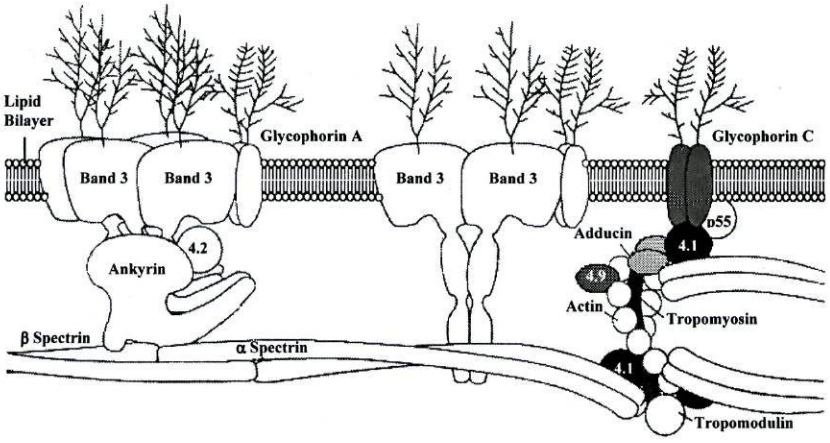


Figure 4–1. Schematic model of the red blood cell membrane structure. The membrane consists of a two molecule thick lipid sheath (rich in phospholipids and cholesterol) fixed to a protein network. Cytoplasmic domains of integral membrane proteins interact with cytoskeletal proteins. (Adapted from Lux and Palek, 1995)

Naturally occurring anti-band 3 antibodies, involved in RBC clearance, present low affinity for band 3 monomer, preferring the binding to band 3 protein oligomers (Lutz, 1992). This enables anti-band 3 antibodies to differentiate mature RBCs from senescent or damaged red cells. In addition, it seems that anti-band 3 antibodies gain functionality by a firm bivalent binding to oligomerised band 3 protein (Lutz, 1992). Thus, the increase of high molecular weight aggregates (HMWAg) in old and/or damaged RBCs may play a role in their removal.

Older and/or damaged RBCs are also associated with an increase in membrane bound haemoglobin (MBH), indicative of the increased amount of denatured haemoglobin (Santos-Silva *et al.*, 1998). As haemoglobin begins to denature, it binds to the cytoplasmic pole of band 3, promoting the aggregation of band 3 and an increased binding of IgG, which may ultimately trigger RBC removal (Low *et al.*, 1985).

In a previous study (Santos-Silva *et al.*, 1995), an abnormal band 3 profile and a suggestive involvement of neutrophils in RBC damage were found in patients at risk for cardiovascular disease (CVD) events and in patients who have suffered a myocardial infarction within the last 48 hours. Subsequently, it was found that ischaemic stroke patients present a similar abnormal band 3 profile and higher levels of leukocyte activation products, strengthening the previous hypothesis (Santos-Silva *et al.*, 2002). Moreover, the *in vitro* study

of changes in band 3 profile, imposed by activated neutrophils or by neutrophilic elastase, resembled those reported for CVD and ischaemic stroke, as well as those changes found in older erythrocytes (Figure 4–2) (Santos-Silva *et al.*, 1998). Older and damaged RBCs present higher HMWAg and MBH and lower fragmentation.

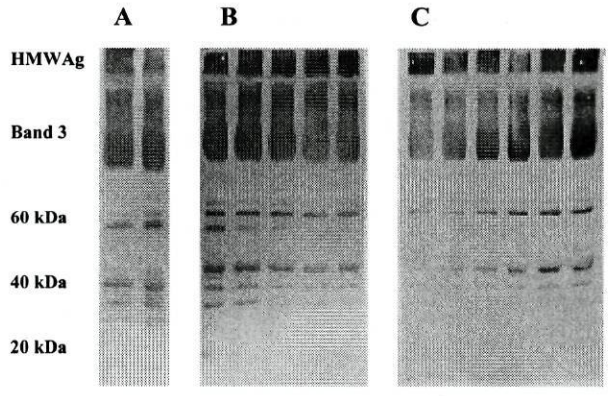


Figure 4–2. Band 3 immunoblots. Monoclonal anti-human band 3 recognises erythrocyte band 3, a 90–100 kDa protein, its aggregates (HMWAg) and proteolytic fragments. A: Dense and less dense RBCs (dense on the left and less dense on the right). B: RBCs incubated with activated neutrophils (control without and with phorbolmyristate acetate, 500/1, 300/1, and 200/1 RBC to granulocyte ratio, from left to right). C: RBCs incubated with elastase (control, 0.05, 0.1, 0.25, 0.3, 1.0 $\mu g/\mu l$, from right to left). (From Santos-Silva *et al.*, 1998)

Therefore, RBC seems to be a useful model of oxidative and proteolytic stress associated with inflammatory conditions.

5. Atherosclerosis –An Inflammatory Disease

Atherosclerosis is a slow progressive disease that begins early in life but usually presents its clinical manifestation in middle age or later. It is a multifactorial disease, involving the interplay of genetic and environmental factors, and is the leading cause of mortality and morbidity in the western world (Singh *et al.*, 2002). Atherosclerosis affects large arteries, being the primary cause of heart disease and stroke (Lusis, 2000).

Atherosclerotic Lesions

The earliest morphologically recognised lesion in atherogenesis is the fatty streak, characterised by an accumulation of cells (mainly macrophages) loaded with lipid (foam cells) just beneath the endothelium (Woolf, 1999). The fatty streak may then progress to more advanced, complex lesions, leading to the formation of fibrolipid or atheromatous plaque, which consists of a central lipid-rich atheromatous core and an overlying dense fibrocellular layer. This layer consists primarily of smooth muscle cells (SMCs) that migrate from the media into the intima where they proliferate and elaborate connective tissue matrix, rich in

collagen, elastin and proteoglycans (Woolf, 1999). Plaques with a massive basal, lipid-rich pool, and a thin sheet of connective tissue are prone to undergoing intimal injury, which is often followed by some degree of thrombosis.

Risk Factors

Numerous risk factors are associated with atherosclerosis. Some of these have an important genetic component while others are largely environmental (Table 5–1) (Lusis, 2000). Among these factors, some special attention will be devoted to systemic inflammation as well as dyslipidaemia.

Table 5–1. Genetic and environmental factors associated with atherosclerosis

Factors with a strong genetic component	Environmental factors
- Systemic inflammation	- High-fat diet
- Dyslipidaemia	- Smoking
- Elevated levels of homocysteine and haemostatic factors	- Low antioxidant levels
	- Lack of exercise
- Elevated blood pressure	- Infectious agents
- Family history	
- Diabetes and obesity	
- Depression and other behavioural traits	
- Gender (male)	
- Metabolic syndrome	

(Adapted from Lusis, 2000)

Inflammation or dyslipidaemia, among other risk factors, can often be participants in endothelial dysfunction, which is known to have an important role in the pathogenesis of atherosclerosis. The endothelium is responsible for a wide variety of functions, including regulation of vascular tone and growth, control of blood coagulation and fibrinolysis and modulation of inflammation (Vapaatalo and Mervaala, 2001). The loss of these physiological functions contributes not only to the development and progression of atherosclerosis but also to the evolution of clinical syndromes (Adams *et al.*, 2000).

Systemic inflammation

There is increasing evidence that atherosclerosis is an inflammatory process (Ross, 1999). However, it is not clear whether inflammation accelerates the atherosclerotic process or is reparative (Weintraub and Harrison, 2000).

Leukocytosis is often associated with atherosclerotic disease and is now accepted as a risk factor for CVD (Ernst *et al.*, 1987; Danesh *et al.*, 1998; Danesh *et al.*, 2000). The correlation between leukocyte count and risk of atherosclerotic disease is plausible because leukocytes make a major contribution to the rheologic properties of the blood, they alter adhesive properties under stress and also participate in endothelial injury (Ernst *et al.*, 1987). Moreover, the recruitment of monocytes and lymphocytes to the artery wall is characteristic of atherosclerosis. The exposure of endothelial cells to some factors, such as Ox-LDL, stimulates endothelial cells to produce a number of pro-inflammatory molecules, including adhesion molecules (Takei *et al.*, 2001) and growth factors (Lusis, 2000). The expression of adhesion molecules mediates the attachment of circulating mononuclear cells to the endothelium and facilitates their migration into the subendothelial space, thus contributing to their accumulation in the vascular wall.

Several studies have also documented associations between higher CRP levels and risk of CVD (Danesh *et al.*, 1998; Lagrand *et al.*, 1999; Danesh *et al.*, 2000; Ridker, 2001). Moreover, among several inflammatory markers that have been shown to predict cardiovascular events [namely IL-6, SAA and soluble intercellular adhesion molecule (ICAM)-1] CRP levels have emerged as the most powerful marker (Blake and Ridker, 2001). In the atherosclerotic process, CRP may be only a marker of vascular inflammation or alternatively, may have a direct role in atherogenesis. Evidence that CRP may have a pathogenic role in atherogenesis is increasing (Yu and Rifai, 2000; Arici and Walls, 2001; Blake and Ridker, 2001; Pepys and Berger, 2001). CRP is involved in complement activation (Bhakdi *et al.*, 1999), in the uptake of native LDL by macrophages (Zwaka *et al.*, 2001) and possibly, foam cell formation (Torzewski *et al.*, 1998; Zwaka *et al.*, 2001). A recent study has also shown the direct pro-inflammatory effect of CRP on human endothelial cells by inducing adhesion molecule expression in these cells (Pasceri *et al.*, 2000). Moreover, CRP is found within atherosclerotic plaques where it may colocalise with lipids (Zhang *et al.*, 1999), enzymatically degraded LDL (Bhakdi *et al.*, 1999) and activated complement (Torzewski *et al.*, 1998) within such plaques. Furthermore, CRP may also contribute to thrombosis by inducing human monocytes to synthesise tissue factor, a potent procoagulant (Cermak *et al.*, 1993). This may be associated with thrombotic complications of atherosclerosis, where CRP

levels are potentially higher. It is also important to note that CRP has been associated with other atherosclerosis-related risk factors: obesity markers (Mendall *et al.*, 1996; Yudkin *et al.*, 1999; Folsom *et al.*, 2001) and insulin resistance, markers of inflammation and acute phase reaction, and smoking (Mendall *et al.*, 1996; Folsom *et al.*, 2001).

Although elevated CRP is a marker of increased risk of atherothrombotic clinical events, little information is available describing genetic determinants of this effect. Baseline plasma CRP levels have very recently been reported to exhibit high heritability (Pankow *et al.*, 2001; Vickers *et al.*, 2002) and there is considerable interest in identifying genetic polymorphisms that are associated with CRP levels. Interleukin-6 (IL-6) regulates CRP gene expression and a promoter polymorphism (–174G/C) of the IL-6 gene, which has been shown to influence IL-6 transcription, was recently reported to be associated with CRP plasma levels, the –174C allele being associated with the higher baseline levels (Vickers *et al.*, 2002). The CRP gene is also an important candidate gene for such an association and therefore, for atherosclerosis. Interestingly, a recently described polymorphism within exon 2 of the CRP gene (1059G/C) (Cao and Hegele, 2000) was shown to be associated with CRP concentrations (Zee and Ridker, 2002). Carriers of this polymorphism in the CRP gene (GC or CC) presented significantly reduced CRP concentrations as compared to non-carriers (GG). However, this polymorphism did not itself predict clinical outcomes (Zee and Ridker, 2002). It would be important to confirm these findings and to investigate whether other genetic polymorphisms are associated with CRP levels and risk of atherosclerotic events.

Dyslipidaemia

Hypercholesterolaemia is a major risk factor for the initiation and progression of atherosclerosis (Witztum, 1993) and LDL, being the principal vehicle for Chol transport in human plasma, plays a central role in atherogenesis (Witztum, 1993). In addition, different LDL particles exhibit distinct properties. Smaller, denser LDL particles are believed to be more atherogenic: they penetrate the arterial wall more easily and demonstrate enhanced affinity for arterial wall proteoglycan (Packard *et al.*, 2000); exhibit reduced receptor binding, which may increase the plasma residence time (Nigon *et al.*, 1991); and are more susceptible to oxidation, at least *in vitro*, than its larger counterparts (Graaf *et al.*, 1991). Once oxidised, LDL is believed to be highly atherogenic, promoting foam cell formation and initiating endothelial dysfunction (Steinberg *et al.*, 1989; Witztum, 1993). Ox-LDL plays an important role in the immune activation that characterises the progression of atherosclerosis (Hörkkö *et al.*, 2000). Ox-LDL impairs endothelial-dependent vascular relaxation, by inactivating

endothelial-derived relaxing factor (nitric oxide or a related molecule) (Chin *et al.*, 1992); it inhibits endothelial prostacyclin synthesis and simultaneously increases endothelin-1 (a vasoconstrictor and mitogenic factor) production and release (Stewart and Monge, 1993). These changes promote platelet activation with a resulting enhanced thromboxane release. Plasma levels of Ox-LDL are markedly elevated in patients with angiographically proven coronary artery disease (Holvoet *et al.*, 2001), and it is becoming clear that circulating levels of Ox-LDL are markers of coronary atherosclerosis. Recently, Hulthe and Fagerberg (2002) demonstrated that circulating Ox-LDL associates with both subclinical atherosclerosis and inflammatory variables (namely TNF-α and CRP), supporting the concept that Ox-LDL may play a role in atherosclerosis development. Although LDL plays a major role in atherogenesis, atherogenicity is actually determined by LDL together with other factors (Rudel and Kesäniemi, 2000).

Lipoprotein (a) (Lp(a)) is also associated with atherosclerosis, being an important inherited and independent risk factor for this disease (Dahlen, 1994). Lp(a) contains the plasminogen-like apolipoprotein (a) bound to LDL. Lp(a), by competing with plasminogen for its binding sites on fibrin clots and endothelial cells, inhibits its activation to plasmin (Miles *et al.*, 1989), imposing a reduction in fibrinolysis (Hajjar *et al.*, 1989). This inhibition of plasminogen activation by Lp(a) also inhibits the activation of latent transforming growth factor-β with the consequent promotion of SMC proliferation (Dahlen, 1994). These features of Lp(a) suggest this lipoprotein has both antifibrinolytic and atherogenic potential. Given its influence on the fibrinolytic process, Lp(a) may increase the likelihood of thrombosis occurring on atheromatous plaques. Moreover, a novel mechanism associating Lp(a) and thrombosis, expressed by Lp(a) capacity in binding and inactivating tissue factor pathway inhibitor, was recently proposed (Caplice *et al.*, 2001).

On the other hand, HDL seems to have a protective role in the development of atherosclerosis (Lusis, 2000; Mertens and Holvoet, 2001; Nofer *et al.*, 2002); its role in reverse Chol transport (Gordon and Rifkind, 1989), in antioxidant (Parthasarathy *et al.*, 1990), anti-aggregatory (inhibition of LDL aggregation) (Khoo *et al.*, 1990), and/or anti-inflammatory effects (Cockerill *et al.*, 2001) is possibly related to its anti-atherogenic capacity. There is a strong inverse relationship between HDL cholesterol (HDLc) concentration and atherosclerotic events (Gordon and Rifkind, 1989), which is in agreement with a possible protective role of HDL in atherogenesis.

Recently, hypertriglyceridaemia and elevated levels of TG-rich lipoproteins (e.g., VLDL and remnants) have re-emerged as risk factors for atheroscleosis (Byrne, 1999). TG-rich

particles may directly damage the endothelium principally via oxidative mechanisms. These particles stimulate endothelial expression of adhesion molecules (such as VCAM-1) and the prothrombotic factor PAI-1 (Stewart and Monge, 1993; Adams *et al.*, 2000). An important consequence of hypertriglyceridaemia, relating to increased atherosclerotic risk, is a shift in the spectrum of LDL subfractions towards smaller, denser species. Moreover, raised TG levels are frequently associated with reduced HDL levels. The combination of a preponderance of small, dense LDL particles with moderately elevated TG and low HDL levels is termed as the atherogenic lipoprotein phenotype and is a significant risk factor for coronary heart disease (Packard *et al.*, 2000).

Genetic variants, such as those associated with apo E gene, may also influence atherogenesis through effects on lipid and/or lipoprotein levels. Apo E is produced mainly in the liver and is a constituent of chylomicrons, chylomicron remnants, liver-derived VLDL and their remnants as well as some Chol-rich subclasses of HDL. When associated with these lipoproteins, apo E serves as a ligand for the LDL (or apo B/E) receptor and the hepatic apo E receptor (or the LDL-receptor related protein) (Wu *et al.*, 1997). In humans, the structural gene *locus* for apo E is polymorphic: three common alleles, designated ε2, ε3, and ε4 which code for three major apo E isoforms E2, E3 and E4, respectively (Wu *et al.*, 1997). The three isoforms differ in their amino acid sequence at positions 112 and 158 and as a consequence, their binding affinities to the LDL receptor are also different. Genetic variation at the apo E *locus* in human populations is an important determinant of plasma lipid levels and relative risk of atherosclerosis. An association of apo E isoforms and atherosclerotic lesions in young people has been reported (Hixson and Group, 1991). Subjects carrying the E2 (E2+) and E4 (E4+) alleles are associated, respectively, with lower and higher Chol levels compared to E3/3 individuals (Dallongeville *et al.*, 1992). Concerning the effect of apo E allele on TG levels, contradictory results are found in the literature. However, a meta-analysis reported that TG levels are higher in E2+ and in E3/4 than E3/3 subjects (Dallongeville *et al.*, 1992). Most patients with type III hyperlipoproteinaemia, a condition characterised by the accumulation in the plasma of triacylglycerol-rich lipoprotein remnants, are homozygous for apo E2 (Wu *et al.*, 1997). Apo E is atheroprotective (Curtiss and Boisvert, 2000) and apo E polymorphism also associates with the plasma concentration of apo E, E2+ presenting the highest values (Larson *et al.*, 2000). Also of interest, is a preliminary study showing that variation in apo E gene *locus* influences serum CRP level, with E4+ presenting the lowest values (Mänttäri *et al.*, 2001).

Section Two: Human Pregnancy

Major adaptations in maternal anatomy, physiology, and metabolism are required for a successful pregnancy. These adaptations profoundly affect nearly every organ system and most are greatly mediated by the hormonal changes initiated before conception and continue throughout pregnancy. Some outstanding modifications occurring in maternal body throughout pregnancy will be described in this section.

6. Uterine Vascular Changes

In the human placenta, the villous trophoblasts consist of two cell layers: an inner layer composed of individual and well-defined cells, the cytotrophoblasts; and an outer and thicker layer comprising a continuous mass of cell plasma containing multiple nuclei with indistinct cell borders, the syncytiotrophoblasts. It is evident that the former cells display highly proliferative and invasive properties, whereas the latter display functions indispensable for the maintenance of pregnancy and foetal growth with little potential for proliferation (Buster and Carson, 2002).

In humans, during normal placentation, the extravillous cytotrophoblast cells extensively colonise the decidua and adjacent myometrium of the placental bed. The human placenta receives its blood supply from the uteroplacental arteries, which are developed by the action of the extravillous cytotrophoblast into the walls of spiral arteries. The conversion of the spiral arteries of the uterus into the uteroplacental arteries occurs in two waves of endovascular invasion by cytotrophoblast: the invasion of the decidual segments of the spiral arteries by a wave in the first trimester (around 6–8 weeks) and the invasion of the myometrial segments by a subsequent wave in the second trimester (around 16–20 weeks) (Starkey, 1993). The invasion process is associated with degeneration of the vascular endothelium, extensive fibrinoid formation and loss of muscoloelastic tissue. This loss of musculature and elasticity is accompanied by a rather dramatic increase in the luminal width and allows uterine arteries to expand as pregnancy progresses.

Thus, during normal pregnancy, the arteries that feed the intervillous space undergo structural physiological remodelling. They lose many of their structural components and increase greatly in diameter. This remodelling of the spiral arteries results in the formation of a low resistance arteriolar system with a dramatic increase in blood supply to the placenta and

growing foetus (Starkey, 1993). Moreover, these remodelled vessels do not respond to humoral or neurogenic stimuli in order to protect the foetus.

The process of cytotrophoblast invasion is accompanied by characteristic changes, which include up-regulated expression of type IV collagenase, human leukocyte antigen (HLA)-G [a nonclassic, truncated major histocompatibility complex (MHC) class I antigen], and human placental lactogen (Branch and Porter, 1999). The invading cytotrophoblasts express a variety of adhesion molecules involved in invasion of the decidual-myometrial interstitium and vasculature, including a decidual-endothelial cell cadherin, platelet-endothelial cell adhesion molecule-1, vascular cell adhesion molecule (VCAM)-1, $\alpha 1$ and $\alpha 4$ integrins, $\beta 1$ integrins, and angiogenesis-related $\alpha V\beta 3$ (Zhou *et al.*, 1997b). HLA-G seems to play a role in modulating the maternal immune response, being involved in the inhibition of natural killer cell activity (Branch and Porter, 1999).

7. Haemodinamic Modifications

Healthy human pregnancy is associated with a fall in vascular resistance, despite increasing cardiac output and blood volume (Gordon, 2002). During gestation, blood pressure decreases until the second trimester, returning to pre-pregnancy levels in the third trimester of gestation (Freund and Arvan, 1990). Pregnancy is also characterised by resistance to pressor effects of vasoconstrictors, by water and sodium retention, and by activation of the renin-angiotensin system (RAS) (Gordon, 2002).

The increment in cardiac output is associated with regional blood flow changes. Blood flow increases in different organs and tissues, being particularly enhanced in the uterus and kidneys.

The major modifications in haemodinamics are primarily related with the necessity to expand uteroplacental blood flow, which is essential for the normal development of the growing foetus. Uterine blood flow increases during gestation from about 50 ml/min in the non-pregnant state to approximately 200 ml/min by the 28^{th} week of pregnancy and approximately 500 ml/min by term (Metcalfe *et al.*, 1955; Assali *et al.*, 1960). Twenty percent of the total uterine blood flow is received by the uterine musculature and eighty percent by the placental area (Gibbs, 1981).

Concerning the kidneys, the effective renal plasma flow (ERPF) rises approximately 75 and 85 percent over non-pregnant levels by 16 and 26 weeks' gestation, respectively (Dunlop, 1981). However, ERPF declines about 25 percent from this new level at 36 weeks' gestation

(Dunlop, 1981). The increase in renal blood flow is associated with an increase in glomerular filtration rate (GFR). GFR rises early in gestation and by the end of the first trimester is 50% higher than in the non-pregnant state, before remaining stable until term (Hill, 1978).

Despite increases of 40–50% in cardiac output, blood flow through the liver remains essentially at the pre-pregnancy level of 1.5 l/min and therefore the relative perfusion of this organ is decreased. This functional stress is well tolerated by the normal liver (Freund and Arvan, 1990).

8. Physiological Changes in Maternal Blood

Acute Phase Proteins

Despite an overall increase in total body protein, the serum total protein and albumin levels fall during pregnancy, primarily as a result of haemodilution (Freund and Arvan, 1990).

The concentrations of several proteins produced by the liver increase in pregnancy, namely APPs such as caeruloplasmin (Haram *et al.*, 1983), α_1-antitrypsin and fibrinogen (Stirling *et al.*, 1984), and CRP (Rebelo *et al.*, 1995). Serum levels of transferrin (a negative APP) also increase in pregnancy (Freund and Arvan, 1990), perhaps as a response to decreased iron stores (Hubel, 1998).

Although α_1-antitrypsin and fibrinogen (Stirling *et al.*, 1984) and caeruloplasmin (Haram *et al.*, 1983) increase consistently as pregnancy progresses, the pattern of change in other APP, namely CRP, remains to be clarified.

CRP may be useful in the diagnosis of infection in women with pre-term rupture of membranes (Hawrylyshyn *et al.*, 1983). Its clinical utility may become even more relevant if changes in its levels throughout normal gestation are better understood. Rebelo *et al.* (1995) reported that mean CRP levels increase early in gestation (first trimester), compared to non-pregnant levels, before remaining constant throughout the second and third trimesters. Another study, reporting no consistent change in CRP levels with gestational age among serially sampled women, was performed only from 22 weeks of gestation onwards (Watts *et al.*, 1991). Romem and Artal (1985) found a positive correlation of CRP levels with gestational age but others did not (Hawrylyshyn *et al.*, 1983). It is important to note that in some studies the sensitivity of the CRP assay used was limited, whilst in others the enrolled individuals were unmatched (cross-sectional studies).

Changes in haptoglobin, another APP, are also controversial. In pregnancy, Rebelo *et al.* (1995) described gradual decreases in haptoglobin levels throughout gestation, being statistically reduced in the second and third trimesters compared to non-pregnant levels, possibly expressing increased haemolysis of RBCs. Higher levels of haptoglobin in the first and third trimesters with a decline around 24 weeks of gestation were also reported (Haram *et al.*, 1983).

Liver Function Tests

Regarding "liver function tests", serum alkaline phosphatase activity rises markedly during the third trimester, with most of this increase being caused by placental production of the heat-stable isoenzyme. However, other tests are unaffected by normal pregnancy, including serum levels of bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase, creatinine phosphokinase, and lactate dehydrogenase (LDH) (Gordon, 2002).

Lipids and Lipoproteins

Human pregnancy is associated with pronounced physiological hyperlipidaemia (Aurell and Cramér, 1966; Potter and Nestel, 1979; Ordovas *et al.*, 1984; Pocovi *et al.*, 1984; Fåhraeus *et al.*, 1985; Desoye *et al.*, 1987; Piechota and Staszewski, 1992), which is believed to be under hormonal control (Desoye *et al.*, 1987; Piechota and Staszewski, 1992; Alvarez *et al.*, 1996). During normal human pregnancy, plasma TG increase 2- to 4-fold while Chol rises 25–50% (Potter and Nestel, 1979; Ordovas *et al.*, 1984; Piechota and Staszewski, 1992; Alvarez *et al.*, 1996). This physiological hyperlipidaemia may help to supply the placenta with Chol for steroid synthesis and to supply the growing foetus with metabolic precursors (Tikkanen, 1999). Despite major modifications in lipids and lipoproteins during normal pregnancy, this phenomenon is well tolerated by the mother and does not seem to be atherogenic (Tikkanen, 1999).

Plasma Chol and LDLc concentrations may fall somewhat in early pregnancy but then rise continuously reaching their highest values in the third trimester of gestation (Fåhraeus *et al.*, 1985; Piechota and Staszewski, 1992). VLDLc also increases progressively with gestational time (Iglesias *et al.*, 1994; Alvarez *et al.*, 1996; Winkler *et al.*, 2000), whereas the increase in HDLc concentration, which reaches a maximum at mid-gestation, is followed by a partial return to lower levels at term (Pocovi *et al.*, 1984; Fåhraeus *et al.*, 1985; Piechota and Staszewski, 1992; Alvarez *et al.*, 1996). HDL_2 rather than HDL_3 is responsible for the

enhancement of HDLc during gestation and the attenuation of HDLc elevation during the second half of pregnancy may be related to the development of insulin resistance (Fåhraeus *et al.*, 1985).

The increase in TG levels is primarily due to an increase in very-low-density lipoprotein (VLDL) concentrations, with increments in both $VLDL_1$ and $VLDL_2$ of 4-fold (on average) by 35 weeks (Sattar *et al.*, 1997b). During gestation, an elevation of TG level is found not only in VLDL, but also in all lipoprotein fractions, namely LDL and HDL (Iglesias *et al.*, 1994; Alvarez *et al.*, 1996; Winkler *et al.*, 2000). Moreover, while the TG to Chol ratio remains relatively stable in VLDL throughout gestation, it increases in both LDL and HDL, suggesting a TG enrichment of these particles (Alvarez *et al.*, 1996). Mechanisms for such changes involve increased hepatic synthesis of TG and/or reduction in maternal TG catabolism. The latter is reflected by reduced activities of circulating lipoprotein lipase (LPL) and hepatic lipase (HL), the effect being more striking for HL than for LPL (Alvarez *et al.*, 1996). The abundance of VLDL-TG drives an accelerated transfer of TG to lipoproteins of higher density by the CETP and the reduced HL activity appears to be responsible for the shift of HDL subclasses towards larger and more buoyant species in late gestation (Alvarez *et al.*, 1996). In one study, gradient gel electrophoresis analysis indicated that in late gestation, 86% of the subjects had a substantial increase of the most buoyant and largest of the HDL species, HDL_{2b} (Silliman *et al.*, 1993).

Although LDL particles also become enriched in TG during gestation, the pattern of change in their size/density remains controversial. The overt discrepancy between the published data on LDL may involve differences in the studied population and/or in the used methodology.

Using equilibrium density gradient centrifugation to separate LDL subfractions by virtue of their density, Winkler *et al.* (2000) reported that dense LDL particles increase in the early stages of pregnancy with no further increase being observed with advancing gestation. In contrast, there is an increase in buoyant LDL as gestation advances (Winkler *et al.*, 2000). However, other studies reported a significant increase of small, dense LDL throughout pregnancy (Sattar *et al.*, 1997b; Hubel *et al.*, 1998b). By using nonequilibrium density gradient ultracentrifugation, to separate LDL subfractions by flotation rate, Sattar *et al.* (1997b) found, in six of ten women, an increase in LDL-III mass at the expense of LDL-II, with the proportion of LDL-I remaining unchanged throughout gestation. Considerable individual variation was shown in the gestational ages and TG levels at which the LDL profile began to change in these six women, suggesting a TG threshold effect (Sattar *et al.*, 1997b).

Hubel *et al.* (1998b), by using nondenaturing gradient polyacrylamide gel electrophoresis (PAGE), which separates LDL by virtue of size, found, in the majority of cases, a progressive decrease in LDL peak particle diameter (LDL-PPD) during normal gestation as TG increased. In this study, no evidence was found of a TG threshold for decreases in LDL-PPD.

It is important to note that LDL-PPD, often reported in the literature (Silliman *et al.*, 1994; Hubel *et al.*, 1998b), provides little information about the LDL profile, as it indicates only the predominant LDL size at the major LDL band. It would be important to measure not only LDL-PPD but also LDL mean particle diameter (LDL-MPD), an indicator of the mean size of the entire LDL population. To address this problem, some authors use the "LDL particle score". This score is a calculation that involves the contribution of the relative proportion of each of the LDL subfractions (Campos *et al.*, 1992); the higher the particle score, the smaller the particle size. However, it would make more sense to use a real measure of particle size to compare with LDL-PPD rather than giving an arbitrary number. The evaluation of LDL size is important because smaller, denser subpopulations of LDL are more susceptible to oxidation (Graaf *et al.*, 1991).

Despite the changes in LDL size during pregnancy, Vriese *et al.* (2001) reported that the oxidative resistance of LDL increases as gestation progresses, which could be partially explained by the concomitant increase in plasma vitamin E levels. However, in the same report, the *in vitro* oxidation rate and formation of conjugated dienes of the LDL subfraction increased throughout gestation, indicating a possible change in the composition of LDL and the amount of substrate available for lipid peroxidation. Curiously, neither this study nor those evaluating LDL size measured circulating levels of Ox-LDL.

Controversy also exists regarding changes in Lp(a) levels. During gestation, some authors found no change in Lp(a) (Winkler *et al.*, 2000), others found a progressive increase in median Lp(a) concentration between 10 and 35 weeks (Sattar *et al.*, 2000), while another study reported that Lp(a) levels reach a peak concentration in the middle of pregnancy and decrease thereafter (Zechner *et al.*, 1986). Two studies, employing cross-sectional analysis, found a lack of association between Lp(a) levels and gestational age (Cachia *et al.*, 1995; Wang *et al.*, 1998). As with the LDL profile during gestation, discrepancies in Lp(a) may also express differences in the studied population or in the method used. Moreover, only longitudinal studies may be useful in evaluating changes in variables presenting non-Gaussian distributions within the population, such as Lp(a).

Uric Acid

Although the potential diagnostic value of serum urate measurements in pregnancy is uncertain, abnormalities in this routinely available test are sufficiently common to merit special attention (Freund and Arvan, 1990). Serum uric acid decreases in early pregnancy, reaching a nadir by 16 weeks, as a result of haemodilution, the rise in GFR and changes in the renal tubular reabsorption and/or secretion of urate (Hill, 1978). After 16 weeks, the serum uric acid concentration increases and by the end of gestation it attains the pre-pregnancy levels. The rise in uric acid levels is caused by increased renal tubular reabsorption of urate (Hill, 1978).

Lipid Peroxidation *versus* Serum or Plasma Antioxidants

A review of the literature indicates that lipid peroxides are increased in pregnant women compared with non-pregnant controls (Little and Gladen, 1999). During pregnancy, elevations in lipid peroxides appear by the second trimester and may taper off later in gestation (Little and Gladen, 1999). Uncontrolled production of lipid peroxides can result in oxidative stress. In normal pregnancy, increased lipid peroxidation may (Uotila *et al.*, 1991) or may not (Rebelo *et al.*, 1995) be controlled by adequate serum antioxidative response.

Using the brain homogenate auto-oxidation bioassay, a progressive increase in serum antioxidant activity has been observed during the course of normal human gestation (Davidge *et al.*, 1992). This assay primarily detects the preventive antioxidants transferrin and caeruloplasmin, as it measures the ability of serum to inhibit the auto-oxidation of brain tissue homogenate or phospholipid liposomes, which is ferrous ion (Fe^{2+})-dependent (Hubel, 1998). As referred previously, concentrations of both transferrin and caeruloplasmin increase in normal pregnancy, and moreover, serum iron concentrations fall during the course of normal gestation, leading to decreased transferrin saturation and increased apotransferrin (iron-free transferrin) (Hubel, 1998).

Other studies have evaluated the concentration of specific antioxidants. Plasma or serum levels of α-tocopherol were reported to increase during pregnancy (Wang *et al.*, 1991a; Oostenbrug *et al.*, 1998; Vriese *et al.*, 2001). Other lipid soluble antioxidants, such as vitamin A (Oostenbrug *et al.*, 1998; Vriese *et al.*, 2001) and β-carotene (Oostenbrug *et al.*, 1998), decrease in plasma along pregnancy. Moreover, not only are serum vitamin C levels decreased late in gestation, although without statistical significance (Rebelo *et al.*, 1995), but plasma GPX activity also seems to decline progressively as pregnancy advances (Behne and Wolters, 1979; Zachara *et al.*, 1993).

Considering all these changes, it would be important to evaluate the total antioxidant status of plasma and/or serum during gestation.

Plasma Volume and RBC

Maternal blood volume increases progressively until 30 to 34 weeks and then plateaus until delivery. The increase in blood volume results from a rise in both plasma volume and RBC mass, the latter being driven by higher erythropoietin levels (Cleary and Duggan, 1991; Wintrobe *et al.*, 1993).

The increase in plasma volume being higher than the increase in the red cells, leads to an apparent drop in the haemoglobin (Hb) concentration and haematocrit (Ht), usually referred as the "physiological anaemia of pregnancy", a misnomer (Wintrobe *et al.*, 1993). Maximum haemodilution seems to occur in the middle of gestation, followed by a gradual, significant increase in RBC count, Ht, and Hb concentration until delivery (Milman *et al.*, 2000). This increase is explained by the fact that whereas plasma volume expands at a steady pace until it plateaus at 30 weeks' gestation, the erythrocyte mass grows progressively until term. A greater increase in RBC mass may occur if iron supplements are taken (Wintrobe *et al.*, 1993); Milman *et al.* (2000) reported that placebo-treated women present significantly lower haematologic indices compared to iron-treated women.

In pregnancy, it is difficult to distinguish between an apparent and an effective erythrocyte modification. Although some studies claim that in pregnancy there is a change in RBC profile towards younger species (Tian *et al.*, 1998) and that erythropoiesis increases with gestational age (Choi and Pai, 2001), the evaluation of erythrocyte enzyme activities as indicators of RBC age has given rise to controversial results. Some authors found a significant increase in erythrocyte GPX activity during pregnancy (n = 20) (Uotila *et al.*, 1991). Others, in a smaller study (n = 10) in which they failed to detect significant increases in GPX, reported significant increases in erythrocyte superoxide dismutase activity (Carone *et al.*, 1993). Another group found that RBC GPX activity started to decrease after the 30th week of pregnancy and was significantly lower before delivery than during the 10th week of pregnancy (Zachara *et al.*, 1993).

Human pregnancy is associated with an increased oxidative stress (Rebelo *et al.*, 1995), which may account for some erythrocyte damage in pregnancy and consequent acceleration of RBC ageing. Curiously, levels of thiobarbituric acide reactive substances in RBC haemolysates were reported to be significantly increased in the third trimester of gestation

compared with previous trimesters and compared with values obtained in non-pregnant women (Carone *et al.*, 1993).

It would be important to find a marker, independent of the "physiological anaemia", which could provide information about the effective erythrocyte changes during pregnancy. The study of erythrocyte band 3 profile, a cumulative marker of oxidative and/or proteolytic damage, and MBH may provide new data about erythrocyte changes in pregnancy. Indeed, no information exists in the literature about pregnancy-induced modifications in erythrocyte membrane band 3, the RBC senescent neoantigen.

Leukocytes and the Immune System

The number of white blood cells (WBCs) increases in pregnant women in response to high levels of cortisol and possibly other factors (Cleary and Duggan, 1991). The rise in WBC count is mainly due to an increase in neutrophils (Cleary and Duggan, 1991; Rebelo *et al.*, 1995). Neutrophil count increases markedly between the first and the second trimesters and remains high in the third trimester (Rebelo *et al.*, 1995). Thus, a neutrophilic leukocytosis occurs in pregnancy.

A successful pregnancy is dependent on maternal tolerance to paternal antigens. Despite an immuno-nonreactivity to foreign antigens, pregnancy is not a state of immunodeficiency. It is however a state of altered immune functions. Investigations on systemic immune function, focused on lymphocyte function, have demonstrated a weakening of cell-mediated immunity and an enhanced humoral-mediated immunity (Gordon, 2002). Total T and B lymphocyte numbers are not altered substantially during pregnancy but within the subpopulation of T-cells, an increase in the ratio of suppressor/helper cells has been observed (Feinberg and Gonik, 1991); T helper 1 cells and natural killer cells decline, whereas T helper 2 cells increase (Gordon, 2002). Despite enhanced humoral-mediated immunity, levels of the immunoglobulins A, G, and M are not significantly different from pre-pregnancy values (Feinberg and Gonik, 1991).

The decrease in cellular immunity may explain the improvement of various chronic inflammatory diseases during pregnancy, particularly rheumatoid arthritis (Gordon, 2002), and the fact that pregnant women present increased susceptibility to specific intracellular infections (Feinberg and Gonik, 1991). Pregnancy-associated depression of polymorphonuclear leukocytes (PMNs) function may also account for this phenomenon (Crouch *et al.*, 1995). However, it is still uncertain whether PMNs are primed and/or activated in normal pregnancy. The measurement of oxygen radical production by PMNs or

neutrophils, with (respiratory burst activity) or without (basal response) an external stimulus (Tsukimori *et al.*, 1993; Crouch *et al.*, 1995; Sacks *et al.*, 1998; Crocker *et al.*, 1999; Zusterzeel *et al.*, 2001), as well as the investigation of cell surface adhesion molecule expression on those cells (Crouch *et al.*, 1995; Sacks *et al.*, 1998; Crocker *et al.*, 1999) has lead to controversial findings. Studies involving indirect measurements of neutrophil activation, by measuring circulating levels of granular components derived from neutrophils, have also produced conflicting results. Greer *et al.* (1989) found higher concentrations of plasma elastase in women with normal pregnancies (in the third trimester of gestation) than in normal non-pregnant women, suggesting increased neutrophil activation and degranulation in normal pregnancy. Rebelo *et al.* (1995) found reduced plasma levels of elastase in the first and second trimesters of gestation, compared with non-pregnant values, and no differences in the third trimester of gestation. This study also reported that plasma lactoferrin concentration as well as its relation to the number of neutrophils was significantly increased in all three trimesters of gestation. A recent study, by examining non-pregnant and pregnant women in third trimester of gestation, failed to confirm these findings on lactoferrin (Crocker *et al.*, 1999).

Haemostatic System

Profound changes in coagulation and fibrinolytic mechanisms occur during normal human pregnancy (Stirling *et al.*, 1984; Åstedt, 1993; Higgins *et al.*, 1998), which may be explained by hormonal and lipid changes in pregnancy (Sattar *et al.*, 1999). A hypercoagulable state develops in normal pregnancy, which seems to be a physiological adaptation for maintaining the placento-uterine interface and/or to prevent major haemorrhage during and after placental separation. This hypercoagulable state may also contribute to the increased susceptibility of pregnant women to thrombotic disorders (Letsky, 1999).

Plasma levels of several coagulation factors increase markedly throughout pregnancy, including factors I (fibrinogen), VII, VIII and X (Stirling *et al.*, 1984) and von Willebrand factor (vWf) (Sattar *et al.*, 1999). The rise in fibrinogen contributes to the observed increase in erythrocyte sedimentation rate (Freund and Arvan, 1990). Concerning natural anticoagulants, antithrombin III (Stirling *et al.*, 1984) and protein C (Clark *et al.*, 1998b) activities seem to remain relatively unchanged, whereas total and free protein S show a progressive fall throughout pregnancy (Clark *et al.*, 1998b). Activated protein C (APC) sensitivity ratio (APC:SR), which is the ratio of the clotting time in the presence and the absence of APC, was shown to progressively decline throughout pregnancy, and moreover, by

the third trimester, 38 percent of women were found to have an acquired APC resistance (APC:SR < 2.6) (Clark *et al.*, 1998b). The result of these physiological changes is to alter the usual balance between the procoagulants and anticoagulants in favour of the factors promoting blood clotting. The prothrombin time, activated partial thromboplastin time, and thrombin time all fall slightly (Gordon, 2002).

Regarding fibrinolytic factors (see Figure 8–1 for a general review of the fibrinolytic system), higher concentrations of tissue type plasminogen activator (t-PA) antigen (Halligan *et al.*, 1994; Sattar *et al.*, 1999) and plasminogen activator inhibitors (PAIs) types 1 (Declerck *et al.*, 1988; Estelles *et al.*, 1989; Cadroy *et al.*, 1993; Halligan *et al.*, 1994) and 2 (Estelles *et al.*, 1989; Halligan *et al.*, 1994) are reported. Changes in PAIs may explain the decrease in fibrinolytic activity during pregnancy described elsewhere (Stirling *et al.*, 1984). Moreover, as referred above, the APP α_1-antitrypsin (now called α_1-antiprotease) also increases markedly in pregnancy (Haram *et al.*, 1983; Stirling *et al.*, 1984), possibly contributing to the reduction in fibrinolysis.

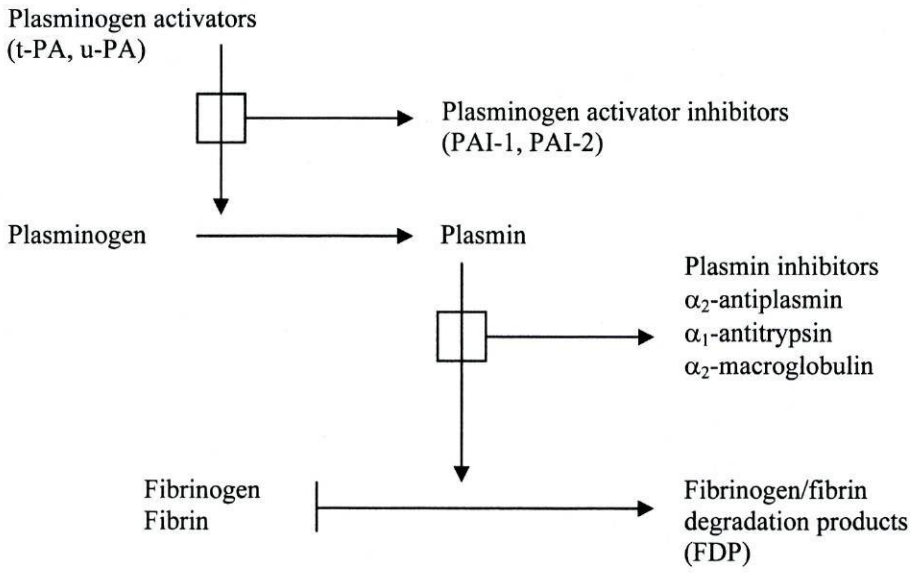


Figure 8–1. The fibrinolytic system. Plasminogen activators (PAs), serine proteases of either blood vessel/tissue-type (t-PA) or urokinase type (u-PA), convert the inactive proenzyme, plasminogen, into the active protease plasmin. Plasmin degrades fibrin but also fibrinogen into fibrin degradation products (FDP) of various molecular sizes. There are two highly specific plasminogen activator inhibitors (PAIs)- one of endothelial type (PAI-1) and the other of placental type (PAI-2). There are also inhibitors of plasmin: α_2-antiplasmin, α_1-antitrypsin and α_2-macroglobulin. (Adapted from Åstedt, 1993)

Despite the marked impairment in fibrinolytic potential, the fibrinolytic system remains functionally active, as fibrin degradation products (FDPs), usually assayed as cross-linked fibrin fragments (D-dimer), also increase during gestation (Ballegeer *et al.*, 1987; Cadroy *et al.*, 1993; Sattar *et al.*, 1999). The evaluation of D-dimer can be used as a measure of the formation (via thrombin) and lysis (via plasmin) of fibrin.

Changes in platelet count may also occur. Although the data regarding changes in platelet count during pregnancy are still somewhat unclear, most recent studies show a decline in its count during pregnancy possibly caused by increased destruction (Gordon, 2002). Not only may mild decreases in the mean platelet count occur in pregnancy but also some women may develop gestational thrombocytopenia, which is thought to result from accelerated platelet consumption (Gordon, 2002).

Section Three: Pre-eclampsia as a Pathologic Paradigm of Human Pregnancy

Maternal hypertensive disorders are the most common medical complications of pregnancy. Pre-eclampsia (PE) is a hypertensive disorder characteristic of human pregnancy that subsides after delivery. PE is unpredictable in its onset and progression and is a major cause of maternal and foetal morbility and mortality. This condition is much more than hypertension, being better defined as a syndrome: a conjunction of features that occur together.

Although the precise cause of PE remains unknown, the placenta has been suggested as the focus of all manifestations. PE can occur in hydatidiform mole, where the uterus contains only disordered placental tissue. Thus, a foetus is not necessary for its development, only the placenta (Redman, 1993). The regression of the illness occurs when the uterus is emptied of placental tissue.

Considering the complexity of PE, this section will describe some of the most important aspects regarding this multisystem disorder.

9. Characterisation

Definition

Two definitions of PE are widely used in clinical practice and research: one proposed by the American College of Obstetricians and Gynaecologists (ACOG) and the other by the International Society for the Study of Hypertension in Pregnancy (ISSHP) (Zhang *et al.*, 1997).

The ISSHP defines PE, also called "gestational proteinuric hypertension", as hypertension and proteinuria developing after 20 weeks of pregnancy, during labour or the puerperium in a previously normotensive non-proteinuric woman (Davey and MacGillivray, 1988). The ISSHP defines hypertension as either (1) one measurement of diastolic blood pressure (DBP) equal to or greater than 110 mmHg on any one occasion or (2) two or more consecutive measurements of DBP equal to or greater than 90 mmHg, 4 or more hours apart. Proteinuria is defined as either (1) one 24-hour urine collection with a total protein excretion of 300 mg or more or (2) two random clean-catch or catheter urine specimens, collected 4 or

more hours apart, with 2+ (1 g albumin/l) or more on reagent strip or 1+ (0.3 g albumin/l) if specific gravity less than 1.030 and pH lower than 8.

According to the ACOG, PE was traditionally defined as hypertension accompanied by proteinuria, oedema, or both, usually occurring after 20 weeks' gestation (or earlier in the case of trophoblastic diseases such as hydatidiform mole or hydrops) (NHBPEP Working Group, 1990). In this disorder, the Committee on Terminology of the ACOG defined hypertension as an increase by at least 30 mmHg in systolic or 15 mmHg in diastolic blood pressure, when compared with the values obtained before 20 weeks of gestation; or a sustained blood pressure of at least 140/90 mmHg after 20 weeks of gestation, if prior blood pressure was unknown. The elevated blood pressure must be observed on at least two occasions 6 or more hours apart. Proteinuria is defined as the excretion of 300 mg or more, of protein in a 24h urine collection specimen. This usually agrees with 30 mg/dl (1+ on dipstick testing) or higher in a random specimen of urine. Oedema is diagnosed as clinically evident swelling or as a rapid increase of weight.

This definition of the ACOG had some imprecisions. Indeed, oedema is neither sufficient nor necessary to confirm the diagnosis of PE, since many normal pregnant women present with generalised oedema late in pregnancy and oedema may be absent in women with PE and eclampsia (Sibai, 2002). Moreover, the use of the increments in blood pressure to define hypertension in pregnancy has been criticised (Branch and Porter, 1999). The ACOG definition would be imprecise because of the natural tendency of blood pressure to fall during the second trimester and to rise during the third trimester. The ACOG reviewed the definition of PE: oedema was removed from the definition of PE and the increments in blood pressure are no longer used to define hypertension in pregnancy. Nevertheless, in clinical practice, a rising blood pressure still merits increased surveillance, especially if accompanied by proteinuria and hyperuricaemia (NHBPEP Working Group, 2000).

At present there is universal agreement that oedema should not be considered as part of the diagnosis of PE, which is primarily defined as gestational hypertension plus proteinuria (Sibai, 2002; Roberts *et al.*, 2003). Moreover, in the absence of proteinuria, PE is highly suspected when gestational hypertension is present associated with the following symptoms: headache, blurred vision and abdominal pain and/or with abnormal laboratory test results, such as low platelet counts and abnormal liver enzyme values (NHBPEP Working Group, 2000).

PE has a clinical spectrum ranging from mild to severe forms and can lead to two life-threatening complications: HELLP syndrome and eclampsia (NHBPEP Working Group, 1990).

Severe Forms of PE

PE has an unpredictable course, ranging from slow to fulminant progression of mild PE to severe PE. Therefore, even mild PE is potentially dangerous. Early onset is often associated with severe PE.

The degree of hypertension and proteinuria are frequently used to define the severity of PE (Branch and Porter, 1999). According to some authors, the two criteria that are primarily taken into account in the diagnosis of severe PE are: blood pressure of ≥ 160 mmHg systolic or ≥110 mmHg diastolic (recorded on at least two occasions, at least 6 hours apart, with the patient at rest) and proteinuria of ≥ 5 g *per* 24 hours (Robson, 1999; Sibai, 2002). However, other signs and symptoms can also be considered: oliguria (≤ 400 ml in 24 hours), cerebral or visual disturbances, epigastric pain, nausea, and vomiting, pulmonary oedema, impaired liver function of unclear aetiology, thrombocytopenia.

HELLP Syndrome

HELLP syndrome, a variant of severe PE with a typical laboratory triad, has received considerable attention. H (haemolysis) stands for microangiopathic haemolytic anaemia; EL (elevated liver enzymes) for liver dysfunction and LP (low platelet count) for thrombocytopenia (Rath *et al.*, 2000). Although HELLP syndrome is considered a variant of severe PE, its severity is reflected in its laboratory parameters and not in the clinical parameters that typically reflect the severity of PE (blood pressure and proteinuria) (Magann and Martin, 1999). It is important to emphasise that HELLP syndrome may be present in patients with no hypertension and with no or minimal proteinuria (Saphier and Repke, 1998; Rath *et al.*, 2000; Vigil-De Gracia, 2001a). Patients may present signs and symptoms such as headache, right upper quadrant or epigastric pain, haematuria, nausea and/or vomiting (Rath *et al.*, 2000; Vigil-De Gracia, 2001a; Vigil-De Gracia, 2001b).

It should be highlighted that there is considerable debate regarding the diagnosis of this syndrome. Although authorities agree that haemolysis, liver dysfunction, and thrombocytopenia must all be present for the diagnosis, they disagree over specific criteria. For instance, there is some controversy about what constitutes an abnormal serum level of AST (Sibai, 2002). Regarding haemolysis, although a lot of altered parameters [such as

abnormal peripheral blood smear with characteristic RBC morphology (e.g., schistocytes, burr cells), increases in total bilirubin and LDH] may be used in its diagnosis, it was claimed that the determination of serum haptoglobin is the most sensitive method, although it should always be accompanied by the evaluation of CRP (Rath *et al.*, 2000). Reflecting the differences in the diagnostic criteria used, HELLP syndrome has an incidence in PE of 2 to 12 percent (Sibai, 2002).

Table 9–2 describes two classification systems used for categorisation and comparison of patient groups with HELLP syndrome: the Mississippi triple-class system and the Tennessee complete-incomplete system (Magann and Martin, 1999).

Table 9–2. Mississippi and Tennessee classification systems for HELLP syndrome

Mississippi	Tennessee
THROMBOCYTOPENIA	COMPLETE
Class 1: platelets ≤ 50,000/μl	Platelets < 100,000/μl
Class 2: platelets > 50,000 – ≤ 100,000/μl	LDH ≥ 600 IU/l
Class 3: platelets > 100,000 – ≤ 150,000/μl	AST ≥ 70 IU/l
HAEMOLYSIS+HEPATIC DYSFUNCTION	INCOMPLETE
LDH ≥ 600 IU/l	Only one or two of above present
AST and/or ALT ≥ 40 IU/l	
All must be present to qualify	

LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase. (Adapted from Magann and Martin, 1999)

Patients with class 1 HELLP syndrome (according to the Mississippi classification) and those with complete expression of HELLP syndrome (according to the Tennessee classification) represent the more severe forms and are associated with a higher frequency of maternal complications (Magann and Martin, 1999). A high and significant association of HELLP syndrome and eclampsia was reported (Vigil-De Gracia, 2001a).

Eclampsia

Eclampsia is the occurrence of seizures or coma in a patient with PE that cannot be attributed to other causes (Branch and Porter, 1999). Although a rarity in developed countries, it has a great incidence in the third world, with a high maternal and foetal mortality (Jamelle, 1997).

Seizures may occur antepartally, during the *intrapartum* or in the *postpartum* period. In eclamptic women, headaches, epigastric pain, hyperreflexia, and haemoconcentration usually precede convulsions. However at times, sudden convulsions occur in asymptomatic women with only mildly increased blood pressure (Branch and Porter, 1999). Indeed, a wide spectrum of signs and symptoms are exhibited in women who develop eclampsia (Sibai, 2002).

Symptoms

PE is a multisystem disorder characterised by poor blood perfusion to almost all organs. Thus, the occurrence of a wide variety of symptoms is not surprising, including cerebral (such as headache and mental confusion), visual (such as scotomata and blurred vision), and gastrointestinal (such as nausea and epigastric pain) symptoms (Roberts, 1984). As referred above, some of these symptoms are important indicators of the severity of the disorder. However, it is important to emphasise that the majority of women with early PE are asymptomatic (Roberts, 1984).

Incidence and Risk Factors

PE has an incidence of 3 to 7 percent in nulliparas and 0.8 to 5 percent in multiparas (Sibai, 2002). Thus, nulliparity is a risk factor for PE. Other risk factors such as a family history of PE or eclampsia, obesity, multi-foetal gestation, PE and/or poor outcome (intrauterine growth retardation, abruptio, foetal death) in previous pregnancy, new paternity, extremes of maternal age (younger than 20 and older than 35 years of age), pre-existing medical-genetic conditions (chronic hypertension, renal disease, diabetes mellitus, thrombophilias), nonimmune or alloimmune foetal hydrops, and hydatidiform mole are also described (Branch and Porter, 1999; Sibai, 2002). Moreover, results obtained in a recent study (Qiu *et al.*, 2003) suggest that women's family history of hypertension and type 2 diabetes is also a risk factor for PE.

Maternal and Perinatal Outcome with PE

PE is associated with significant maternal and perinatal mortality and morbidity worldwide, particularly in developing countries (Myatt and Miodovnik, 1999). Poor maternal and

perinatal outcomes are mainly associated with patients who suffer from early onset of PE (Myatt and Miodovnik, 1999) and those experiencing severe forms of PE (Sibai, 2002).

HELLP syndrome is often associated with serious maternal complications such as abruptio placentae, acute renal failure and disseminated intravascular coagulation (DIC) (Vigil-De Gracia, 2001a). The most serious complication is hepatic rupture, being responsible for a maternal mortality of up to 50% and a foetal mortality of 60–70% (Rath *et al.*, 2000).

PE is commonly associated with intrauterine growth restriction (IUGR) (Schjetlein *et al.*, 1997; Schjetlein *et al.*, 1999). IUGR is an important cause of infant mortality and morbidity and predisposes individuals to diseases during adulthood, such as coronary heart disease and diabetes (Newnham, 1998; Regnault *et al.*, 2002).

PE: Similarities with Atherosclerosis

Endothelial lesions in PE resemble the beginning of atherosclerosis, although the clinical course of PE is more acute than atherosclerosis, a chronic disease. Moreover, similarities in the pathophysiology of both disorders seem to exist, namely the involvement of oxidative stress (Roberts and Hubel, 1999) and endothelial dysfunction (Roberts, 1998).

Although PE does not cause later CVD, it is associated with increased risk compared with women who have been pregnant without PE (Roberts, 1998). This finding supports the existence of common risk factors between the two disorders and some of those risk factors have already been identified, which include obesity, diabetes, and hypertension. Also of interest is the fact that some changes in lipid and lipoprotein profile observed in PE are identical to those observed in dyslipidaemia predisposing to atherosclerosis. In addition, atherosclerosis is accepted as an inflammatory disease (Ross, 1999) and it was postulated that PE represents an exacerbated form of inflammation compared with normal pregnancies (Redman *et al.*, 1999). However, the well-established inflammatory characteristics of atherosclerosis do not seem to be so clear in PE.

10. Pathological and Pathophysiological Changes

The pathological and early pathophysiological changes observed in PE indicate that the disorder is present before it is clinically evident (usually in late pregnancy) (Roberts, 1998). Moreover, they also indicate that the increase in blood pressure is not the pathogenetic factor of primary importance, being rarely responsible for the multiorgan dysfunction.

Pathological Changes

The pathological changes in this disorder, observed during autopsy in women who died of eclampsia and even in biopsy specimens from women with PE, are of ischaemic nature (NHBPEP Working Group, 2000).

Uterine Vascular Changes

One of the major clinical pathologic features of PE is the shallow trophoblast invasion and inadequate spiral artery remodelling (Redman, 1993). In PE, the second wave of trophoblastic invasion into the myometrial segments of the spiral arteries is inhibited. Remodelling of the uterine spiral arteries is limited to their decidual portions, the myometrial segments of these arteries remaining anatomically intact (with their endothelial linings and muscular walls), undilated (remaining relatively narrow-bore, high-resistance vessels) and persistent to their potential for vasoconstriction.

In addition, some arteries of the uteroplacental bed are affected by a process termed acute atherosis, a lesion characterised by the accumulation of foam cells or lipid-laden macrophages, fibrinoid necrosis, fibroblast proliferation and a perivascular infiltrate. Lp(a) is found in the walls of placental bed spiral arteries that have undergone atherosis (Meekins *et al.*, 1994), and immunoglobulin and complement components are deposited in those lesions (Redman, 1993). The affected arteries may become partially or completely blocked (Zeek and Assali, 1950).

The failure of trophoblastic invasion and the presence of acute atherosis result in a reduction of blood supply to the foetal-placental unit. The clinical consequences may be foetal growth impairment, significant foetal hypoxia, or, in extreme cases, foetal death. Related to the severity of the disorder, the risk of placental abruption is also increased (Branch and Porter, 1999).

These findings on uterine vessels are typical of, but not specific to PE, as they can also be found in normotensive pregnancies complicated with IUGR (Redman, 1993).

Placenta

The failure of trophoblast invasion in the spiral arteries and the absence of the physiological adaptations have been regarded as responsible for the pathological changes in the placenta. Placentas from women with PE display an increased frequency of placental infarcts and altered morphology evidenced by abnormal cytotrophoblast proliferation and increased

formation of syncytial knots. Recently, Ishihara *et al.* (2002) demonstrated that the syncytiotrophoblast in human term placentas complicated by severe PE show increased apoptosis, which may result from decreased expression of Bcl-2 protein, an apoptosis-inhibiting gene product, in the syncytiotrophoblasts of those placentas.

It is important to note that some PEc women appear to have a normal placenta (Zhang *et al.*, 1997).

Renal Changes

A highly characteristic, although not pathognomonic, renal lesion, indicative of maternal endothelial damage, is observed in PE and is termed glomerular endotheliosis. The primary pathological change occurs in endothelial cells: these cells, which line the glomerular capillaries, are greatly increased in size and may occlude the capillary lumen. Despite this, the basement membrane is relatively normal, although it contains some inclusions, while the epithelial cell podocytes are not modified (Roberts, 1998). These changes are more consistently correlated with proteinuria than with hypertension.

Hepatic Changes

Pathological changes described in the liver include periportal haemorrhages, ischaemic lesions and fibrin deposition (NHBPEP Working Group, 2000). In patients with HELLP syndrome, fibrin deposition in the sinusoids of the liver is associated with parenchymal necrosis, haemolysis, elevated liver enzymes and upper epigastric pain. Also, in patients with HELLP syndrome, immunohistochemical analysis of liver biopsies revealed high amounts of leukocyte elastase and tumour necrosis factor (TNF)-α protein in the areas of liver cell necrosis (Wetzka *et al.*, 1999).

Pathophysiological Changes

PE is a multisystem disorder characterised by poor blood perfusion to virtually every organ, which is secondary to intense vasoconstriction due to an increased sensitivity of the vasculature to pressor agents such as angiotensin II, adrenaline and noradrenaline. There is no consistent data about the increase in the concentration of the usual pressor agents in PE. The organ perfusion is further compromised by the formation of microthrombae, resulting from the activation of the coagulation cascade (Roberts, 1998).

The magnitude of vasoconstriction is indicated by the status of volume regulatory hormones. Despite reduced plasma volume, pre-eclamptic (PEc) women have increased atrial naturetic peptide and reduced renin concentrations. These changes, which are indicative of volume overfill, suggest that despite the reduced vascular content (plasma volume) characteristic of the disorder, the vascular compartment is even more reduced (Roberts and Redman, 1993).

Additionally, although sodium retention is a predominant feature of the pathophysiology of PE, the oedema of PE is also due to "leaky vasculature". Suppression of the RAS is a well-documented feature of PE and may be a consequence rather than a cause of impaired sodium excretion (Roberts, 1998). The well-known changes in the RAS do not seem to be found in patients with HELLP syndrome (Bussen *et al.*, 1998).

11. Aetiology and Pathogenesis

PE has been termed the "disease of theories", reflecting the misunderstanding that surrounds the aetiology and pathogenesis of PE (Branch and Porter, 1999). However, there are three main aetiologic factors believed to be involved in the development of PE: 1) deficient trophoblast invasion, 2) immune maladaptation, and 3) genetic predisposition (Dekker and Sibai, 1998; Branch and Porter, 1999; Wilson *et al.*, 2003). Moreover, there are well-accepted pre-disposing factors (referred above) as well as information supporting the contribution of several areas in the pathogenesis of the disorder. For instance, there is evidence to suggest that endothelial dysfunction and oxidative stress are implicated in the pathophysiology of PE.

Deficient Trophoblast Invasion

A possible reason for PE is deficient trophoblast invasion, which may be caused by a failure of the cytotrophoblast cells to express the adhesion molecules necessary for the normal remodelling of the maternal spiral arteries (Zhou *et al.*, 1997a).

Angiogenic growth factors, as regulatory molecules in placental development and function, are also potential candidates involved in deficient trophoblast invasion (Regnault *et al.*, 2002). The altered uteroplacental angiogenesis may be a consequence of abnormal expression of vascular endothelial growth factor (VEGF) isoforms (Regnault *et al.*, 2002; Tsatsaris *et al.*, 2002).

Immune Maladaptation

Epidemiologic studies indicate a possible involvement of immunologic factors in the pathogenesis of this disorder. Actually, PE has a higher incidence in the first pregnancies, seldom recurring in subsequent pregnancies. The risk may also increase with changing paternity (Li and Wi, 2000) and when condoms are used as a primary form of contraception (Taylor, 1997). On the other hand, an extended length of sexual cohabitation (without barrier contraceptives) before conception (Robillard *et al.*, 1999) and history of prior blood transfusion (Taylor, 1997) are associated with a lower risk of PE.

Also consistent with this hypothesis is the fact that there are increased levels of inflammatory cytokines in the placenta and maternal circulation in PE, as well as evidence of increased natural killer cells and neutrophil activation (Taylor, 1997).

Moreover, placental tissues from pregnancies complicated with PE may express less or different HLA-G proteins, resulting in the breakdown of maternal tolerance to the placenta (Taylor, 1997). It has been postulated that acute atherosis may be an immunologically mediated lesion (Redman, 1993). The atherotic change is quite similar to the vascular changes of allograft rejection.

Genetic Predisposition

PE has a higher incidence within families with a history of such a disorder, suggesting a genetic predisposition to PE (Chesley *et al.*, 1968; Sutherland *et al.*, 1981; Cincotta and Brennecke, 1998). Despite this, the pattern of inheritance of PE remains uncertain. Most data suggest that maternal genotype is the major responsible for the susceptibility, but some studies have ascribed an influence to the foetus and thus a contribution of the father (Cooper *et al.*, 1988; Lie *et al.*, 1998). Supporting this theory, the changes in paternity seem to be a risk factor for PE in multiparous women (Li and Wi, 2000). It is not clear whether the maternal genotype or the foetal genotype, or even some combination of both, is the cause of the genetic predisposition (Wilson *et al.*, 2003).

Endothelial Dysfunction

Dysfunction of the endothelial cell is proposed as an important mechanism in the pathogenesis of PE (Roberts, 1998). This may explain many of the pathophysiological changes observed in this syndrome, such as increased sensitivity to pressors, activation of coagulation, and loss of vascular integrity. Evidence exists that endothelial dysfunction is an

early event in PE, suggesting that endothelial dysfunction is a possible cause rather than a result of PE (Granger *et al.*, 2001; Roberts and Lain, 2002).

Although there is morphological evidence of endothelial injury in PE, this finding cannot be generalised for all vascular beds. It seems more appropriate to consider the altered endothelial function in PE as endothelial dysfunction rather than damage or injury (Roberts, 1998). The mechanism by which endothelial cell dysfunction arises is not yet well established.

Oxidative stress

Oxidative stress (an imbalance between oxidant and antioxidant forces in favour of oxidants) provides a plausible explanation for the endothelial dysfunction and subsequent pathophysiological changes of PE (Hubel, 1998; Walsh, 1998). It has been hypothesised that hypoxia at the foetal-maternal interface results in the generation of free radicals that may lead to oxidative stress dependent on the maternal constitution (Roberts and Hubel, 1999). Reactive oxygen species or their metabolites ultimately compromise the "defensive" vasodilatory, anti-aggregatory and barrier functioning of the vascular endothelium.

Abundant evidence of oxidative stress in blood and tissues of women with PE support this hypothesis (Roberts *et al.*, 2003). Further support comes from a small study, in which the administration of antioxidants (vitamins C and E) from early gestation (16–22 weeks) to women at increased risk of PE reduced the frequency of the disorder (Chappell *et al.*, 1999b). However, this finding requires confirmation in larger studies. Moreover, trials are needed to show whether vitamin supplementation affects the occurrence of PE in low-risk women.

Pathogenesis of PE –The Prevailing Hypothesis

One of the most popular current hypotheses regarding the pathogenesis of PE is based in the "hypoperfusion" model (Zhang *et al.*, 1997). This hypothesis may result from a combination of the already described factors and/or features. In PE, immune maladaptation or some defect in the cytotrophoblasts themselves might lead to inadequate cytotrophoblast invasion with the resulting lack of physiological change in the spiral arteries. Consequent ischaemia may result in the liberation of circulating factor(s) by the underperfused trophoblast into the maternal circulation, producing endothelial dysfunction and ultimately leading to PE (Granger *et al.*, 2001). Alternatively, formed blood elements such as platelets and neutrophils may be activated by passage through the intervillous space and secondarily act on endothelium

(Roberts, 1998). Cytokines and products of oxidative stress are candidate molecules involved in endothelial dysfunction (Sattar *et al.*, 1996; Scalera, 2003).

It is evident that the component of pregnancy leading to the disorder is the placenta. Evidence implicating the placenta as the central focus in the aetiology of PE is the recovery that patients experience following delivery. However, reduced placental perfusion *per se* is not sufficient to account for the disorder, as a great proportion of women show no evidence of IUGR. Moreover, PE seems to be more common in obese and insulin resistant women, who have large babies (Roberts, 1998). Thus, it is possible that pre-existing maternal constitutional factors (genetic/environmental) interact with reduced placental perfusion to contribute to the pathogenesis of PE (Roberts and Hubel, 1999). Maternal factors, such as obesity, insulin resistance, dyslipidaemia, hyperhomocisteinaemia, hypertension, coagulopathies and inflammation are independent risk factors for endothelial dysfunction. They may also work as risk factors for PE.

12. Laboratory Findings in Maternal Blood

Considering the wide and high number of laboratory findings reported in PE, the following summarises some of those. It is also important to note that major changes in the studied variables occur in severe PE, with mild forms presenting minimal or no changes (Roberts, 1984).

Endothelium Function and Haemostasis

Multiple markers of vascular endothelial damage or dysfunction have been reported in PE, including decreased circulating concentrations of prostacyclin (Wang *et al.*, 1991b), and elevated levels of endothelin-1 (Nova *et al.*, 1991; Barden *et al.*, 1997; Rogers and Thorp, 1997), cellular fibronectin (Friedman *et al.*, 1995; Shaarawy and Didy, 1996; Powers *et al.*, 1998; Bellart *et al.*, 1999; Islami *et al.*, 2001), thrombomodulin (Shaarawy and Didy, 1996; Kobayashi *et al.*, 1998), vWf (Friedman *et al.*, 1995), and circulating cell adhesion molecules [namely intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, E-selectin (Austgulen *et al.*, 1997; Krauss *et al.*, 1997), and platelet endothelial cell adhesion molecule-1 (Krauss *et al.*, 1997)]. Many of those markers (such as cellular fibronectin, thrombomodulin, ICAM-1, and VCAM-1) may be predictors of PE, since elevations in their concentrations were detected at weeks to months before the clinical manifestation of PE (Granger *et al.*, 2001). In addition, some of those biomarkers may also

reflect the severity of the disorder. Endothelin-1 levels were reported to be significantly higher in PEc pregnancies with HELLP syndrome than those without the syndrome (Nova *et al.*, 1991).

Rodgers *et al.* (1988) found that serum from PEc women is more cytotoxic than that of healthy pregnant women to endothelial cells *in vitro*. Thus, in PE a circulating factor may be responsible for the modifications observed in the endothelial cell.

The endothelial cell damage or activation is believed to play a central role in PE, and may underlie the haemostatic changes observed in this syndrome (Roberts, 1998; Robson, 1999). While normal endothelial cells participate in the regulation of haemostasis, disturbed vascular cells may express prothrombotic properties promoting pathologic events (Rodgers, 1988; van Hinsbergh, 2001). The imbalance of haemostasis observed in normal pregnancy seems to be further pronounced in PE (Roberts, 1984; Perry and Martin, 1992; Branch and Porter, 1999). PE is characterised by an enhanced hypercoagulable state, with activation of the coagulation cascade. The activation of haemostasis is evidenced by platelet activation (release of platelet contents, namely β-thromboglobulin), increased platelet size (indicating increased platelet turnover), consumption of pro-coagulants, reduced anti-thrombin III, increased thrombin-antithrombin complexes and increased fibrin degradation products (Perry and Martin, 1992). In addition, thrombocytopenia is a common haematologic abnormality in women with PE, whereas plasma fibrinogen levels are rarely reduced in this disorder (although hypofibrinogenaemia may occur in severe cases) (Sibai, 2002).

The fibrinolytic system is also altered in PE. A significant increase in PAI-1 antigen (Estelles *et al.*, 1989; Estelles *et al.*, 1991; Halligan *et al.*, 1994; Friedman *et al.*, 1995) and PAI-1 activity (Estelles *et al.*, 1989; Estelles *et al.*, 1991; Shaarawy and Didy, 1996; Schjetlein *et al.*, 1997; Schjetlein *et al.*, 1999) as well as t-PA antigen (Estelles *et al.*, 1991; Friedman *et al.*, 1995) has been observed in PE and these may be other markers of endothelial dysfunction. On the other hand, PAI-2 levels are decreased both in PE (Estelles *et al.*, 1989; Halligan *et al.*, 1994; Schjetlein *et al.*, 1997; Schjetlein *et al.*, 1999) and IUGR (Estelles *et al.*, 1991) and may be a marker of placental function, as its concentrations seem to correlate with birth weight (Estelles *et al.*, 1991). D-dimer levels show a tendency to be elevated in PE, particularly in severe PE (Schjetlein *et al.*, 1997; Schjetlein *et al.*, 1999). Of interest, Bellart *et al.* (1999) found a significantly higher level of D-dimer in PE but they also found a higher ratio of fibrinopeptide A to D-dimer, indicating that the activation of coagulation is associated with relative hypofibrinolysis.

Acute Phase Proteins and Liver Function Tests

It is already described that in cases with HELLP syndrome the activities of LDH, ALT, and AST are dramatically increased. Even in women with PE without HELLP syndrome, mean activities of both AST and ALT may be significantly higher compared with normal pregnancies (Rebelo *et al.*, 1996). In contrast, haptoglobin is decreased in cases of HELLP syndrome but the mean levels of this APP seem to be similar in normal pregnant women and in PEc women without HELLP (Rebelo *et al.*, 1996).

Albumin and transferrin are two negative APPs and both albumin (Barden *et al.*, 1997; Barden *et al.*, 2001) and total transferrin (diferric transferrin and apotransferrin) levels (Hubel *et al.*, 1996a) are decreased in PE. Caeruloplasmin, a positive APP, was reported to be significantly higher in PEc women (although ferroxidase activity of caeruloplasmin was not higher) (Vitoratos *et al.*, 1999) but others did not find differences in its concentration (Hubel *et al.*, 1996a).

An APP that has merited special attention from investigators is CRP. The advent of reproducible, high-resolution CRP assays, has allowed clinicians to explore the potential role of low-grade inflammation in predicting inflammatory diseases. Such approach was attempted in PE but no consistent conclusions were obtained. A significant association between elevated CRP levels in the first trimester and subsequent development of PE was recently proposed (Wolf *et al.*, 2001), although such association was mitigated after adjustment for body mass index (BMI). The authors concluded that inflammation could be part of a causal pathway through which obesity predisposes to PE. In contrast to those findings, another recent study reported that serum levels of CRP, at 23–25 weeks of gestation, were similar in pregnant women who subsequently developed PE and in women without complications of pregnancy (Savvidou *et al.*, 2002). Others, by using less sensitive CRP assays, found significantly higher CRP values in women with established PE but no reference was made to maternal weight or BMI (Rebelo *et al.*, 1996; Teran *et al.*, 2001).

Lipids and Lipoproteins

In complicated pregnancies the mechanisms regulating physiological hyperlipidaemia may disrupt. Hypertensive disorders of pregnancy are associated with increased TG levels (Potter and Nestel, 1979; Kokia *et al.*, 1990). There is considerable agreement that PE is associated with higher free fatty acids (Hubel *et al.*, 1996b; Murai *et al.*, 1997; Hubel *et al.*, 1998a) and TG levels (Rosing *et al.*, 1989; Hubel *et al.*, 1996b; Murai *et al.*, 1997; Sattar *et al.*, 1997a; Hubel *et al.*, 1998a; Ware-Jauregui *et al.*, 1999; Wetzka *et al.*, 1999; Wakatsuki *et al.*, 2000),

although it seems that HELLP syndrome is not accompanied by the same degree of hypertriglyceridaemia (Wetzka *et al.*, 1999). The raised TG concentrations in PE are associated with markedly elevated concentrations of VLDL in the circulation (Sattar *et al.*, 1997a; Wetzka *et al.*, 1999). Moreover, free fatty acids and TG seem to be elevated in early pregnancy in women predisposed to develop PE (Hubel, 1998).

There is also some consistent evidence that mean levels of Chol (Rosing *et al.*, 1989; Hubel *et al.*, 1996b; Sattar *et al.*, 1997a; Ware-Jauregui *et al.*, 1999; Wetzka *et al.*, 1999; Wakatsuki *et al.*, 2000) and LDLc (Hubel *et al.*, 1996b; Ware-Jauregui *et al.*, 1999; Wetzka *et al.*, 1999; Wakatsuki *et al.*, 2000) are similar in normal and PEc pregnancies. However, the pattern of change in other variables is still controversial.

It has been observed that TG levels in the LDL fraction are higher in pregnancy-induced hypertension patients compared to controls (Franz and Wendler, 1992). In PE, others have also reported that TG levels are higher in other lipoprotein subclasses, especially VLDL, IDL, and HDL (Wetzka *et al.*, 1999). In addition, in this disorder, it was described that the TG content relative to the apo B is significantly increased in the LDL fraction (Wetzka *et al.*, 1999), and that the LDL profile is dominated by the most buoyant LDL, with the most dense LDL being significantly decreased (Winkler *et al.*, 2003). It has been speculated that TG enrichment of lipoproteins in PE may be caused by an increased insulin resistance or by reduced maternal lipase activities (reduced TG catabolism), resulting in the accumulation of buoyant lipoproteins (Wetzka *et al.*, 1999). In contrast to these reports, others found no differences in LDL content of TG and that pre-heparin HL activity and small, dense LDL were increased in PE (Sattar *et al.*, 1997a). In agreement with this, LDL-PPD was reported to be significantly decreased in PE relative to normal pregnancy (Hubel *et al.*, 1998a). Moreover, in PE, LDL particles were shown to be more prone to *in vitro* oxidation, as assessed by incubation with copper ions and evaluated by monitoring the kinetics of conjugated diene formation (Wakatsuki *et al.*, 2000); none of those reports has evaluated circulating levels of Ox-LDL.

When antibodies to Ox-LDL were measured, higher titres of serum autoantibodies to an epitope of Ox-LDL, malondialdehyde-conjugated LDL (MDA-LDL), were described in PEc patients relative to controls admitted for term labour (Branch *et al.*, 1994). However, a negative report for PEc patients with or without HELLP syndrome also exists (Armstrong *et al.*, 1994). Others, by measuring antibodies against both MDA-LDL and copper-oxidised LDL, found antibody levels to copper-oxidised LDL, but not against MDA-LDL, to be significantly higher in PE compared with normotensive controls (Uotila *et al.*, 1998).

Similarly, no agreement exists concerning changes in HDLc and Lp(a) levels in PE. Some authors found no differences in HDLc between normal and PEc women (Hubel *et al.*, 1996b; Wetzka *et al.*, 1999; Wakatsuki *et al.*, 2000), whereas others reported significantly lower values for PE (Rosing *et al.*, 1989; Sattar *et al.*, 1997a; Ware-Jauregui *et al.*, 1999). Concerning Lp(a), it has been claimed that circulating levels of Lp(a) are markedly increased in PE compared to normal pregnant controls, and that the concentrations of Lp(a) are associated with the severity of the disorder (Wang *et al.*, 1998; Mori *et al.*, 2003). A recent study reported that plasma Lp(a) levels are significantly higher in PE compared to controls in the third trimester of gestation, but increased levels of Lp(a) were not detected before the appearance of the disorder in pregnant women at risk (Bar *et al.*, 2002). Sattar *et al.* (2000) and Wetzka *et al.* (1999) did not find differences in Lp(a) levels between PEc and normal pregnant groups. In addition, Leerink *et al.* (1997) found no differences either in Lp(a) levels or in apolipoprotein(a) phenotype between non-pregnant women with or without a history of PE.

Uric Acid

It is widely accepted that uric acid levels are higher in PE compared with normotensive controls (Hubel *et al.*, 1996b; Rebelo *et al.*, 1996; Barden *et al.*, 1997; Murai *et al.*, 1997; Hubel *et al.*, 1998a; Wakatsuki *et al.*, 2000). Moreover, several authors suggest that high serum uric acid correlates with the severity of PE and with foetal outcome (Hill, 1978; Many *et al.*, 1996). Thus, uric acid levels are frequently elevated in severe PE and HELLP syndrome (Freund and Arvan, 1990). Although the most commonly accepted explanation for hyperuricaemia of PE is an increased reabsorption and a decreased excretion of uric acid in the proximal tubules, some mechanisms do occur in PE that can lead to increased production of uric acid and thus may also contribute to hyperuricaemia (Many *et al.*, 1996). In severe forms of PE, serum uric acid elevations appear to be out of proportion to the degree of associated renal failure and may result from extensive tissue necrosis occurring in some patients (Freund and Arvan, 1990).

Considering the hypothesis of increased production of uric acid in PE, it has been suggested that elevated levels of uric acid may be a marker of free radical generation, which contributes to the increased oxidative stress observed in this syndrome (Many *et al.*, 1996). On the other hand, uric acid can act as an antioxidant (Halliwell and Gutteridge, 1990) and therefore increased levels may also have a protective role (Many *et al.*, 1996).

Lipid Peroxidation *versus* Serum or Plasma Antioxidants

In PE, evidence suggesting an exacerbation of lipid peroxidation is supported by reports demonstrating elevated levels of conjugated dienes (Uotila *et al.*, 1993), malondialdehyde (Uotila *et al.*, 1993; Hubel *et al.*, 1996b; Wu, 1996; Gratacós *et al.*, 1998a; El-Salahy *et al.*, 2001), and F_2-isoprostanes (Barden *et al.*, 2001).

A decrease in the antioxidant defences has also been reported in PE. It has been reported that serum total antioxidant status levels (Shaarawy *et al.*, 1998) and plasma/serum vitamin E levels (Gratacós *et al.*, 1998a; El-Salahy *et al.*, 2001) are significantly lower in PE than that in healthy pregnant women. Using the brain homogenate auto-oxidation bioassay, the antioxidant activity of serum was reduced by approximately half in women with PE compared with pregnant controls (Davidge *et al.*, 1992). This primarily expresses a decrease in the preventive antioxidants caeruloplasmin and transferrin. It seems that the plasma of PEc women shows a loss of caeruloplasmin ferroxidase activity and a reduction in total iron binding capacity (Vitoratos *et al.*, 1999). PE is characterised by increased serum iron (Hubel *et al.*, 1996a; Vitoratos *et al.*, 1999) and decreased total transferrin with concomitant increased transferrin saturation and consequent decreased iron-binding reserve (apotransferrin, unsaturated iron-binding capacity) (Hubel *et al.*, 1996a).

Confounding these observations, some studies found no differences in circulating levels of conjugated dienes (Poranen *et al.*, 1996), lipid hydroperoxide (Morris *et al.*, 1998; Diedrich *et al.*, 2001), malondialdehyde (Davidge *et al.*, 1992; Morris *et al.*, 1998), and isoprostanes (Morris *et al.*, 1998). Furthermore, no differences were found in vitamin E (Uotila *et al.*, 1993; Rebelo *et al.*, 1996; Morris *et al.*, 1998), vitamin C (Rebelo *et al.*, 1996) or selenium levels (Uotila *et al.*, 1993). In addition, others reported that vitamin E (Schiff *et al.*, 1996), GPX activity (Uotila *et al.*, 1993) and the peroxyl radical trapping capacity (Poranen *et al.*, 1996) are higher in the plasma/serum of PEc patients. A possible explanation for the vitamin E increase is the marked hyperlipoproteinaemia characteristic of PE (Hubel, 1997). The rise in peroxyl radical trapping capacity is speculated to be due mainly to the increased concentration of uric acid in PE (Hill, 1978).

Plasma Volume and RBC

In severe PE, reduction in plasma volume may be indicated by a rapid increase in Ht, whereas in mild PE the Ht is not usually elevated. Indeed, some studies have demonstrated no differences in mean Ht between mild PE and healthy pregnancies (Hubel *et al.*, 1996a; Hubel *et al.*, 1996b; Rebelo *et al.*, 1996; Teran *et al.*, 2001).

Regarding antioxidant defences, lower erythrocyte glutathione levels (Knapen *et al.*, 1999) and higher erythrocyte GPX activity (Uotila *et al.*, 1993; Diedrich *et al.*, 2001) were reported in patients with PE; the highest GPX activities were described in patients with HELLP syndrome (Diedrich *et al.*, 2001). However, no significant differences were found in the activities of glutathione reductase, glutathione S-transferase or superoxide dismutase in RBCs between healthy pregnant women and PEc women with or without HELLP (Diedrich *et al.*, 2001). In PE, increased GPX activity and decreased glutathione suggest a protective role against increased oxidative stress, implicating an enhanced turnover of glutathione (Knapen *et al.*, 1999).

A reduced RBC deformability was also observed in women with PE and/or IUGR and may contribute to the reduced microcirculation in these pathologies (Schauf *et al.*, 2002). Reduced RBC deformability may be induced by changes in membrane properties initiated by high levels of free oxygen radicals (Schauf *et al.*, 2002).

Moreover, oxidative damage and changes in membrane deformability may result in enhanced haemolysis. However, data on erythrocyte susceptibility to lysis in PE is somewhat conflicting. Spickett *et al.* (1998) found that erythrocytes from PEc patients undergo more lysis than erythrocytes from control pregnant women when incubated with hypotonic saline. *In vivo*, the described increase in serum iron (Hubel *et al.*, 1996a; Vitoratos *et al.*, 1999) is compatible with a higher (but silent) haemolysis or an increased RBC turnover in PE (Hubel, 1998). In contrast, others found a greater resistance to haemolysis in PE, which was accompanied by lower levels of plasma haemoglobin (Rebelo *et al.*, 1996).

RBC morphology is also altered in PE, particularly in HELLP syndrome. Fragmented RBCs are presented in patients with HELLP syndrome and the fragmentation of RBCs results from high-velocity passage through the damaged endothelium where a fibrin matrix forms (Magann and Martin, 1999).

Leukocytes and the Immune System

Leukocytes have been implicated in PE. However, the exact changes in these cells in PE remain to be determined.

PEc women present with a significantly elevated leukocyte count (mainly due to neutrophil count) compared with normal pregnant women, but this finding is not consistent in published research (Rebelo *et al.*, 1996; Barden *et al.*, 1997; Kobayashi *et al.*, 1999; Teran *et al.*, 2001).

Confusing results have been obtained when assessing PMN or neutrophil priming and/or activation, either by evaluating cell surface adhesion molecule expression (Barden *et al.*, 1997; Sacks *et al.*, 1998; Crocker *et al.*, 1999) or by measuring oxygen radical production in those cells (Tsukimori *et al.*, 1993; Sacks *et al.*, 1998; Crocker *et al.*, 1999; Zusterzeel *et al.*, 2001).

In addition, several reports using indirect techniques have shown that neutrophils are activated in PE through demonstrating significantly higher plasma levels of elastase (Greer *et al.*, 1989; Halim *et al.*, 1996; Kobayashi *et al.*, 1998) and defensins (Prieto *et al.*, 1997), but none of those reports calculated the ratios of neutrophil activation products *per* number of neutrophils. Moreover, there is no agreement concerning lactoferrin changes in this syndrome (Rebelo *et al.*, 1996; Prieto *et al.*, 1997; Crocker *et al.*, 1999).

Cytokines may also be involved in this process. Granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates granulo-monocytopoiesis (Edwards, 1994a) and both GM-CSF and TNF-α are capable of priming and activating neutrophils (Edwards, 1994b). Some authors found increased plasma levels of TNF-α in PEc women (Visser *et al.*, 1994; Teran *et al.*, 2001; Serin *et al.*, 2002), whereas others found no differences (Tsukimori *et al.*, 1993; Greer *et al.*, 1994). Regarding GM-CSF, Greer *et al.* (1994) and Tsukimori *et al.* (1993) found no differences in its level between normal and PEc pregnancies, whereas Gratacós *et al.* (1998b) reported significantly lower values for PE. Thus, conflicting data also exists when evaluating inflammatory cytokines. It would be interesting to measure cytokines together with products released from activated neutrophils.

The number of white blood cells (WBCs) was significantly higher in patients with HELLP syndrome than in patients with severe PE without HELLP syndrome; moreover, WBC count increased in proportion to the HELLP syndrome severity (Terrone *et al.*, 2000). In addition, plasma TNF-α concentrations were reported to be significantly higher in HELLP syndrome when compared to patients with PE without HELLP (Visser *et al.*, 1994).

Genetics

Considering that a family history of PE is a risk factor of PE, several studies have evaluated a possible link between PE and specific genetic mutations.

Several genetic mutations were studied, including a molecular variant of angiotensinogen, factor V Leiden mutation, which causes resistance to activated protein C,

and mutations in methyltetrahydrofolate reductase and the prothrombin gene. However, no consistent findings were reported (Lachmeijer *et al.*, 2002; Sibai, 2002; Wilson *et al.*, 2003).

Some attempts were made to define the immunogenetics of PE. Although the expression of trophoblast HLA-G is reduced in PE, HLA-G genotypes were not found to differ among PEc and normal pregnant women (Taylor, 1997). Moreover, the analysis of a TNF-α promoter polymorphism (–308G/A) has led to conflicting results. Indeed, one study found an association between the TNF1 (TNF –308G) allele and PE, suggesting a major role for TNF-α in mediating endothelial dysfunction in PE, but others failed to confirm this association (Lachmeijer *et al.*, 2002).

Also, apo E and CRP genes are candidate genes associated with this disorder. Indeed, PE is associated with abnormal lipid profiles and possibly a higher inflammatory response to pregnancy. No previous studies have investigated the frequency of CRP alleles, of the 1059G/C polymorphism, among women with PE. On the other hand, although earlier reports have already focused on the association of apo E with this disorder, conclusions remain controversial. Some authors have noticed a higher frequency of the ε2 and ε4 alleles in women with PE (Williams *et al.*, 1996; Nagy *et al.*, 1998), but these findings have not been corroborated by others (Chikosi *et al.*, 2000; Makkonen *et al.*, 2001).

13. Therapy

The only efficacious therapy for PE is the delivery. Thus, in pregnancies with advanced gestational age (at or near term), in which the cervix is favourable, labour should be induced. In PE remote from term a much more difficult problem is presented. The decision between immediate delivery and expectant management will depend on the severity of the disorder and the length of gestation (Branch and Porter, 1999). The primary considerations of therapy must always be the safety of the mother and thereafter, the delivery of a live, mature newborn, who will not require intensive and prolonged neonatal care.

Bed rest, anticonvulsant and antihypertensive therapies are frequently used in PE, especially in the presence of severe forms (Kelley, 1999).

The treatment of hypertension remote from delivery is for maternal benefit, preventing intracraneal haemorrhage and damage to other vital organs. However no evidence exists to suggest that such therapy is beneficial to the foetus (NHBPEP Working Group, 1990). If therapy is elected, drugs such as hydralazine (vasodilator), methyldopa (central adrenergic

antagonist), labetalol (α and β-blocker), and nifedipine (calcium channel blocker) are frequently used.

Magnesium sulphate is the drug of choice to prevent or treat eclamptic convulsions (Kelley, 1999). Magnesium sulphate has antihypertensive properties (magnesium ion is a direct smooth-muscle relaxant at relatively high concentrations) and has a vasodilating effect on the middle cerebral artery (Kelley, 1999). The large Magpie trial (The Magpie Trial Collaborative Group, 2002) showed that magnesium sulphate reduced the risk of eclampsia in women with severe PE, but it is uncertain whether the administration of magnesium sulphate to patients with well-defined mild PE presents clinical benefits (Scott, 2003).

In HELLP syndrome, *antepartum* administration of corticosteroids improves platelet count, reduces liver enzyme abnormalities, and prolongs latency to delivery (O'Brien *et al.*, 2000).

PART II

RESEARCH PROJECT

Section One: Objectives

The aim of this project was to assess circulating biochemical variables in the maternal body during human pregnancy in a Portuguese population in order to evaluate and/or clarify:

- The effect of pregnancy on such variables, by comparing non-pregnant and normal pregnant women;
- Changes in those variables throughout gestation, by studying normal pregnant women throughout gestation;
- Changes occurring in PE, by comparing normal pregnant women and women with PE;
- The association of genetic variants not only with levels of some of those variables but also with PE.

Several biochemical variables were quantified in order to detect changes occurring in different tissues. In particular, attention was given to variables that might be altered as a consequence of the inflammatory process resulting from implantation, in both normal and PEc pregnancies.

The evaluation of some APPs, including CRP and haptoglobin, was performed. As APPs are produced by the liver, transaminases (aspartate aminotransferase and alanine aminotransferase) were quantified to assess liver function. Moreover, APPs are involved in haemostasis and variables related to coagulation (fibrinogen and platelets) and to fibrynolysis (t-PA, PAI-1, and D-dimer) were determined. Inflammation induces modifications in lipid and lipoprotein metabolism, and therefore the traditional lipid profile, together with the quantification of LDL size (peak and mean particle diameters) and Ox-LDL were also performed. Leukocytes, as major participants in inflammation, were quantified (total and differential count) and neutrophil activation was assessed by evaluating the plasma levels of elastase and lactoferrin. The erythrocyte was used as a model of cumulative damage imposed by the inflammatory process. The determination of erythrocyte GPX activity (indicator of RBC age), of serum total bilirubin and haptoglobin (indicators of RBC haemolysis), and of membrane bound haemoglobin and erythrocyte band 3 profile (indicators of oxidative and proteolytic stress) were all tested. In order to assess renal function, uric acid was determined,

which can also be interpreted as a contributor to the total antioxidant status. Moreover, the evaluation of uric acid was also performed taking in consideration that it may act as a marker of the severity of PE.

The determination of the referred hepatic enzymes as well as of platelet count and serum haptoglobin is also justified by the definition of HELLP syndrome used in this work.

In the genetic study, two polymorphic regions were selected (one associated with the CRP gene and the other with the apo E gene) in order to investigate their potential involvement with PE. Another intention was to evaluate how both polymorphic regions may influence circulating variables (CRP, lipids and lipoproteins) in pregnant women.

Section Two: Materials and Methods

14. Biological Material

Studied Population

The studies were carried out on non-pregnant and pregnant (normal and PEc) women between October 1998 and October 2001. The protocol was approved by the Committee on Ethics of the University Hospital S. João, Porto, and informed consent was obtained from each participating subject. All pregnant women were clinically evaluated by the medical staff of the Obstetrics Service, Hospital S. João.

- For the control group of pregnancy, nulliparous women were selected, who were normotensive, without known medical disorders and not under any medication (in particular hormonal).
- For the study of normal pregnancy, primigravid women were selected, who were normotensive and without known medical disorders, at different stages of gestation (different gestational ages). Normal pregnancy was diagnosed on the basis of clinical and ultrasound findings. The pregnant women were divided into three groups based on gestational age: first trimester (gestation 1.0–13.0 weeks), second trimester (gestation 13.1–25.0 weeks), and third trimester (gestation 25.1–40.0 weeks). Considering the erythropoietic demands in pregnancy, pregnant women were supplemented with appropriate nutrients, therefore avoiding confounding factors in erythrocyte changes. A daily folic acid (10 mg) supplementation during the first trimester of gestation and a daily supplementation of folic acid (1 mg) plus ferrous sulphate (90 mg of elemental iron) from the second trimester onwards, were used.
- A group of PEc women in the third trimester of gestation was also studied. For this group, primigravid women were selected following the ACOG definition of PE (gestational hypertension accompanied by proteinuria and/or oedema) (NHBPEP Working Group, 1990). Diagnosis of HELLP syndrome was made according to the Mississippi triple classification. After evaluation of patients at

emergency, women with no indication of immediate delivery were admitted and considered on a case by case basis, for administration of anti-hypertensive therapy and a low salt diet. Patients presenting medical disorders other than PE were excluded from the study. None of the evaluated women presented signs of haemorrhage or foetal suffering at admission.

Performed Studies

Normal Pregnancy

To evaluate the different parameters throughout pregnancy, two different studies were performed: a cross-sectional and a longitudinal.

i) Cross-sectional Study

In the cross-sectional study four independent groups were involved: non-pregnant (n = 24), pregnant women in the first (n = 64), second (n = 48) and third (n = 67) trimesters. In this study, the inclusion of a non-pregnant (control) group also allowed the evaluation of the effect of pregnancy on the referred parameters.

ii) Longitudinal Study

In the longitudinal study twenty-three healthy pregnant women were sequentially evaluated throughout the three trimesters of gestation.

PEc Pregnancy

To compare normal and PEc women, a cross-sectional study was performed. PEc women studied in the third trimester of gestation (n = 51) were compared with the matched controls obtained in the cross-sectional study of normal pregnancy.

Sample Collection

Blood samples were always collected in the morning (9.00–11.00 am) and on a non-fasting basis. Blood obtained by venipuncture was kept at 4°C until processed within 2 h of collection:

- Serum was obtained and aliquots were made and immediately stored at –70°C until assayed.

- Whole blood (collected in tubes containing EDTA) was used for haematological procedures. This was also used to obtain plasma and the buffy-coat, both required to perform laboratory determinations. The buffy-coat as well as aliquots of whole blood and plasma were stored at –70°C until assayed.

15. Methods

Acute Phase Proteins and Liver Function Tests

C-Reactive Protein (CRP)

The quantification of serum CRP was made using a high-sensitivity, two-site enzyme-linked immunoassay (ELISA), following an adaptation of the method of Highton and Hessian (1984).

The ELISA plate was coated with a polyclonal antibody against human CRP (Rabbit anti-human CRP, Dako). The plate was incubated (100 μl/well) with this capture antibody, diluted to a protein concentration of 10 μg/ml in coating buffer (0.01 M phosphate buffer, 0.145 M NaCl; pH 7.2), for at least an overnight period, at 4°C. The plate was washed with a buffer [0.01 M phosphate buffer, 0.5 M NaCl, 0.1% (w/v) Tween 20; pH 7.2] three times, with a 3 min soak between each wash. 100 μl of sample (diluted 1:100 in wash buffer) and standards (Human serum CRP calibrator, Dako) in the following concentrations: 0.1070, 0.0535, 0.0214, 0.0107, 0.0054 and 0.0011 mg/l (dilutions made in wash buffer) were applied to the plate in duplicate and incubated for 16 h at 4°C. The plate was washed three times as described above and incubated with the second antibody (Peroxidase-conjugated rabbit anti-human CRP, Dako) –in each well, 100 μl of the antibody, diluted 1:3,000 in wash buffer, was applied. Following an incubation of 60 min at room temperature, the wash procedure was repeated, as described above.

The revelation was made by using *o*-phenylenediamine (OPD tablets, 2 mg, for ELISA, Dako) in the presence of H_2O_2. For each ELISA plate, the following solution was prepared: 4 OPD tablets dissolved in 12 ml of distilled water, to which 5 μl 30% H_2O_2 was added before 100 μl of this substrate solution was applied *per* well. The enzymatic reaction was developed for a period of 15 min in darkness and was stopped by the addition of 0.5 M H_2SO_4 (100 μl/well).

The reaction was quantified spectrophotometrically in an ELISA plate reader (MRX, Dynatech) at 490 nm with background correction at 620 nm. The software of the ELISA plate

reader plotted the absorbance (ordinate) as a function of standard CRP concentrations (abcissa), in a Log/Lin axes scaling. The results were automatically calculated using a sigmoid curve fit ($y = ((A - D)/(1 + (x/C)^B)) + D$) and considering the sample dilution.

Haptoglobin

Serum haptoglobin was measured by immunonephelometry on a Dade Behring nephelometer (BN II), using protein standards and reagents (N Antiserum to Human Haptoglobin) from the manufacturer. The haptoglobin in the sample forms immune complexes with the specific antibody. A beam of light is scattered by the complexes formed. The intensity of scattered light is proportional to the concentration of protein in the sample. The result is evaluated by using a standard of known concentration.

Aspartate Aminotransferase (AST or ASAT; Formerly Glutamate Oxaloacetate Transaminase, GOT) and Alanine Aminotransferase (ALT or ALAT; Formerly Glutamate Pyruvate Transaminase, GPT) Activities

The activities of AST and ALT in serum were evaluated by kinetic tests at 340 nm in an auto-analyser (Cobas Mira S, Roche) using commercially available kits (AST and ALT, ABX Diagnostics, respectively). The determination of transaminase activity followed reactions A and B for AST and reactions C and D for ALT.

$$\text{L-Aspartate} + \alpha\text{-ketoglutarate} \xrightleftharpoons{\text{AST}} \text{oxaloacetate} + \text{L-glutamate} \qquad \text{(A)}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{MDH}} \text{L-malate} + \text{NAD}^+ \qquad \text{(B)}$$

MDH: Malate dehydrogenase

$$\text{L-Alanine} + \alpha\text{-ketoglutarate} \xrightleftharpoons{\text{ALT}} \text{pyruvate} + \text{L-glutamate} \qquad \text{(C)}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{LDH}} \text{L-lactate} + \text{NAD}^+ \qquad \text{(D)}$$

LDH: Lactate dehydrogenase

The NADH oxidation, catalised by MDH or LDH, was quantified by measuring the decrease in the absorbance at 340 nm, which is proportional to the transaminase activity.

Lipids and Lipoproteins

The quantification of serum lipids, lipoproteins and apolipoproteins was performed in an auto-analyser (Cobas Mira S, Roche) using commercially available kits.

Triglycerides (TG)

TG concentrations were determined by an enzymatic colorimetric test (Triglycerides, ABX Diagnostics) using the GPO/PAP method.

$$TG \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + ATP \xrightarrow{GK} \text{glycerol-3-P} + ADP$$

$$\text{Glycerol-3-P} + O_2 \xrightarrow{GPO} \text{dihydroxyacetone phosphate} + H_2O_2$$

$$2\ H_2O_2 + \text{4-chlorophenol} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + 4\ H_2O$$

GK: Glycerol Kinase

GPO: Glycerol phosphate oxidase

The colour intensity of the quinoneimine produced, measured spectrophotometrically at 505 nm, is directly related to the TG concentration in the sample.

Cholesterol (Chol)

Serum Chol concentrations were determined by an enzymatic colorimetric test (Cholesterol, ABX Diagnostics) using the CHOD/PAP method.

$$\text{Chol esters} \xrightarrow{CE} \text{Chol} + \text{fatty acids}$$

$$\text{Chol} + O_2 \xrightarrow{ChOD} \text{cholest-4-en-3-one} + H_2O_2$$

$$2\ H_2O_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + 4\ H_2O$$

CE: Cholesterol esterase

ChOD: Cholesterol oxidase

The colour intensity of the quinoneimine formed, determined spectrophotometrically at 500 nm, is directly proportional to the concentration of Chol.

HDL Cholesterol (HDLc)

HDLc level was measured in serum, by using an enzymatic colorimetric test (HDL Cholesterol Direct, ABX Diagnostics). In the first step, synthetic polyanions are absorbed by LDL and VLDL, and transform these lipoproteins into a stable form. In the second step, free HDL particles are solubilised by a detergent, allowing the quantification of the HDLc by a standard enzymatic method in the presence of cholesterol oxidase (ChOD) and cholesterol esterase (CE).

LDL Cholesterol (LDLc)

LDLc concentration in serum was measured by using an enzymatic colorimetric test (LDL Cholesterol Direct, ABX Diagnostics). In the first stage, non-LDL lipoproteins are solubilised by a detergent. The Chol released from HDL, VLDL, and chylomicrons is transformed by the ChOD and CE into an inactive, colourless product. In the next stage, a second detergent dissolves LDL lipoproteins, releasing the Chol from the LDL fraction. The latter reacts with ChOD and CE in the presence of a chromogen. The colour development is proportional to the LDLc concentration in the sample.

Apolipoprotein A-I (apo A-I), Apolipoprotein B (apo B) and Lipoprotein (a) (Lp(a))

Apo A-I, apo B and Lp(a) serum levels were evaluated by immunoturbidimetric assays (Apolipoprotein A1, Apolipoprotein B and Lipoprotein (a), ABX Diagnostics, respectively). Human apo A-I, apo B and Lp(a) precipitate with specific antiserums and the absorption of radiation by the suspension is determined turbidimetrically at 340 nm (fixed time methods), being positively correlated with the concentrations of the evaluated parameters. The assay for Lp(a) presents a limited sensitivity and therefore, only Lp(a) concentrations above 8.8 mg/dl could be assessed.

Oxidised LDL (Ox-LDL)

Ox-LDL was measured directly in plasma by using a two-site enzyme immunoassay (Oxidised LDL ELISA, Mercodia). This assay is based on the direct sandwich technique in which two monoclonal antibodies, directed against separate antigenic determinants on the oxidised apo B molecule, are used. During incubation, Ox-LDL reacts with anti-Ox-LDL

antibodies bound to the microtitration well. After a washing procedure, to remove non-reactive plasma components, a second antibody (peroxidase conjugated anti-apo B) recognises the Ox-LDL bound to the solid phase. After a second incubation and a simple washing step, that removes unbound enzyme labelled antibody, the bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint, which is read spectrophotometrically at 450 nm.

LDL Size and Relative Proportion of LDL I, LDL II and LDL III

LDL peak particle diameter (LDL-PPD), LDL mean particle diameter (LDL-MPD) and the relative proportion of 3 LDL subfractions (LDL I, II and III) were quantified by gradient gel electrophoresis, after isolation of LDL by ultracentrifugation.

i) Isolation of LDL by Sequential Flotation Ultracentrifugation

LDL (*d* 1.019–1.063 g/ml) was isolated according to the method of Havel *et al.* (1955). The density solutions were prepared from stock solutions at *d* 1.006 and *d* 1.182 g/ml of NaBr in 0.195 M NaCl, 0.001% (w/v) EDTA. Densities were measured in a Paar Scientific density meter. In centrifuge tubes 12.8 μl *d* 1.182 g/ml solution were mixed with 160 μl of plasma and overlaid with 67.2 μl *d* 1.019 g/ml solution. Tubes were centrifuged at 42,000 rpm for 18 h at 10°C in a Beckman Ti 42.2 rotor in a Beckman L8-60M ultracentrifuge. The top 80 μl (containing VLDL and IDL) was removed. The remaining infranatant was transferred to a clean centrifuge tube, mixed with 58.8 μl *d* 1.182 g/ml and overlaid with 21.2 μl *d* 1.063 g/ml solution. Following overnight centrifugation (42,000 rpm, 18 h, 10°C), LDL was obtained in the upper 80 μl.

ii) Gradient Gel Electrophoresis

Electrophoresis was carried out, as described by Nichols *et al.* (1986). Commercially available polyacrylamide slab gels containing a linear gradient of 2–16% (Alamo Gels, San Antonio, Texas) were used. The buffer system used within the electrophoresis apparatus (GE-4, Pharmacia) was 90 mM Tris Base, 80 mM boric acid, 2.5 mM EDTA, pH 8.3. The gels were pre-equilibrated at 70 V for 20 min. Samples were mixed (2:1) with the tracking dye [40% (w/v) sucrose, 0.01% (w/v) bromophenol blue, in electrophoresis buffer] and 10 μl of each mixture was loaded into the different wells of the gel. Diameter calibration standards were applied on separate wells of the gel. These standards included three samples prepared by

density gradient ultracentrifugation with different LDL peak particle diameters (LDL I, II and III), which had previously been calibrated against markers: seradyn latex (38.0 nm), thyroglobulin (17.0 nm) and ferritin (12.2 nm). Samples and standards were electrophoresed at 20 V for 20 min, 70 V for 30 min and finally at 120 V for 24 h. Gels were fixed in 10% (w/v) sulphosalicylic acid for 30 min, stained with 0.1% (w/v) Coomassie Brilliant Blue in methanol:acetic acid:distilled water (4:1:5, v/v/v) for 1 h, and destained, for at least 24 h, in 7.5% (v/v) acetic acid, 5% (v/v) methanol.

iii) Evaluation of LDL Size

The gels were scanned by laser densitometry (Bio-Rad Multi-Analyst^{TM}/PC Version 1.1). LDL-PPD was reported as the size of the major LDL fraction (predominant LDL size at maximum optical density). LDL-MPD was calculated to give the mean diameter at the entire LDL particles profile. To achieve this, each LDL profile peak was sliced into ten portions and the peak area under the curve (volume) was calculated. For each portion, the particle size was calculated using the known reference sizes of LDL I, II and III. Then, the frequency for each particle was calculated (size X volume). Finally, the sum of frequencies divided by the sum of volumes gave the Mean Particle Diameter (MPD).

Haematologic Variables

Basic Haematological Study

RBC count, Ht, Hb concentration, mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), red cell distribution width (RDW), total WBC count and platelet count were evaluated using an automatic blood cell counter (ABX Micros 60-OT). Blood cell morphology and WBC differential count were evaluated in Wright stained blood films (Dacie and Lewis, 1991).

Glutathione Peroxidase (GPX) Activity

The GPX activity was determined in the whole blood (EDTA) after sample thaw in a water bath at 25°C. The activity of the enzyme was determined in an auto-analyser (Cobas Mira S, Roche) by kinetic test at 340 nm using a commercial kit (Ransel, RS 505, Randox), according to the following reaction:

$$2\ GSH + ROOH \xrightarrow{GPX} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+ + 2\,GSH$$

GSH/GSSG: glutathione, reduced/oxidised
ROOH: cumene hydroperoxide
GR: glutathione reductase
NADPH/ $NADP^+$: nicotinamide adenine dinucleotide phosphate, reduced/oxidised

The NADPH oxidation, proportional to GPX activity, is quantified by measuring the decrease in absorbance at 340 nm.

Percentage of Erythrocyte Membrane Bound Haemoglobin (%MBH)

The evaluation of %MBH was performed after isolation, preparation of erythrocyte membrane suspensions and calculation of its protein concentration.

i) Isolation of Erythrocytes

Erythrocytes were isolated from plasma and other blood cells by centrifugation on a discontinuous double density gradient. Solutions used for this purpose consisted of ficoll and a radiopaque medium, adjusted to a density of 1.077 and 1.119 (Histopaque® 1077 and 1119, Sigma, respectively). In this procedure, an equal volume (3 ml) of Histopaque 1077 was layered on the Histopaque 1119. Afterwards, 3 ml of fresh blood (EDTA) was layered on the upper medium. After centrifugation at 700 *g* for 30 min, at room temperature, erythrocytes were placed at the bottom of the tube, perfectly isolated from platelets and leukocytes. Erythrocytes were transferred to centrifuge tubes (50 ml capacity) and washed twice with saline at 4°C, by successive centrifugations at 720 *g* for 5 min.

ii) Preparation of Erythrocyte Membranes

The isolated and washed RBCs were immediately lysed, according to the method of Dodge *et al.* (1963). The erythrocyte containing tubes were filled with 5 mM phosphate buffer, pH 8.0 (Dodge buffer) with phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, at a final concentration of 0.1 mM, in order to inhibit endoproteases. The tubes were placed in ice for 10 min. Following centrifugation at 20,440 *g* for 10 min and at 4°C, the supernatant was discarded. The first wash of the membranes used Dodge buffer with PMSF (0.1 mM) and the tubes were again placed in ice for 10 min before being centrifuged as described above. A further two washes were performed using Dodge buffer. After the last centrifugation, the

supernatant was discarded as much as possible. The obtained membrane suspensions were homogenised and aliquoted for storage at –70°C. These aliquots were used to assess the protein concentration, %MBH and the erythrocyte band 3 profile.

iii) Protein Quantification of the RBC Suspensions

Protein concentration of the membrane suspensions was determined according to the method of Bradford (1976).

iv) Quantification of MBH

%MBH was measured spectrophotometrically, after membrane protein dissociation: 100 μl of the sample with 700 μl Triton X-100 [5% (w/v) in Dodge buffer]. The absorbance was read at 415 nm (wavelength of maximum absorbance of Hb) and at 700 nm (background of other proteins) against a blank (800 μl solvent) in a double beam UV-Visible spectrophotometer. The %MBH was calculated by the formula: $(A_{415nm}-A_{700nm})$/ protein concentration (μg/μl).

Erythrocyte Membrane Protein Band 3 Profile

Erythrocyte band 3 profile was performed by electrophoresis followed by Western Blotting and densitometry analysis.

i) Sample Preparation

The volume of RBC membranes suspension (prepared in the last step) containing 100 μg of protein was treated with an equal volume of a solubilisation buffer [0.125 M Tris Base (pH 6.8), 4% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue] and if necessary, the final volume (100 μl) was completed with Dodge buffer. Sample treatment was completed by heat denaturation in a boiling water bath for 5–7 min. A molecular weight standard (Prestained SDS-7B molecular weight markers, Sigma) was also prepared following the manufacturer's instructions and denatured prior to electrophoresis.

ii) Electrophoresis

Samples (20 μl/lane) and standard (10 μl/lane) were submitted to polyacrylamide gel electrophoresis containing SDS (SDS-PAGE), using the discontinuous Laemmli system (1970). A 9% polyacrylamide separating gel, in 0.375 M Tris Base (pH 8.8), 0.1% (w/v) SDS,

and a 4.5% polyacrylamide stacking gel, in 0.125 M Tris Base (pH 6.8), 0.1% (w/v) SDS, were used. The gel (8 cm width x 11 cm length x 1.5 mm thick) was mounted in an electrophoresis chamber (SE 280 –Hoefer Scientific Instruments) which was filled with buffer [0.025 M Tris Base (pH 8.3), 0.192 M glycine, 0.1% (w/v) SDS] and placed in the cold (4°C). The electrophoresis was performed at 12 mA (stacking gel) until it reached the running gel and then at 22 mA (running gel), until the bromophenol blue line reached 2 cm from the bottom of the gel. The stacking gel was removed and discarded.

iii) Transfer to Nitrocellulose

Sample proteins and weight markers were electrophoretically transferred from SDS gels to a nitrocellulose sheet (Nitrocellulose membrane, electrophoresis grade, 0.2 μm pore size, Sigma), as described elsewhere (Towbin *et al.*, 1979). This was performed in a tank (TE 22, Hoefer Scientific Instruments) filled with transfer buffer [0.025 M Tris Base (pH 8.3), 0.192 M glycine, 20%(v/v) methanol], connected to a cooling system (10°C), and with continuous stirring. An electric field of 200 mA was applied for 2 h.

iv) Immunostaining

On completion of the transfer, the nitrocellulose membrane was removed and soaked overnight at 4°C, under slow regular shaking, in wash buffer [0.1% (v/v) Triton X-100, in PBS (phosphate-buffered saline) pH 7.0] containing 5% (w/v) milk powder (Molico magro), to block additional reactive sites on the nitrocellulose. The membrane was then washed twice in wash buffer, for 10 min periods, under gentle agitation at room temperature.

In the first incubation, a monoclonal antibody (mouse IgG2a isotype) against human band 3 protein (Monoclonal anti-human band 3, Sigma) was used. This antibody recognises an epitope located in the cytoplasmic pole of the band 3 molecule (Czerwinski *et al.*, 1988) and therefore band 3 protein (90–100 kDa), several lower molecular mass peptides migrating in SDS-PAGE gels in the regions of 55–60, 38–42 and 21–26 kDa (designated by fragments of 60, 40 and 20, respectively) as well as band 3 high molecular weight aggregates (HMWAg). The anti-band 3 was added (diluted 1:3,000) and incubated for 4 h. The nitrocellulose was then washed three times, as described above. This was followed by incubation with the second antibody (Anti-mouse IgG, whole molecule, peroxidase conjugated, Sigma) for 1 h (dilution 1:4,000). A further four washes were followed [the last two only using PBS (pH 7.0)]. The incubations with antibodies were carried out at room

temperature and under slow regular shaking; the dilutions of the antibodies were prepared with 0.5% (w/v) milk powder (Molico magro) in wash buffer.

Hydrogen peroxide and horseradish peroxidase (HRP) colour developer reagent were used to develop the immunoblot. A freshly made solution, containing 99 ml of PBS (pH 7.0) and 1ml of 5% (w/v) 4-chloro-1-naphthol in absolute ethanol was prepared, filtered and to which 30 μl of 30% H_2O_2 was added. Colour development was stopped by the addition of distilled water. The band 3 profile was quantified by densitometry (Cybertech CS1) and the percentages of HMWAg, band 3 monomer and proteolytic fragments (Pfrag) were calculated.

Total Bilirubin (Tbil)

Quantification of Tbil serum levels was performed in an auto-analyser (Cobas Mira S, Roche) by a colorimetric test using a commercial kit (Bilirubin total and direct, ABX Diagnostics). Tbil was determined in the presence of dimethyl sulfoxide (DMSO) by diazo reaction with diazotised sulphanic acid. The intensity of the developed colour, measured at 550 nm, is proportional to Tbil concentration.

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

Plasma GM-CSF was quantified using a commercial sandwich enzyme immunoassay (Quantikine HS, Human GM-CSF, R&D Systems). Samples and standards are added into the wells of a microplate, coated with a monoclonal antibody specific for human GM-CSF. Any present GM-CSF is allowed to bind to the immobilised antibody and unbound substances are washed away. This is followed by the incubation of the wells with enzyme-linked antibody specific for GM-CSF. Following a wash procedure to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. After an incubation period, an amplifier solution is added to the wells and the colour develops in proportion to the amount of GM-CSF bound in the first step. After stopping the reaction, the intensity of the colour is measured at 490 nm (background correction at 650 or 690 nm).

Elastase

The concentration of elastase in plasma was determined by a heterogenous enzyme immunoassay (PMN Elastase, 2h version, Merck). This kit is specific for the determination of elastase from polymorphonuclear leukocytes (PMNelastase) in complex with α1-proteinase inhibitor (α1 PI) in plasma. In the first step, the PMNelastase-α1 PI complex in the sample

binds, by its PMNelastase end, to the antibodies pre-attached to the wall of the tube. In the second stage of incubation, antibodies marked with alkaline phosphatase (AP) bind to the α1 PI end of the complex. After washing out the excess AP-marked antibodies, the enzymatic activity of the immuno-complexed AP is measured spectrophotometrically (405 nm) upon addition of 4-nitrophenyl phosphate. The intensity of the colour developed is directly proportional to the concentration of PMNelastase-α1 PI.

Lactoferrin

Levels of plasma human lactoferrin were evaluated by ELISA (Bioxytech lactof-EIA, Oxis International). Samples are incubated in the wells of a microplate, previously coated with a monoclonal antibody to lactoferrin. The lactoferrin-monoclonal antibody complex is detected by a biotinylated-monoclonal antibody to lactoferrin. The final step of the assay is an amplification based on a biotin-avidin coupling in which avidin has been covalently linked to HRP. The amount of lactoferrin is measured enzymatically upon addition of OPD at 420 nm.

Fibrinogen

Plasma fibrinogen was measured by immunonephelometry on a Dade Behring nephelometer (BN ProSpec), using protein standards and reagents (N Antiserum to Human Fibrinogen) from the manufacturer.

Fibrin Fragment D-dimer (D-dimer) and Tissue Type Plasminogen Activator (t-PA)

The levels of D-dimer and t-PA antigen in plasma were evaluated by ELISA using kits from Biopool (TintElize® D-dimer and TintElize® t-PA, respectively). Both assays follow the double antibody principle. Plasma sample or standard is added to a microplate well which is coated with antibodies against the evaluated antigen (D-dimer or t-PA). Following an incubation period, a second antibody (anti-D-dimer or anti-t-PA), HRP labelled, is added. After another incubation period, the wells are emptied and washed (to remove unbound conjugate). A peroxidase substrate (OPD/H_2O_2) is then added and the yellow colour developed, proportional to the amount of D-Dimer or t-PA present in the sample, is quantified at 492 nm.

Plasminogen Activatior Inhibitor Type 1 (PAI-1) Activity

PAI-1 activity was measured in plasma using a two-stage, indirect enzymatic assay (Spectrolize®/pL PAI, Biopool). In the first stage, a fixed amount of t-PA is added to the sample and allowed to react with the PAI-1 present. The sample is then acidified to destroy α2-antiplasmin and other potential plasmin inhibitors, which would otherwise interfere with the assay. Subsequently, the sample is diluted. In the second stage, the residual t-PA activity is measured by adding the sample to a mixture of glu-plasminogen, poly-D-lysine and chromogenic substrate at neutral pH. The residual t-PA activity in the sample will catalyse the conversion of plasminogen to plasmin, which in turn hydrolyses the chromogenic substrate. The intensity of colour developed, proportional to the amount of t-PA activity in the sample, is measured at 405 nm (background correction at 492 nm).

Genetic polymorphisms

Isolation of DNA from Blood

Genomic DNA was extracted from WBCs by the proteinase K/salt precipitation method (Olerup and Zetterquist, 1992; Gaffney and Campbell, 1994). In a 1.5 ml eppendorf tube, 150 μl of sample (buffy-coat) were mixed with 1 ml red cell lysis buffer [0.32 M sucrose, 12 mM Tris.HCl (pH 7.5), 5 mM $MgCl_2.6H_2O$, 1% (v/v) Triton X-100]. The tube was left on ice for 1 min or so. After centrifugation at 11,000 *g* for 1 min, the supernatant was removed. The pellet was washed once with 1 ml of sterile water (11,000 *g*, 1 min) and resuspended in 80 μl 5x proteinase K buffer [375 mM NaCl, 120 mM EDTA (pH 8.0)], 220 μl sterile water, 40 μl 10% (w/v) SDS and 30 μl proteinase K (10mg/ml) before being incubated at 55°C for 20 min inverting occasionally. After the addition of 150 μl 4 M NaCl, the tube was mixed and left on ice for 5 min approximately. Precipitated proteins were spun down (11,000 *g*, 5 min) and the supernatant was transferred to another eppendorf containing 1 ml of absolute ethanol. The tube was mixed and stored at –20°C for 15 min. Following centrifugation (11,000 *g*, 2 min), the supernatant was removed and the tube centrifuged again briefly to facilitate removal of all liquid using a yellow tip. The resulting DNA pellet was dissolved in 100 μl sterile TE buffer [10 mM Tris.Hcl (pH 7.6), 1 mM EDTA (pH 8.0); pH 7.6]. DNA was stored at –20°C until used.

*Mae*III Polymorphism in the CRP Gene

CRP genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as previously described (Cao and Hegele, 2000).

A fragment of 744 bp in the exon 2 of the CRP gene was amplified. Each amplification was performed in a thermal cycler (HYBAID – TouchDown) using 1 μl of DNA in a volume of 10 μl containing 1x PCR buffer [20 mM $(NH_4)_2SO_4$, 75 mM Tris.HCl (pH 8.8), 0.01% (v/v) Tween, 1.5 mM $MgCl_2$], 1 μM of each oligonucleotide primer (F: 5'-GATC-TGTGTGATCTGAGAAACCTCT-3' and R: 5'-GAGGTACCAGAGACAGAGACGTG-3'), 0.2 mM dNTPs (2'-deoxyribonucleoside 5'-triphosphates: dATP, dCTP, dGTP, and dTTP), and 0.25 U of *Taq* polymerase (AB). The reaction mixture was heated to 95°C for 5 min for denaturation and subjected to 30 amplification cycles by primer annealing (57°C for 30 s), extension (72°C for 30 s), and denaturation (95°C for 30 s), followed by a final extension at 72°C for 8 min.

The PCR products (10 μl) were subjected to restriction enzyme analysis by digestion with 0.4 U of the restriction endonuclease *Mae*III at 55°C for 3 h, in the buffer recommended by the manufacturer (Incubation Buffer for *Mae*III, Roche). After separation by electrophoresis, on 2% agarose gel (10 cm width x 13 cm length x 7 mm thick) in 1x TAE buffer, at 170 mA, the digested DNA fragments were stained with ethidium bromide (0.0005%, w/v) for 5 min and visualised by UV illumination. The sizes of *Mae*III fragments were estimated by comparison with known size markers (GeneRuler™ 100 bp DNA ladder, MBI Fermentas).

*Mae*III cleavage site

5' – ↓ G T N A C – 3'

3' – C A N T G ↑ – 5'

In the polymorphism, less common allele (C) looses a *Mae*III site:

Digestion of less common 1059C allele:	434 and 310 bp	(CC)
Digestion of both alleles:	434, 310, 233 and 201 bp	(GC)
Digestion of more common 1059G allele:	310, 233 and 201 bp	(GG)

*Hha*I Polymorphisms in the Apo E Gene

ApoE genotyping was also performed by PCR-RFLP using the method of Hixson and Vernier (1990), with some modifications. For the PCR reaction, a HotStarTaq DNA Polymerase (Qiagen) was used in the recommended PCR buffer (containing Tris.HCl, KCl, $(NH_4)_2SO_4$, $MgCl_2$; pH 8.7). The Hot Start enzyme requires an initial 15 min step at 95°C to be activated.

A 244 bp fragment located in the exon 4 of the apo E gene was amplified using oligonucleotide primers that flank positions 112 and 158 in the referred exon (F4: 5'-ACAGAATTCGCCCCGGCCTGGTACAC-3' and F6: 5'-TAAGCTTGGCACGGCT-GTCCAAGGA-3'). The PCR reaction was carried out in a thermal cycler (HYBAID – TouchDown) using 1 μl of DNA in a volume of 20 μl containing 1x PCR Buffer (HotStarTaq polymerase Buffer, with a final concentration of 2.0 mM $MgCl_2$), 1 μM of each primer, 10% (v/v) DMSO, 0.2 mM dNTPs and 0.5 U of HotStarTaq DNA Polymerase. The PCR conditions were 95°C for 15 min, and subsequently 31 cycles at 95°C for 45 s, 60°C for 1 min, and 72°C for 2 min, and finally at 72°C for 8 min.

The PCR products (20 μl) were digested with 10U of *Hha*I in the recommended buffer (React 2, Gibco BRL), for 3 h at 37°C. Each reaction mixture was loaded onto an 8% polyacrylamide gel (10 cm width x 7 cm length x 1.5 mm thick) in 1x TBE buffer [89 mM Tris Base, 89 mM Boric acid, 2 mM EDTA (pH 8.0); pH 8.0] and electrophoresed at 40 mA. The gel was stained with ethidium bromide (0.0005%, w/v) for 2 min and DNA fragments were visualised by UV illumination. The sizes of *Hha*I fragments were estimated by comparison with known size markers (10 bp DNA ladder, Invitrogen life technologies).

*Hha*I Cleavage site: 5' – G CG↓C – 3'

3' – C↑GC G – 5'

Differentiation: *Hha*I cuts codons for arginine residues at positions 112 and 158:

If both restriction cutting sites present in both alleles –E4/E4	72, 19, 48 and 35 bp
(The 19 bp fragment is too small for detection)	
If restriction cutting site at 158 present in both alleles –E3/E3	91, 48 and 35 bp
If both restriction cutting sites absent in both alleles –E2/E2	91 and 83 bp

The gel patterns obtained for E2/E3, E2/E4 and E3/E4 genotypes are combinations of the homozygous fragments.

*Hha*I also cuts at non-polymorphic sites in the apo E gene, producing a non-polymorphic 38 bp fragment (visualised on the gel) and other common fragments that are not detected due to their smaller sizes.

Other Biochemical Parameters

Uric Acid

The quantification of uric acid in serum was performed in an auto-analyser (Cobas Mira S, Roche) by an enzymatic colorimetric test, using a commercial kit (Uric acid PAP, ABX Diagnostics).

$$\text{Uric acid} + 2\ H_2O + O_2 \xrightarrow{\text{uricase}} \text{allantoin} + CO_2 + H_2O_2$$

$$H_2O_2 + \underset{\text{Sulphonic acid}}{\text{3,5-dichloro-2-hydroxybenzene}} + \underset{}{\text{4-amino-antipyrine}} \xrightarrow{\text{peroxidase}} \text{quinonemine} + HCl + 2\ H_2O$$

The amount of quinonemine formed is evaluated spectrophotometrically at 520 nm.

Total Antioxidant Status (TAS)

Serum levels of TAS were assessed in an auto-analyser (Cobas Mira S, Roche) using a commercial kit (Total Antioxidant Status, Randox).

$$HX\text{-}Fe^{III} + H_2O_2 \longrightarrow {}^{\cdot}X - [Fe^{IV} = 0] + H_2O$$

$$ABTS^{®} + {}^{\cdot}X - [Fe^{IV} = 0] \longrightarrow ABTS^{®,+} + HX\text{-}Fe^{III}$$

$HX\text{-}Fe^{III}$: metmyoglobin

${}^{\cdot}X - [Fe^{IV} = 0]$: ferrylmyoglobin

$ABTS^{®}$: 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]

The radical cation $ABTS^{®,+}$, which has a relatively stable blue-green colour, is measured at 600 nm. Antioxidants in the added sample cause suppression of this colour production to a degree that is proportional to their concentration.

16. Statistical Analysis

Specific statistic analysis was applied to each of the performed studies, using the Statistical Package for Social Sciences (SPSS, version 10.0) for Windows. However, in a general basis:

- Kolmogorov-Smirnov analysis was used to test if the results were normally distributed. The results normally distributed (ND), presented as mean ± SD for continuous variables, were compared using parametric tests. The results not-normally distributed (NND), presented as median (interquartile range (IQR)) for continuous variables, were compared using non-parametric tests.
- Moderate and extreme outliers were defined as cases with values between 1.5 and 3 IQRs or with more than 3 IQRs respectively, from the first and third quartiles.
- The strength of the association between the variables was estimated by the Pearson's correlation coefficient (r; for data ND), or Spearman's rank correlation coefficient (r_s; for data NND).
- Significance was accepted at P less than 0.05.

Normal Pregnancy

i) Cross-sectional Study

For continuous variables ND, comparisons between trimesters were performed by one-way analysis of variance (ANOVA). When statistical significance was obtained, multiple comparisons between groups were performed by one-way ANOVA supplemented with Tukey's honestly significant difference (HSD) *post hoc* test. For continuous variables NND, differences between trimesters were evaluated by the Kruskal-Wallis test. For single comparisons (two groups), the Student's unpaired t test (for continuous variables ND) or Mann-Whitney U test (for continuous variables NND) were used.

ii) Longitudinal Study

For continuous variables ND, longitudinal changes during pregnancy were evaluated by repeated measures ANOVA (Huynh-Feldt adjustment). When differences reached statistical significance, multiple comparisons between trimesters were made using the Bonferroni adjustment. For continuous variables NND, the Friedman's test was used. When statistical significance was achieved, pairwise comparisons between trimesters were performed using the Wilcoxon signed rank sum test, with adjustment of P values for multiple comparisons.

PEc Pregnancy

Normal and PEc groups were compared using the Student's unpaired *t* test (for continuous variables ND) or Mann-Whitney *U* test (for continuous variables NND). Adjustment of statistical differences for confounding factors was performed using analysis of covariance, after log transformation (when necessary). The distribution of subjects with respect to genotypes and to some other variables (presented as proportions) was analysed using the chi-squared (χ^2) test or Fisher's exact test. Multiple comparisons between groups with different genotypes were performed by one-way ANOVA supplemented with Tukey's HSD *post hoc* test.

Section Three: Results

17. Normal Pregnancy

On a general basis, the results concerning the evaluation of normal pregnancy are presented (for each of the topics) in the following order: first those obtained in the cross-sectional study and then those of the longitudinal study. In the cross-sectional study, the changes imposed by pregnancy are presented (comparison of non-pregnant with pregnant women) as well as the changes occurring throughout pregnancy. The longitudinal study presents the data obtained with pregnant women who were followed during the three trimesters of gestation.

Clinical Data

The clinical characteristics presented by the healthy non-pregnant women and by the pregnant women in the first, second and third trimesters, included in the cross-sectional study, are summarised in Table 18–1. Mean age did not differ between groups, except for non-pregnant controls. During pregnancy, as gestational age increased, a significant rise in maternal body weight was observed, while no significant changes in the systolic or diastolic blood pressure were detected.

Similar findings, i.e. rise in body weight and no change in blood pressure, were observed in the longitudinal study (Table 18–2).

Uric Acid and TAS

Uric acid level, but not TAS, was significantly lower in the first trimester of gestation compared with non-pregnant controls (Table 18–3).

During gestation (cross-sectional study), uric acid increased progressively throughout gestation, whereas TAS showed a tendency to decrease (Table 18–3).

In the longitudinal study, decreases in the TAS reached statistical significance (Table 18–4). Changes in uric acid were more pronounced at the end of gestation, while changes in TAS were more marked at middle gestation.

Uric acid and TAS were generally positively correlated in each group of both the cross-sectional and longitudinal studies (Table 18–5).

APPs and Liver Function Tests

When compared with non-pregnant controls, pregnant women in the first trimester presented with significantly higher levels of CRP and haptoglobin (Table 18–6). No significant differences were found for AST or ALT.

During gestation, no significant changes were observed in AST, ALT, CRP or haptoglobin, neither in the cross-sectional study (Table 18–6) nor in the longitudinal study (Table 18–7). However, mean haptoglobin level showed a tendency to decline in the cross-sectional study and a tendency to rise in the longitudinal study.

In the longitudinal study, sixteen women (69.6%) presented increases in haptoglobin from the first to the second trimester but only eight of them showed further increments in the third trimester. Regarding CRP levels, twelve cases (52.2%) presented fluctuations throughout gestation, whereas seven (30.4%) showed progressive reductions and four (17.4%) progressive increments throughout pregnancy.

Table 18–1. Clinical characteristics of the participants in the cross-sectional study

	Non-pregnant	*Pregnant*			*Statistics*			
	N P	**1st trimester**	**2nd trimester**	**3rd trimester**	***P*** NP/1st	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*24*	*64*	*48*	*67*				
Age (years)	22.8 ± 2.6	27.7 ± 5.3	26.3 ± 6.3	26.2 ± 4.8	<0.001	NS	NS	NS
Gestational age (weeks)	–	10.1 ± 2.0	22.3 ± 2.8	34.0 ± 2.8	–	<0.001	<0.001	<0.001
Weight (kg)	61.7 ± 8.5	62.1 ± 9.2	67.1 ± 9.6	73.9 ± 9.9	NS	<0.05	<0.001	<0.001
Blood pressure (mmHg)								
Systolic	NM	115 ± 13	118 ± 11	119 ± 11	–	NS	NS	NS
Diastolic	NM	59 ± 10	58 ± 10	62 ± 9	–	NS	NS	NS

Values are mean ± SD; NS, not significant; NM, not measured.

Table 18–2. Clinical characteristics of the participants in the longitudinal study

	Pregnant			*Statistics*		
	1st trimester	**2nd trimester**	**3rd trimester**	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*23*	*23*	*23*			
Age (years)	26.7 ± 6.7	–	–			
Gestational age (weeks)	10.0 ± 2.0	23.6 ± 1.8	34.7 ± 1.8	<0.001	<0.001	<0.001
Weight (kg)	62.9 ± 8.5	68.6 ± 8.8	73.9 ± 9.1	<0.001	<0.001	<0.001
Blood pressure (mmHg)						
Systolic	116 ± 12	117 ± 8	119 ± 12	NS	NS	NS
Diastolic	61 ± 10	60 ± 10	63 ± 8	NS	NS	NS

Values are mean ± SD; NS, not significant.

Table 18–3. Uric acid and TAS levels (cross-sectional study)

	Non-pregnant	*Pregnant*			*Statistics*			
	N P	**1st trimester**	**2nd trimester**	**3rd trimester**	***P*** NP/1st	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*24*	*64*	*48*	*67*				
Uric acid (mg/dl)	3.7 ± 0.5	2.9 ± 0.7	3.2 ± 0.9	4.0 ± 1.1	<0.001	NS	<0.001	<0.001
TAS (mmol/l)	1.24 ± 0.09	1.22 ± 0.11	1.20 ± 0.13	1.18 ± 0.17	NS	NS	NS	NS

Values are mean ± SD; NS, not significant.

Table 18–4. Uric acid and TAS levels (longitudinal study)

	Pregnant			*Statistics*		
	1st trimester	**2nd trimester**	**3rd trimester**	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*23*	*23*	*23*			
Uric acid (mg/dl)	3.0 ± 0.7	3.3 ± 0.8	4.3 ± 1.0	<0.01	<0.001	<0.001
TAS (mmol/l)	1.23 ± 0.11	1.15 ± 0.10	1.12 ± 0.13	<0.01	NS	<0.01

Values are mean ± SD; NS, not significant.

Table 18–5. Correlations between uric acid and TAS

r, Pearson's correlation coefficient;
NS, not significant.

	Cross-sectional study	Longitudinal study
Controls	$r = 0.419$, $n = 24$, $P<0.05$	
1st trimester	$r = 0.383$, $n = 64$, $P<0.01$	$r = 0.373$, $n = 23$, NS
2nd trimester	$r = 0.251$, $n = 48$, NS	$r = 0.441$, $n = 23$, $P<0.05$
3rd trimester	$r = 0.357$, $n = 67$, $P<0.01$	$r = 0.482$, $n = 23$, $P<0.05$
Total	$r = 0.260$, $n = 203$, $P<0.001$	$r = 0.161$, $n = 69$, NS

Table 18–6. Acute phase proteins and liver function tests (cross-sectional study)

	Non-pregnant	*Pregnant*			*Statistics*			
	N P	**1st trimester**	**2nd trimester**	**3rd trimester**	***P*** NP/1st	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*24*	*64*	*48*	*67*				
AST (U/l)	20 (15–26)	22 (19–28)	23 (20–29)	25 (21–34)	NS	NS	NS	NS
ALT (U/l)	16 (13–20)	16 (12–21)	16 (14–21)	16 (11–20)	NS	NS	NS	NS
CRP (mg/l)	0.47 (0.28–1.50)	3.76 (1.39–6.88)	3.83 (2.87–6.87)	3.18 (2.19–4.81)	<0.001	NS	NS	NS
Haptoglobin (mg/dl)	104.0 ± 42.3	134.5 ± 69.1	129.1 ± 64.9	116.5 ± 55.9	<0.05	NS	NS	NS

Values are mean ± SD or median (IQR); NS, not significant.

Table 18–7. Acute phase proteins and liver function tests (longitudinal study)

	Pregnant			*Statistics*		
	1st trimester	**2nd trimester**	**3rd trimester**	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*23*	*23*	*23*			
AST (U/l)	25 (19–30)	25 (20–28)	25 (21–32)	NS	NS	NS
ALT (U/l)	17 (14–22)	16 (14–20)	12 (10–19)	NS	NS	NS
CRP (mg/l)	2.88 (1.38–8.12)	3.14 (2.40–5.42)	2.97 (2.25–4.04)	NS	NS	NS
Haptoglobin (mg/dl)	102.4 ± 38.4	127.7 ± 51.0	133.1 ± 70.7	NS	NS	NS

Values are mean ± SD or median (IQR); NS, not significant.

Lipids and Lipoproteins

Figure 18–1 shows the densitometric analysis of a LDL band profile, indicating the corresponding LDL mean and peak particle diameters as well as the relative proportion of LDL I, II and III.

Table 18–8 presents lipids and lipoproteins obtained in the cross-sectional study. Compared to the non-pregnant group, TG levels were significantly increased in the first trimester, whereas LDLc concentration, LDLc to apo B and HDLc to apo A-I ratios as well as LDL size (LDL-MPD and LDL-PPD) were significantly reduced.

During gestation, the changes in lipids and lipoproteins were more pronounced between the first and second trimesters than between the second and third trimesters. There was a significant rise in serum levels of TG, Chol, apo B, apo A-I, HDLc, LDLc and Ox-LDL from the first to the second trimester of normal pregnancy and a further increase in TG, Chol, apo B, LDLc and Ox-LDL in the third trimester, with a concomitant decrease in HDLc and apo A-I. As pregnancy progressed from the first to the second trimester, the LDL-MPD and LDL-PPD became significantly smaller, with no further decrease in size in the third trimester. There was a significant increase in the proportion of LDL III between the first and the third trimesters. This is presented in Figure 18–2A that shows the appearance of low particle size bands, corresponding to smaller denser LDL, as pregnancy advances. Chol to HDLc and LDLc to apo B ratios increased as pregnancy progressed (Table 18–8). An inverse behaviour was observed in the HDLc to apoA-I ratio. All the groups presented a wide inter-individual variation in Lp(a) levels. Throughout gestation, Lp(a) concentration showed a trend to rise from the first to the third trimester, although with no statistical significance.

Figure 18–1. The densitometric analysis of a LDL band profile. The corresponding LDL mean (MPD) and peak (PPD) particle diameters are indicated as well as the relative proportion of LDL I, II and III. In this example, LDL II presents the major contribution; the relative proportion of LDL I is greater than LDL III and, therefore, MPD is higher than PPD.

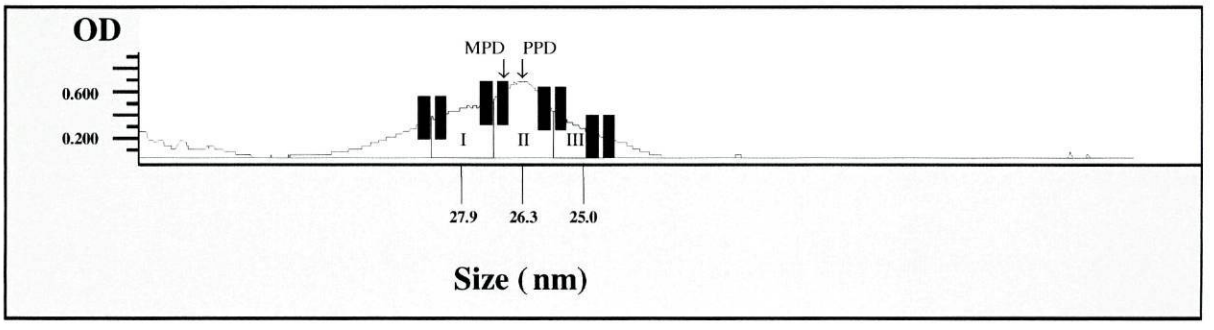


Table 18–8. Lipids and lipoproteins (cross-sectional study)

	Non-pregnant	*Pregnant*			*Statistics*			
	N P	**1st trimester**	**2nd trimester**	**3rd trimester**	***P*** NP/1st	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*24*	*64*	*48*	*67*				
TG (mg/dl)	59.7 ± 20.1	113.6 ± 40.6	147.7 ± 38.1	185.8 ± 38.4	<0.001	<0.001	<0.001	<0.001
Chol (mg/dl)	179.9 ± 32.2	176.0 ± 30.5	252.2 ± 46.9	285.1 ± 63.6	NS	<0.001	<0.01	<0.001
Apo B (mg/dl)	71.4 ± 16.1	77.0 ± 15.8	112.4 ± 24.4	126.8 ± 31.0	NS	<0.001	<0.01	<0.001
Apo A-I (mg/dl)	159.5 ± 32.6	171.8 ± 37.5	224.2 ± 29.0	211.5 ± 47.5	NS	<0.001	NS	<0.001
HDLc (mg/dl)	57.6 ± 11.1	54.8 ± 13.7	69.8 ± 12.7	61.8 ± 14.2	NS	<0.001	<0.01	<0.01
LDLc (mg/dl)	104.5 ± 31.4	84.0 ± 19.5	122.7 ± 31.0	145.9 ± 44.5	<0.01	<0.001	<0.01	<0.001
Lp(a) (mg/dl)								
Number of cases < 8.8 (%)	7 (29.2)	25 (39.1)	21 (43.7)	20 (29.9)				
Median (IQR)	16.8 (<8.8–43.4)	14.2 (<8.8–44.2)	15.6 (<8.8–54.7)	17.1 (<8.8–49.6)	NS	NS	NS	NS
Chol/HDLc	3.19 ± 0.63	3.22 ± 0.60	3.65 ± 0.81	4.62 ± 1.21	NS	<0.05	<0.001	<0.001
LDLc/Apo B	1.47 ± 0.35	1.08 ± 0.15	1.09 ± 0.15	1.14 ± 0.15	<0.001	NS	NS	NS
HDLc/Apo A-I	0.36 ± 0.04	0.32 ± 0.05	0.31 ± 0.04	0.29 ± 0.04	<0.001	NS	NS	<0.05
LDL-MPD (nm)	27.11 ± 0.49	26.66 ± 0.56	26.36 ± 0.46	26.34 ± 0.52	<0.001	<0.01	NS	<0.01
LDL-PPD (nm)	27.05 ± 0.70	26.60 ± 0.57	26.19 ± 0.55	26.09 ± 0.63	<0.01	<0.01	NS	<0.001
LDL I (%)	33.64 ± 9.81	22.66 ± 12.33	19.99 ± 9.68	21.45 ± 8.78	<0.001	NS	NS	NS
LDL II (%)	42.31 ± 8.11	46.80 ± 8.31	45.16 ± 6.23	41.66 ± 7.44	<0.05	NS	<0.05	<0.001
LDL III (%)	24.05 ± 7.74	30.54 ± 13.37	34.84 ± 11.27	36.89 ± 12.94	<0.01	NS	NS	<0.05
Ox-LDL (U/l)	40.1 ± 8.4	42.8 ± 11.1	60.4 ± 18.1	71.9 ± 24.3	NS	<0.001	<0.01	<0.001

Values are mean ± SD unless otherwise indicated; NS, not significant.

Table 18–9. Lipids and lipoproteins (longitudinal study)

	Pregnant			*Statistics*		
	1st trimester	**2nd trimester**	**3rd trimester**	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*23*	*23*	*23*			
TG (mg/dl)	112.5 ± 35.3	154.0 ± 31.7	198.0 ± 46.0	<0.001	<0.01	<0.001
Chol (mg/dl)	177.9 ± 33.4	254.6 ± 47.4	282.6 ± 72.3	<0.001	<0.01	<0.001
Apo B (mg/dl)	80.6 ± 17.8	114.7 ± 26.9	123.3 ± 34.7	<0.001	NS	<0.001
Apo A-I (mg/dl)	172.0 ± 34.8	213.8 ± 20.7	196.4 ± 25.9	<0.001	<0.01	<0.05
HDLc (mg/dl)	53.6 ± 11.9	63.3 ± 8.4	56.1 ± 10.3	<0.01	<0.01	NS
LDLc (mg/dl)	84.8 ± 23.1	127.9 ± 33.0	145.4 ± 44.5	<0.001	<0.01	<0.001
Lp(a) (mg/dl)						
Number of cases < 8.8 (%)	6 (26.1)	2 (8.7)	2 (8.7)			
Median (IQR) (n=21)	33.8 (9.8–65.0)	49.8 (18.8–96.7)	47.1 (25.1–103.1)	<0.001	<0.01	<0.001
Chol/HDLc	3.41 ± 0.68	4.07 ± 0.87	4.90 ± 1.06	<0.01	<0.001	<0.001
LDLc/Apo B	1.05 ± 0.19	1.12 ± 0.18	1.18 ± 0.16	NS	NS	NS
HDLc/Apo A-I	0.32 ± 0.05	0.30 ± 0.03	0.29 ± 0.04	NS	NS	<0.05
LDL-MPD (nm)	26.60 ± 0.54	26.15 ± 0.47	25.95 ± 0.43	<0.001	<0.05	<0.001
LDL-PPD (nm)	26.47 ± 0.50	25.92 ± 0.62	25.62 ± 0.62	<0.001	<0.01	<0.001
LDL I (%)	19.49 ± 14.78	18.95 ± 9.25	17.59 ± 7.35	NS	NS	NS
LDL II (%)	59.51 ± 11.04	49.64 ± 6.40	43.34 ± 6.61	<0.01	<0.01	<0.001
LDL III (%)	21.00 ± 11.43	31.41 ± 10.65	39.07 ± 12.07	<0.01	<0.01	<0.001
Ox-LDL (U/l)	42.4 ± 11.3	55.3 ± 15.7	61.2 ± 18.8	<0.001	<0.05	<0.001

Values are mean ± SD unless otherwise indicated; NS, not significant.

Figure 18–2. LDL gel profile at the first (1st), second (2nd) and third (3rd) trimesters of normal pregnancy. A, cross-sectional study; B and C, two longitudinal cases.

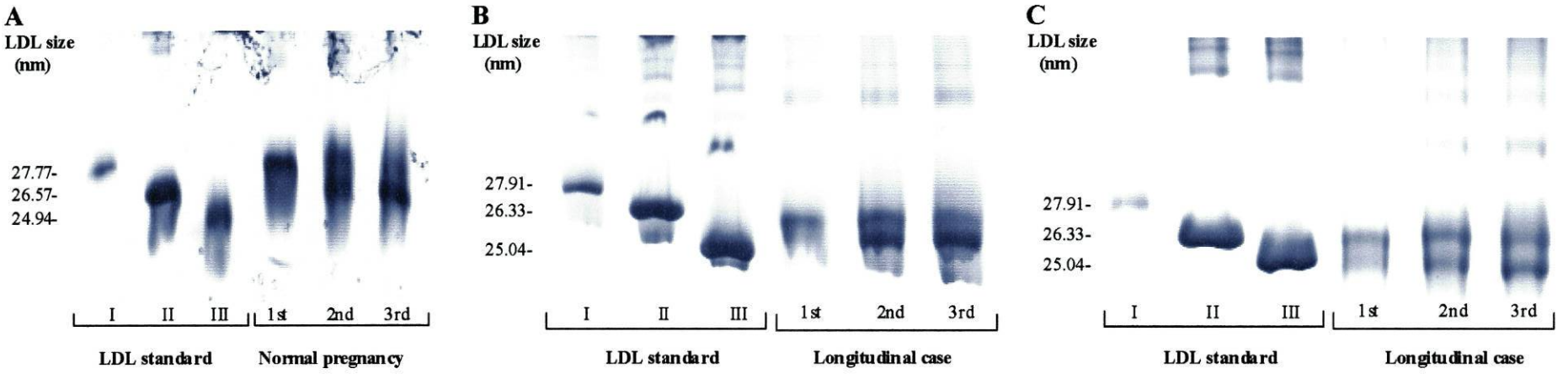
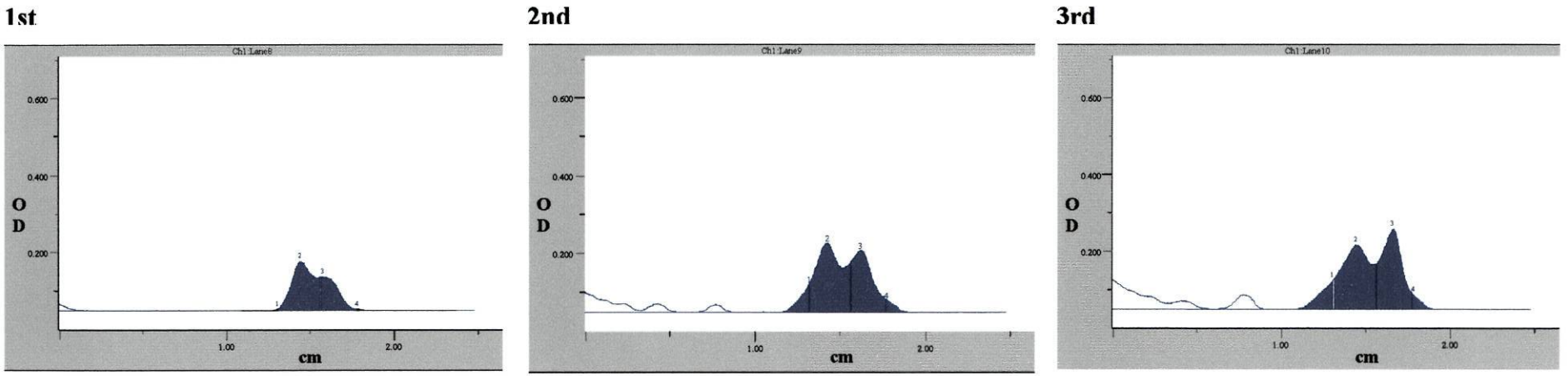


Figure 18–3. Densitometric analysis of the LDL band profile of the longitudinal case presented in Figure 18–2C. The first (1st), second (2nd) and third (3rd) trimesters are presented.



In the longitudinal study, similar changes in lipid and lipoprotein profile were found (Table 18–9). However some changes were more pronounced, particularly the reduction in LDL size and the rise in Lp(a).

Both LDL-PPD and LDL-MPD decreased progressively as pregnancy progressed, with significant changes between each trimester. To illustrate these changes, the LDL gel profiles of two longitudinal cases are shown in Figures 18–2B and 18–2C. The densitometric analysis of the LDL band profile of one of these cases is presented in Figure 18–3. In this case, it is clear that the LDL-PPD only changes in the last trimester, whereas LDL-MPD decreases progressively. It can also be observed that optical density (OD) increases throughout gestation, indicating an increment in LDL level.

From the 23 longitudinal cases studied, only two (8.7%) showed no changes in LDL size throughout gestation. In the 21 cases demonstrating reductions in LDL size, one case presented no changes in LDL-PPD from the first to the second trimester (the case presented in Figure 18–3), and another case showed changes neither in LDL-MPD nor in LDL-PPD from the second to the third trimester. The individual distribution of LDL-MPD, by gestational age at sampling, is presented in Figure 18–4A, and clearly shows the reduction in LDL size throughout pregnancy.

Regarding the analysis of Lp(a), two cases were eliminated as they presented a Lp(a) level lower than 8.8mg/dL (the lower limit of sensitivity of the used method) in all examined periods. Again, a wide inter-individual variation in Lp(a) concentration was observed in all examined periods. Lp(a) increments in each individual, from the first to the third trimester, were also different (increment range: from 6 to almost 400%). Median Lp(a) level (n = 21) increased significantly from the first to the second trimester and remained high in the third one. Four individual cases (19.0%) showed a small decrease in Lp(a) levels from the second to the third trimester. The individual distribution of Lp(a) values, by gestational age at sampling, is presented in Figure 18–4B, showing the wide variation of Lp(a) in each trimester, but also the rise of Lp(a) in pregnancy.

Using either all the results of the cross-sectional study (203 samples), or those of the longitudinal study (69 samples), a significant inverse correlation was obtained between TG and LDL size (Figures 18–5A and 18–5B, respectively) and a significant positive correlation was obtained between TG and Ox-LDL (Figures 18–5C and 18–5D, respectively). Similar correlations were obtained when different groups were evaluated separately, although

statistical significance was only achieved in the first trimester of gestation, in both cross-sectional and longitudinal studies, for TG and LDL size.

In all groups (of both cross-sectional and longitudinal studies), a significant positive correlation was observed between Ox-LDL and LDLc (Table 18–10).

Furthermore, in the longitudinal study, the relative increments in Ox-LDL levels between trimesters (first/second plus second/third) correlated inversely with relative decreases in LDL-MPD (r = –0.397, P<0.01), LDL-PPD (r = –0.334, P<0.05) and TAS levels (r = –0.347, P<0.05).

Table 18–10. Correlations between Ox-LDL and LDLc

	Cross-Sectional Study	Longitudinal study
Controls	r = 0.407, n = 24, P<0.05	
1st Trimester	r = 0.424, n = 64, P<0.01	r = 0.471, n = 23, P<0.05
2nd Trimester	r = 0.608, n = 48, P<0.001	r = 0.753, n = 23, P<0.001
3rd Trimester	r = 0.694, n = 67, P<0.001	r = 0.866, n = 23, P<0.001
Total	r = 0.741, n = 203, P<0.001	r = 0.819, n = 69, P<0.001

r, Pearson's correlation coefficient.

Figure 18–4. Individual distribution of LDL-MPD (A) and Lp(a) (B) values, by gestational age at sampling, in the longitudinal study of normal pregnancy. In figure B, values lower than 8.8mg/dl are presented below the dotted line; two longitudinal cases were eliminated as they presented a Lp(a) level lower than 8.8mg/dl in all examined periods.

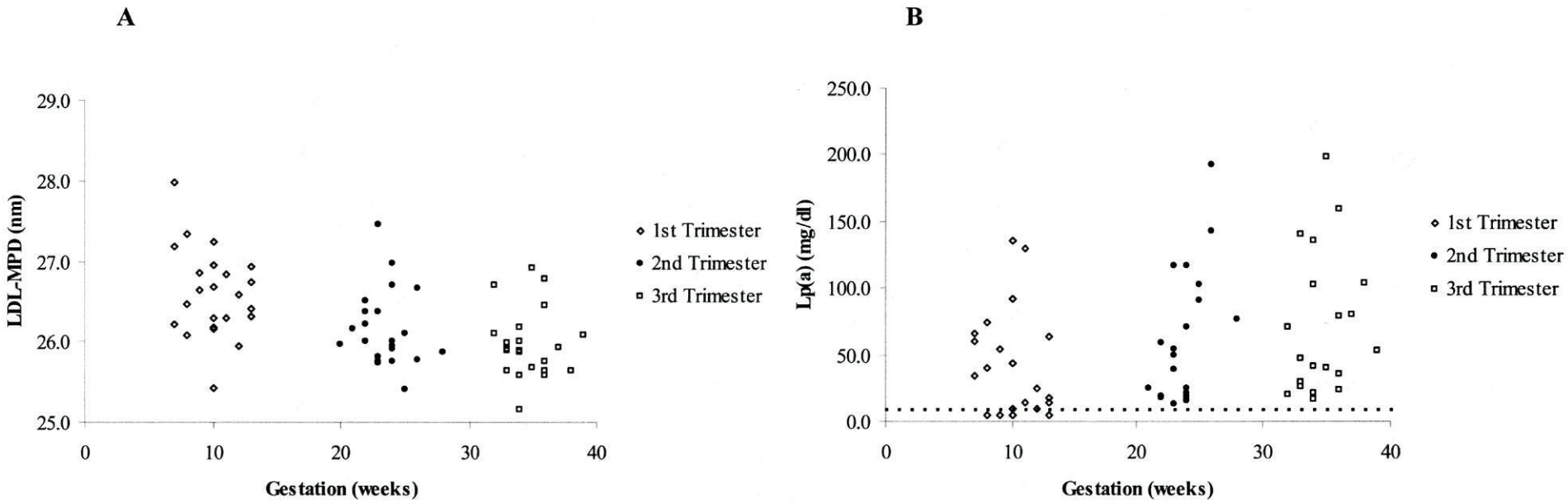
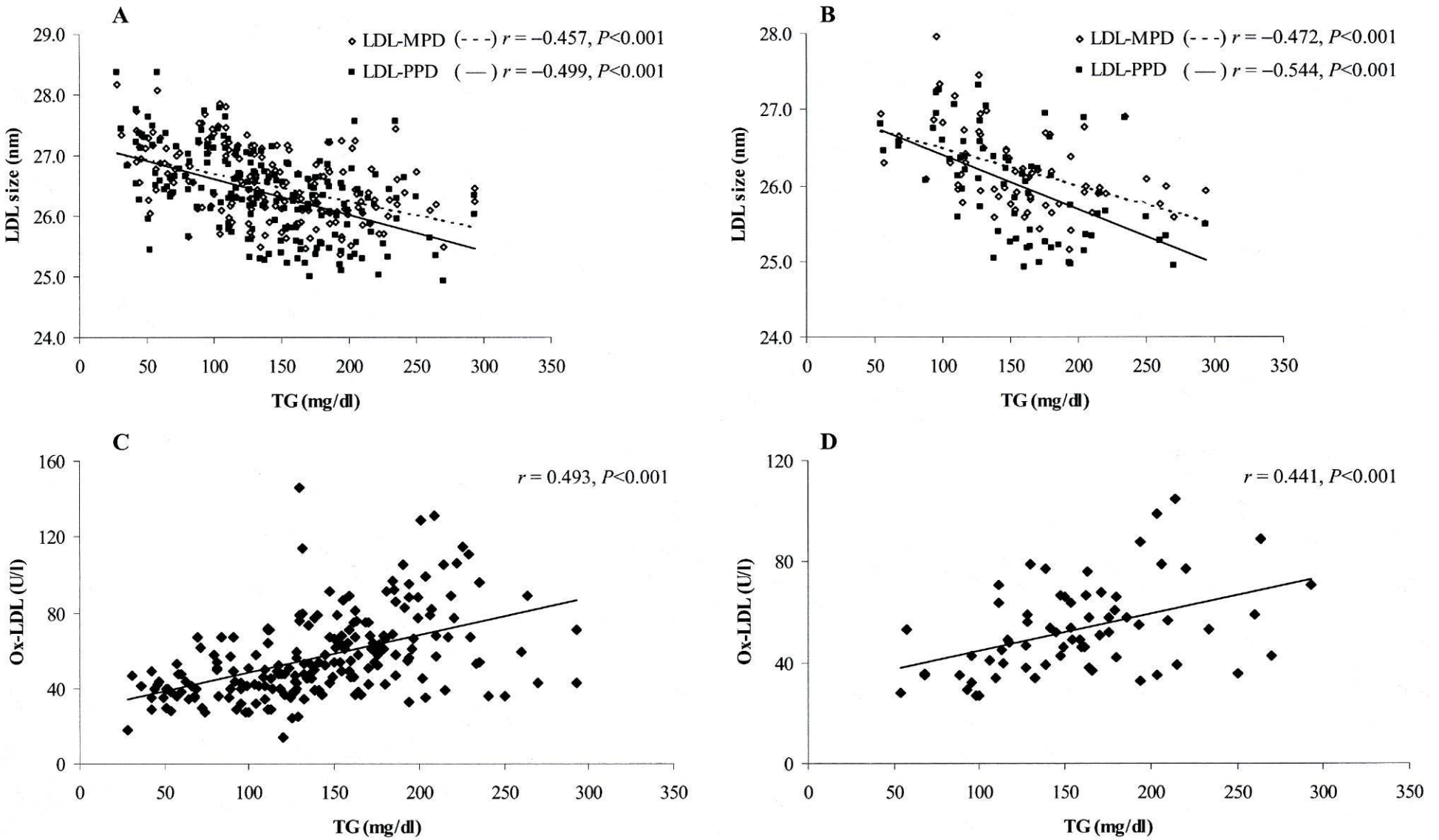


Figure 18–5. Correlations between TG and LDL size in the cross-sectional and longitudinal studies (A and B, respectively) and between TG and Ox-LDL (C and D, respectively). *r*, Pearson's correlation coefficient.



Haematologic Variables

Leukocyte Count and Neutrophil Activation

Data regarding leukocytes and neutrophil activation in the cross-sectional and longitudinal studies are presented in Tables 18–11 and 18–12, respectively.

Mean WBC and neutrophil counts, and median GM-CSF level were significantly higher in pregnant women compared with non-pregnant controls (Table 18–11). Mean lactoferrin and elastase levels were somewhat higher in the first trimester of gestation, although without statistical significance. The elastase to neutrophil ratio and the lactoferrin to neutrophil ratio were significantly reduced in the first trimester of gestation compared with non-pregnant women.

During gestation, WBC count increased significantly until the second trimester and remained high in the last trimester (Table 18–11). The neutrophil count showed a similar pattern and the number of cases that presented a shift to the left in the differential leukocyte count (cases with metamyelocytes) increased as pregnancy advanced. No significant changes were observed in mean lactoferrin and elastase levels. However, the elastase to neutrophil and the lactoferrin to neutrophil ratios decreased significantly from the first to the second trimester.

In the longitudinal study, increments in WBC and neutrophil counts were observed in all longitudinal cases. In contrast, no consistent changes were observed in lactoferrin or elastase levels. Moreover, in the second and third trimesters, two longitudinal cases presented with metamyelocytes.

When all samples were evaluated together in the cross-sectional study, a significant positive correlation was observed between lactoferrin and elastase concentrations ($r = 0.389$, $P<0.001$). Actually, a significant positive correlation was observed between lactoferrin and elastase levels for all the studied groups when evaluated separately, except in the third trimester of normal pregnancy ($r = 0.182$, not significant).

Table 18–11. Total and differential leukocyte count and neutrophil activation (cross-sectional study)

	Non-pregnant	*Pregnant*			*Statistics*			
	N P	**1st trimester**	**2nd trimester**	**3rd trimester**	***P*** NP/1st	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*24*	*64*	*48*	*67*				
WBC (x 10^9/l)	6.38 ± 1.41	8.45 ± 2.19	10.09 ± 2.45	9.60 ± 2.49	<0.001	<0.01	NS	<0.05
Neutrophils (x 10^9/l)	3.70 ± 1.43	5.94 ± 1.73	7.59 ± 2.00	7.02 ± 2.01	<0.001	<0.001	NS	<0.01
Eosinophils (x 10^9/l)	0.15 ± 0.10	0.11 ± 0.08	0.16 ± 0.14	0.11 ± 0.13	<0.05	NS	NS	NS
Basophils (x 10^9/l)	0.04 ± 0.03	0.03 ± 0.03	0.04 ± 0.03	0.02 ± 0.04	NS	NS	NS	NS
Lymphocytes (x 10^9/l)	2.07 ± 0.50	1.97 ± 0.65	1.83 ± 0.57	1.94 ± 0.58	NS	NS	NS	NS
Monocytes (x 10^9/l)	0.43 ± 0.16	0.41 ± 0.19	0.48 ± 0.22	0.50 ± 0.29	NS	NS	NS	<0.05
Cases with metamyelocytes	None	1 (1.6%)	3 (6.3%)	5 (7.5%)				
Elastase (μg/l)	37.2 ± 18.0	42.1 ± 12.7	40.5 ± 13.0	41.7 ± 12.7	NS	NS	NS	NS
Lactoferrin (μg/l)	244 ± 115	283 ± 110	304 ± 89	305 ± 104	NS	NS	NS	NS
Elastase (fg) / neutrophil	10.6 ± 5.5	8.1 ± 4.7	5.6 ± 1.9	6.3 ± 2.3	<0.05	<0.001	NS	<0.01
Lactoferrin (fg) / neutrophil	73.1 ± 39.7	50.0 ± 18.8	42.3 ± 12.5	43.6 ± 14.9	<0.05	<0.05	NS	NS
GM-CSF (pg/ml)	0.14 (0.04–0.21)	0.34 (0.19–0.52)	0.35 (0.14–0.55)	0.37 (0.25–0.53)	<0.001	NS	NS	NS

Values are mean ± SD or median (IQR) unless otherwise indicated; NS, not significant.

Table 18–12. Total and differential leukocyte count and neutrophil activation (longitudinal study)

	Pregnant			*Statistics*		
	1st trimester	**2nd trimester**	**3rd trimester**	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*23*	*23*	*23*			
WBC (x 10^9/l)	9.04 ± 2.18	10.29 ± 2.26	10.04 ± 2.10	<0.001	NS	<0.05
Neutrophils (x 10^9/l)	6.46 ± 1.64	7.70 ± 1.67	7.37 ± 1.76	<0.001	NS	NS
Eosinophils (x 10^9/l)	0.11 ± 0.07	0.16 ± 0.17	0.14 ± 0.12	NS	NS	NS
Basophils (x 10^9/l)	0.03 ± 0.04	0.03 ± 0.03	0.02 ± 0.04	NS	NS	NS
Lymphocytes (x 10^9/l)	2.03 ± 0.73	1.88 ± 0.60	1.92 ± 0.52	NS	NS	NS
Monocytes (x 10^9/l)	0.40 ± 0.17	0.53 ± 0.24	0.57 ± 0.25	<0.01	NS	<0.01
Cases with metamyelocytes	None	2 (8.7%)	2 (8.7%)			
Elastase (μg/l)	46.2 ± 15.1	39.2 ± 12.2	42.3 ± 14.6	NS	NS	NS
Lactoferrin (μg/l)	317 ± 131	297 ± 87	301 ± 110	NS	NS	NS
Elastase (fg) / neutrophil	7.7 ± 3.1	5.3 ± 1.9	5.9 ± 2.1	<0.01	NS	NS
Lactoferrin (fg) / neutrophil	49.9 ± 19.6	39.7 ± 10.0	39.6 ± 13.2	<0.05	NS	NS
GM-CSF (pg/ml)	0.33 (0.19–0.53)	0.40 (0.21–0.62)	0.35 (0.25–0.52)	NS	NS	NS

Values are mean ± SD or median (IQR) unless otherwise indicated; NS, not significant.

Haemostatic Data

The haemostatic data obtained in the cross-sectional and longitudinal studies are presented in Tables 18–13 and 18–14, respectively.

However, the evaluation of fibrinogen, t-PA, PAI-1 and D-dimer along pregnancy was only performed in the longitudinal study (Table 18–14). All these haemostatic substances increased throughout pregnancy. In the third trimester PAI-1 activity was three times that of the first trimester and the same finding occurred for D-dimer. Platelet count decreased significantly at the end of gestation. Moreover, in the cross-sectional study five pregnant women presented with thrombocytopenia (platelets < 100,000/μl) in the last trimester (Table 18–13).

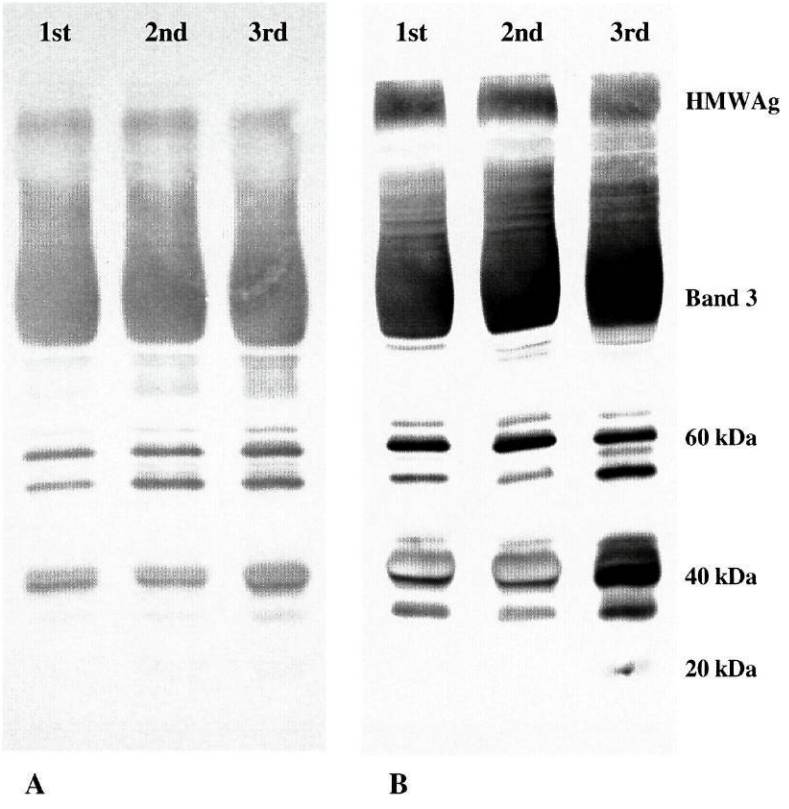
Erythrocyte Study

When compared with non-pregnant controls, pregnant women had significantly reduced RBC count, Ht, and levels of Hb and Tbil (Table 18–15). Considering the erythrocyte membrane band 3 profile, HMWAg values and the HMWAg to monomer ratio were significantly reduced, whereas Pfrag values and the Pfrag to monomer ratio were significantly increased in pregnant women.

During pregnancy (cross-sectional study), RBC count, Hb concentration and Ht reached their lowest values in the second trimester, increasing somewhat in the third trimester (Table 18–15). RDW and GPX activity showed a tendency to rise during pregnancy. The haematimetric indexes and MBH did not differ markedly between groups. The Pfrag increased in pregnancy reaching a maximum level in the third trimester of gestation. Band 3 monomer presented a significantly lower value in the third trimester. Moreover, both HMWAg to monomer and Pfrag to monomer ratios increased throughout pregnancy, although statistical significance was only achieved for the latter (first/third trimester).

In the longitudinal study, some significant changes were identified earlier in pregnancy (Table 18–16). Actually, a significant reduction in MBH was observed in the second trimester. Moreover, the band 3 profile was already significantly different in this trimester, presenting a reduction in band 3 monomer and a rise in Pfrag. In 15 of the 22 longitudinal cases studied, rises in Pfrag (%) and reductions in band 3 monomer (%) were observed from the first to the third trimester. For the same interval, 10 cases showed increments in HMWAg. Figures 18–6A and 18–6B present the band 3 profile (immunoblot) observed in two of the

longitudinal studied cases, clearly showing the rise in Pfrag throughout pregnancy, with no reduction in HMWAg. The RDW and GPX activity rose throughout gestation (Table 18–16), but only GPX activity reached a significant rise (third trimester of gestation compared with the first trimester). Tbil showed a tendency to rise (namely in the 25th and 75th percentiles), although without statistical significance.



Figures 18–6 A and B.
Immunoblots for band 3 of two longitudinal cases in the first (1st), second (2nd) and third (3rd) trimesters of human gestation.
Monoclonal anti-human band 3 recognises erythrocyte band 3, a 90–100 kDa protein, its aggregates (HMWAg) and proteolytic fragments (60, 40 and 20 kDa).

Table 18–13. Haemostatic variables (cross-sectional study)

	Non-pregnant	*Pregnant*			*Statistics*			
	N P	**1st trimester**	**2nd trimester**	**3rd trimester**	***P*** NP/1st	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*24*	*64*	*48*	*67*				
Platelets ($10^3/\mu$l)	221 ± 60	214 ± 57	224 ± 52	183 ± 59	NS	NS	<0.001	<0.01
Cases with thrombocytopenia*	None	None	1 (2.1%)	5 (7.5%)				
No. of cases				*56*				
Fibrinogen (g/l)	NM	NM	NM	5.10 ± 1.04	–	–	–	–
t-PA (ng/ml)	NM	NM	NM	8.0 (6.3-10.9)	–	–	–	–
PAI-1 (U/ml)	NM	NM	NM	56.1 ± 16.9	–	–	–	–
D-dimer (ng/ml)	NM	NM	NM	348 (245-502)	–	–	–	–

Values are mean ± SD or median (IQR) unless otherwise indicated; *Platelets < 100,000/μl; NS, not significant; NM, not measured.

Table 18–14. Haemostatic variables (longitudinal study)

	Pregnant			*Statistics*		
	1st trimester	**2nd trimester**	**3rd trimester**	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*21*	*21*	*21*			
Platelets ($10^3/\mu$l)	233 ± 56	226 ± 53	196 ± 53	NS	<0.001	<0.001
Cases with thrombocytopenia*	None	None	None			
No. of cases	*14*	*14*	*14*			
Fibrinogen (g/l)	3.70 ± 1.00	4.32 ± 0.77	5.12 ± 0.89	NS	<0.01	<0.001
t-PA (ng/ml)	5.7 ± 1.4	7.6 ± 1.6	8.5 ± 2.3	<0.01	NS	<0.01
PAI-1 (U/ml)	19.5 ± 3.9	38.1 ± 4.4	68.8 ± 9.6	<0.001	<0.001	<0.001
D-dimer (ng/ml)	122 ± 72	272 ± 201	377 ± 279	<0.05	<0.05	<0.05

Values are mean ± SD unless otherwise indicated; *Platelets < 100,000/μl; NS, not significant.

Table 18–15. RBC count, Hb concentration, Ht, haematimetric indexes, RDW, Tbil concentration, band 3 profile, MBH, and GPX activity (cross-sectional study)

	Non-pregnant	*Pregnant*			*Statistics*			
	N P	**1st trimester**	**2nd trimester**	**3rd trimester**	***P*** NP/1st	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*24*	*64*	*48*	*67*				
RBC (x10^{12}/l)	4.51 ± 0.34	4.21 ± 0.50	3.68 ± 0.34	3.86 ± 0.33	<0.01	<0.001	NS	<0.001
Hb (g/dl)	13.7 ± 0.9	12.9 ± 1.4	11.6 ± 1.0	11.9 ± 1.1	<0.01	<0.001	NS	<0.001
Ht (%)	40.4 ± 2.7	37.5 ± 3.1	33.6 ± 3.1	34.7 ± 2.9	<0.001	<0.001	NS	<0.001
MCV (fl)	89.5 ± 3.0	90.1 ± 4.1	91.5 ± 4.1	90.0 ± 4.3	NS	NS	NS	NS
MCH (pg)	30.3 ± 1.3	30.7 ± 2.4	31.6 ± 1.9	31.0 ± 2.4	NS	NS	NS	NS
MCHC (g/dl)	33.8 ± 0.9	34.1 ± 1.8	34.6 ± 1.2	34.3 ± 1.7	NS	NS	NS	NS
RDW (%)	13.0 ± 0.5	13.3 ± 0.6	13.4 ± 0.6	13.4 ± 0.6	NS	NS	NS	NS
Tbil (mg/dl)	0.61 (0.43–0.84)	0.30 (0.19–0.42)	0.24 (0.16–0.32)	0.22 (0.12–0.36)	<0.001	NS	NS	NS
No. of cases	*24*	*62*	*46*	*60*				
HMWAg (%)	16.5 ± 4.7	10.8 ± 4.5	10.7 ± 5.6	11.9 ± 5.0	<0.001	NS	NS	NS
Monomer (%)	64.4 ± 4.1	66.4 ± 7.8	66.1 ± 7.8	61.6 ± 9.2	NS	NS	<0.05	<0.01
Pfrag (%)	19.0 ± 4.3	22.7 ± 8.8	23.2 ± 9.9	26.5 ± 9.2	<0.05	NS	NS	<0.05
HMWAg/monomer	0.260 ± 0.085	0.166 ± 0.075	0.163 ± 0.084	0.204 ± 0.118	<0.001	NS	NS	NS
Pfrag/monomer	0.298 ± 0.075	0.361 ± 0.179	0.370 ± 0.189	0.467 ± 0.273	<0.05	NS	NS	<0.05
% MBH (x10^{-4})	77.5 ± 22.3	79.3 ± 21.6	74.2 ± 25.8	81.4 ± 30.8	NS	NS	NS	NS
No. of cases	*20*	*39*	*33*	*35*				
GPX (U/g Hb)	57.5 ± 9.4	63.7 ± 13.5	65.3 ± 16.5	69.8 ± 14.9	NS	NS	NS	NS

Values are mean ± SD or median (IQR); NS, not significant.

Table 18–16. RBC count, Hb concentration, Ht, haematimetric indexes, RDW, Tbil concentration, band 3 profile, MBH, and GPX activity (longitudinal study)

	Pregnant			*Statistics*		
	1st trimester	**2nd trimester**	**3rd trimester**	***P*** 1st/2nd	***P*** 2nd/3rd	***P*** 1st/3rd
No. of cases	*23*	*23*	*23*			
RBC (x10^{12}/l)	4.18 ± 0.29	3.66 ± 0.27	3.86 ± 0.32	<0.001	<0.01	<0.001
Hb (g/dl)	12.7 ± 0.8	11.3 ± 0.8	11.6 ± 1.0	<0.001	NS	<0.001
Ht (%)	37.1 ± 2.3	33.0 ± 2.3	34.4 ± 2.8	<0.001	NS	<0.001
MCV (fl)	89.0 ± 5.1	90.7 ± 4.4	89.3 ± 4.8	NS	NS	NS
MCH (pg)	30.5 ± 2.4	30.8 ± 1.8	30.2 ± 2.0	NS	NS	NS
MCHC (g/dl)	34.2 ± 1.2	34.1 ± 0.8	33.8 ± 0.8	NS	NS	NS
RDW (%)	13.2 ± 0.6	13.4 ± 0.6	13.6 ± 0.7	NS	NS	NS
Tbil (mg/dl)	0.31 (0.19–0.42)	0.29 (0.24–0.50)	0.34 (0.27–0.65)	NS	NS	NS
No. of cases	*22*	*22*	*22*			
HMWAg (%)	11.2 ± 3.2	10.8 ± 5.8	11.4 ± 5.0	NS	NS	NS
Monomer (%)	69.0 ± 7.8	63.3 ± 5.6	62.8 ± 5.8	<0.05	NS	<0.01
Pfrag (%)	19.9 ± 7.6	26.0 ± 7.6	25.8 ± 8.3	<0.05	NS	<0.05
HMWAg/monomer	0.166 ± 0.057	0.172 ± 0.092	0.182 ± 0.079	NS	NS	NS
Pfrag/monomer	0.304 ± 0.154	0.419 ± 0.139	0.424 ± 0.173	<0.05	NS	<0.05
% MBH (x10^{-4})	78.0 ± 21.9	64.4 ± 24.1	67.4 ± 27.2	<0.05	NS	NS
No. of cases	*12*	*12*	*12*			
GPX (U/g Hb)	61.4 ± 16.2	68.5 ± 15.4	74.0 ± 15.0	NS	NS	<0.05

Values are mean ± SD or median (IQR); NS, not significant.

18. Pre-eclampsia (PE)

Clinical Data

In the third trimester of gestation, 45 PEc women (88.2%) presented with proteinuria (≥1+) and 42 (82.4%) presented with oedema; two women presented with class 3 HELLP syndrome [haptoglobin = 29 and 18 mg/dl; AST = 120 and 877 U/l; ALT = 190 and 675 U/l; platelets = 145,000 and 126,000/μl, respectively]; one women presented with partial HELLP syndrome since she was diagnosed with haemolysis (haptoglobin = 8 mg/dl), elevated liver enzymes (AST = 283 U/l; ALT = 487 U/l), and a normal platelet count (227,000/μl). Table 19–1 compares healthy and PEc women in the third trimester of gestation. PEc women presented with higher systolic and diastolic blood pressure and body weight. No significant difference was found between maternal age and gestational age at sampling in both groups.

Table 19–1. Clinical characteristics of normal pregnant and pre-eclamptic (PEc) women

	3rd trimester		
	Normal	**PEc**	***P***
No. of cases	*67*	*51*	
Age (years)	26.2 ± 4.8	28.0 ± 5.5	NS
Gestational age (weeks)	34.0 ± 2.8	34.4 ± 3.6	NS
Weight (kg)	73.9 ± 9.9	80.1 ± 14.8	<0.05
Blood pressure (mmHg)			
Systolic	119 ± 11	152 ± 12	<0.001
Diastolic	62 ± 9	92 ± 9	<0.001
Cases with proteinuria (≥+)	0 (0%)	45 (88.2%)	<0.001
Cases with 1+	–	17 (33.3%)	
Cases with 2+	–	11 (21.6%)	
Cases with 3+	–	10 (19.6%)	
Cases with 4+	–	7 (13.7%)	

Values are mean ± SD unless otherwise indicated; NS, not significant.

Uric Acid and TAS

Mean uric acid level was significantly higher in PE, but no difference was observed in TAS between groups (Table 19–2). In PE, uric acid was positively and significantly correlated with TAS ($r = 0.456$, $n = 51$, $P<0.01$).

Table 19–2. Uric acid and TAS levels of normal pregnant and pre-eclamptic (PEc) women

	3rd trimester		
	Normal	**PEc**	***P***
No. of cases	*67*	*51*	
Uric acid (mg/dl)	4.0 ± 1.1	6.5 ± 1.8	<0.001
TAS (mmol/l)	1.18 ± 0.17	1.17 ± 0.14	NS

Values are mean ± SD; NS, not significant.

APPs and Liver Function Tests

AST and CRP levels were significantly higher in PE, although the significance for CRP was lost after adjustment for maternal body weight (Table 19–3). When HELLP syndrome cases were removed from this analysis, AST levels remained significantly higher in PE [25 (21–34) *versus* 29 (24–41) U/l, $P< 0.05$]; ALT and haptoglobin levels remained without statistical difference between groups.

Table 19–3. Acute phase proteins and liver function tests in normal pregnant and pre-eclamptic (PEc) women

	3rd trimester		
	Normal	**PEc**	***P***
No. of cases	*67*	*51*	
AST (U/l)	25 (21–34)	30 (24–47)	<0.01
ALT (U/l)	16 (11–20)	16 (12–28)	NS
CRP (mg/l)	3.18 (2.19–4.81)	4.83 (2.38–10.14)	<0.05(a)
Haptoglobin (mg/dl)	116.5 ± 55.9	116.6 ± 66.3	NS

Values are mean ± SD or median (IQR); NS, not significant;

(a), not significant after adjustment for maternal weight.

Lipids and Lipoproteins

PEc women exhibited higher mean serum TG concentration and lower apo A-I and HDLc levels compared with healthy pregnant women (Table 19–4). LDLc to apo B ratio and LDL-MPD were also significantly reduced in the pathologic group; the relative proportion of LDL II was significantly higher. All the differences between normal and PEc pregnancies remained significant after adjustment for body weight of the mothers.

Table 19–4. Lipids and lipoproteins in normal pregnant and pre-eclamptic (PEc) women

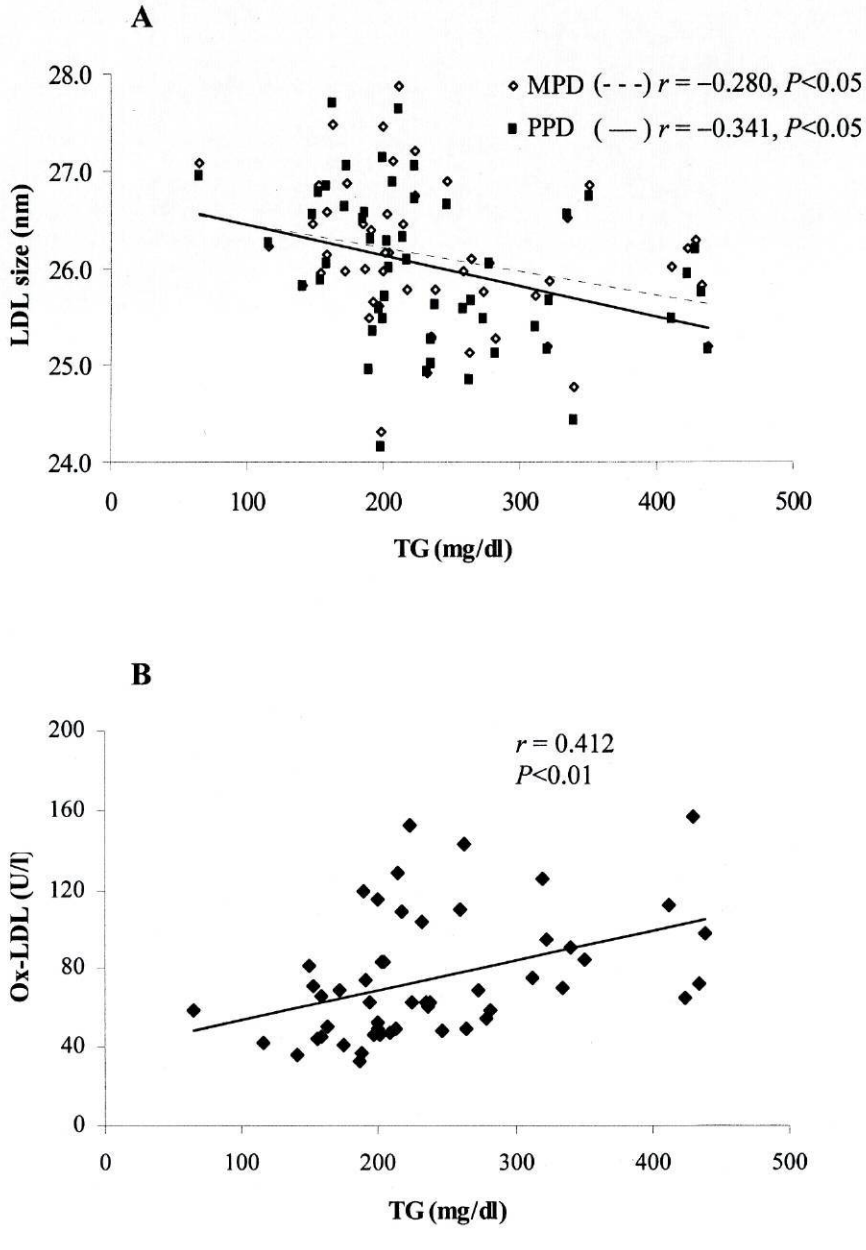
	3rd trimester		
	Normal	**PEc**	***P***
No. of cases	*67*	*51*	
TG (mg/dl)	185.8 ± 38.4	238.8 ± 85.6	<0.001
Chol (mg/dl)	285.1 ± 63.6	268.2 ± 94.7	NS
Apo B (mg/dl)	126.8 ± 31.0	130.5 ± 50.6	NS
Apo A-I (mg/dl)	211.5 ± 47.5	188.5 ± 44.1	<0.01
HDLc (mg/dl)	61.8 ± 14.2	54.2 ± 14.5	<0.01
LDLc (mg/dl)	145.9 ± 44.5	140.3 ± 60.9	NS
Lp(a) (mg/dl)			
Number of cases < 8.8 (%)	20 (29.9)	13 (25.5)	
Median (IQR)	17.1 (<8.8–49.6)	21.5 (<8.8–47.9)	NS
Chol/HDLc	4.62 ± 1.21	5.02 ± 1.86	NS
LDLc/Apo B	1.14 ± 0.15	1.05 ± 0.17	<0.01
HDLc/Apo A-I	0.29 ± 0.04	0.29 ± 0.05	NS
LDL-MPD (nm)	26.34 ± 0.52	26.05 ± 0.59	<0.01
LDL-PPD (nm)	26.09 ± 0.63	25.95 ± 0.67	NS
LDL I (%)	21.45 ± 8.78	20.54 ± 10.32	NS
LDL II (%)	41.66 ± 7.44	46.11 ± 11.02	<0.05
LDL III (%)	36.89 ± 12.94	33.36 ± 18.47	NS
Ox-LDL (U/l)	71.9 ± 24.3	75.1 ± 31.5	NS

Values are mean ± SD unless otherwise indicated; NS, not significant.

In PE, the TG levels correlated inversely and significantly with LDL-MPD and LDL-PPD (Figure 19–1A), and positively and significantly with Ox-LDL concentration (Figure 19–1B).

As happened with normal pregnancy (see Table 18–10), a highly significant positive correlation was observed between Ox-LDL and LDLc in PE ($r = 0.770$, $n = 51$, $P<0.001$). In both normal and PEc groups, Ox-LDL correlated neither with TAS nor with uric acid level.

Figure 19–1. Correlation of TG with LDL size (A) and Ox-LDL (B) in PE ($n = 51$). r, Pearson's correlation coefficient.



Haematologic Variables

Leukocyte Count and Neutrophil Activation

Both normal and PEc women presented with a trend to high values of total WBC, although within the reference range (Table 19–5). It must be noticed, however, that neutrophil number was often higher than the reference range, particularly in the case of PEc women. Moreover, a shift to the left in the differential leukocyte count was observed in 21.6% of the PEc cases, and in 7.5% of normal pregnancies. Elastase levels and elastase to neutrophil ratio were significantly higher in the pathologic group; the cases complicated with class 3 or partial HELLP syndrome showed the highest lactoferrin and elastase levels; both cases with class 3 HELLP presented with high levels of GM-CSF (1.49 and 7.95 pg/ml). In the PEc group, elastase and lactoferrin levels were significantly correlated ($r = 0.530$, $n = 51$, $P<0.001$).

Table 19–5. Total and differential leukocyte count and neutrophil activation in normal pregnant and pre-eclamptic (PEc) women

	3rd trimester		
	Normal	**PEc**	***P***
No. of cases	*67*	*51*	
WBC (x 10^9/l)	9.60 ± 2.49	10.23 ± 2.66	NS
Neutrophils (x 10^9/l)	7.02 ± 2.01	7.48 ± 2.48	NS
Eosinophils (x 10^9/l)	0.11 ± 0.13	0.09 ± 0.11	NS
Basophils (x 10^9/l)	0.02 ± 0.04	0.02 ± 0.03	NS
Lymphocytes (x 10^9/l)	1.94 ± 0.58	2.05 ± 0.71	NS
Monocytes (x 10^9/l)	0.50 ± 0.29	0.57 ± 0.25	NS
Cases with metamyelocytes	5 (7.5%)	11 (21.6%)	<0.05
Elastase (μg/l)	41.7 ± 12.7	51.3 ± 23.6	<0.01
Lactoferrin (μg/l)	305 ± 104	267 ± 103	NS
Elastase (fg) / neutrophil	6.3 ± 2.3	7.3 ± 3.1	<0.05
Lactoferrin (fg) / neutrophil	43.6 ± 14.9	38.5 ± 13.8	NS
GM-CSF (pg/ml)	0.37 (0.25–0.53)	0.40 (0.23–0.62)	NS

Values are mean ± SD or median (IQR) unless otherwise indicated; NS, not significant.

Haemostatic Data

Comparisons of haemostatic variables between normal and PEc women are presented in Table 19–6 and in Figure 19–2. In PE, t-PA and D-dimer were not normally distributed and, therefore, the concentrations of those substances are presented as medians (Figure 19–2). The concentration of fibrinogen and the platelet count were similar in normal women and in PE; PAI-1, t-PA, and D-dimer were increased in PE. The median concentration of t-PA almost doubled in PE. In normal pregnancy, four moderate outliers were detected in D-dimer (944, 992, 1113 and 1184 ng/ml). In PE, three outliers were detected in D-Dimer (>2000 ng/ml); one moderate (36.3 ng/ml) and two extreme (45.6 and 75.0 ng/ml) outliers were also identified in t-PA. When outliers were removed from both groups, the PEc group still had significantly higher medians for D-dimer (465 *versus* 337 ng/ml; $P<0.05$) and for t-PA (13.5 *versus* 8.0 ng/ml; $P<0.001$) when compared with the normal one.

A significant positive correlation was found between fibrinogen and t-PA in normal pregnancy, but this correlation was reversed in PE; the same occurred with the positive correlation between D-dimer and t-PA observed in normal pregnancy (Table 19–7). Such correlations were maintained after removal of outliers.

Table 19–6. Haemostatic variables in normal pregnant and pre-eclamptic (PEc) women

	3rd trimester		
	Normal	**PEc**	***P***
No. of cases	*67*	*51*	
Platelets ($10^3/\mu l$)	183 ± 59	191 ± 62	NS
Cases with thrombocytopenia*	5 (7.5%)	4 (7.8%)	NS
No. of cases	*56*	*37*	
Fibrinogen (g/l)	5.10 ± 1.04	5.06 ± 0.87	NS
PAI-1 (U/ml)	56.1 ± 16.9	67.2 ± 29.1	<0.05

Values are mean ± SD unless otherwise indicated; *Platelets < 100,000/μl; NS, not significant.

Figure 19–2. Distribution of D-dimer (A) and t-PA (B) in normal pregnancy (n = 56) and PE (n = 37). The boxes represent the IQR, with the upper and lower edges of the boxes representing the 75th and 25th percentiles, respectively. The central horizontal lines within the boxes represent median levels for each group. The vertical whiskers above and below the boxes represent the range of outlying data points up to 1.5 times the IQR; they extend from the box to the highest and lowest values, excluding outliers.

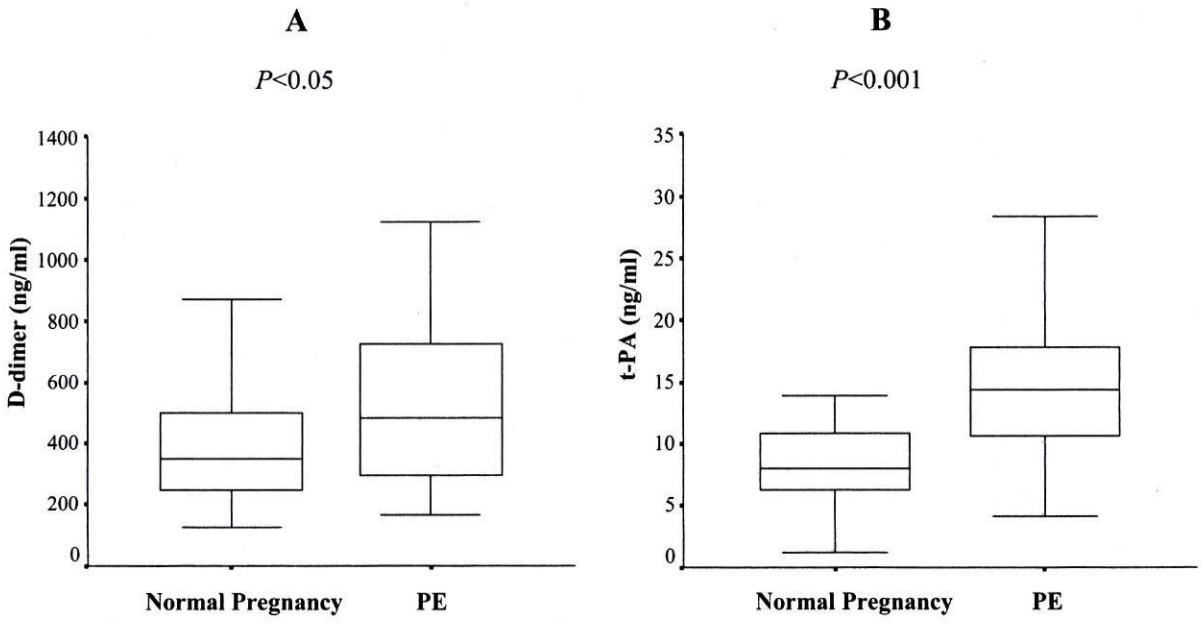


Table 19–7. Correlation of t-PA with fibrinogen and D-dimer in the third trimester of gestation in normal pregnancy and PE

		Fibrinogen	D-dimer
Normal pregnancy (n = 56)	t-PA	$r_s = 0.313, P<0.05$	$r_s = 0.268, P<0.05$
PE (n = 37)	t-PA	$r_s = -0.202$, NS	$r_s = -0.139$, NS

r_S, Spearman's rank correlation coefficient; NS, not significant.

Erythrocyte Study

The RDW, RBC count and Tbil concentration were significantly higher, whereas MCH was significantly lower in PE (Table 19–8). Statistical significance was lost for Tbil when HELLP syndrome cases were removed from the analysis [0.22 (0.12–0.36) *versus* 0.29 (0.20–0.36) mg/dl, not significant]. Similar values were found for band 3 profile, MBH, and GPX.

Table 19–8. RBC count, Hb concentration, Ht, haematimetric indexes, RDW, Tbil concentration, band 3 profile, MBH, and GPX activity in normal pregnant and pre-eclamptic (PEc) women

	3rd trimester		
	Normal	**PEc**	***P***
No. of cases	*67*	*51*	
RBC (x10^{12}/l)	3.86 ± 0.33	4.02 ± 0.37	<0.05
Hb (g/dl)	11.9 ± 1.1	12.0 ± 1.2	NS
Ht (%)	34.7 ± 2.9	35.5 ± 3.8	NS
MCV (fl)	90.0 ± 4.3	88.5 ± 6.2	NS
MCH (pg)	31.0 ± 2.4	30.0 ± 2.5	<0.05
MCHC (g/dl)	34.3 ± 1.7	33.9 ± 1.2	NS
RDW (%)	13.4 ± 0.6	13.9 ± 1.0	<0.01
Tbil (mg/dl)	0.22 (0.12–0.36)	0.30 (0.20–0.36)	<0.05(a)
No. of cases	*60*	*49*	
HMWAg (%)	11.9 ± 5.0	10.7 ± 3.8	NS
Monomer (%)	61.6 ± 9.2	63.6 ± 6.8	NS
Pfrag (%)	26.5 ± 9.2	25.6 ± 8.0	NS
HMWAg/monomer	0.204 ± 0.118	0.170 ± 0.062	NS
Pfrag/monomer	0.467 ± 0.273	0.420 ± 0.179	NS
% MBH (x10^{-4})	81.4 ± 30.8	73.2 ± 24.9	NS
No. of cases	*35*	*48*	
GPX (U/g Hb)	69.8 ± 14.9	71.5 ± 16.8	NS

Values are mean ± SD or median (IQR); NS, not significant; (a), not significant after removal of HELLP cases.

Genetic Polymorphisms

Figures 19–3 and 19–4 show, respectively, gel-separated products of apo E and CRP gene amplification and restriction enzyme digestion using DNA from study participants; homozygotic and heterozygotic combinations of apo E and CRP alleles are represented, respectively, in each of the figures.

In the examined polymorphisms, no differences were found in the distribution of subjects with respect to genotypes between normal and PEc groups (Table 19–9).

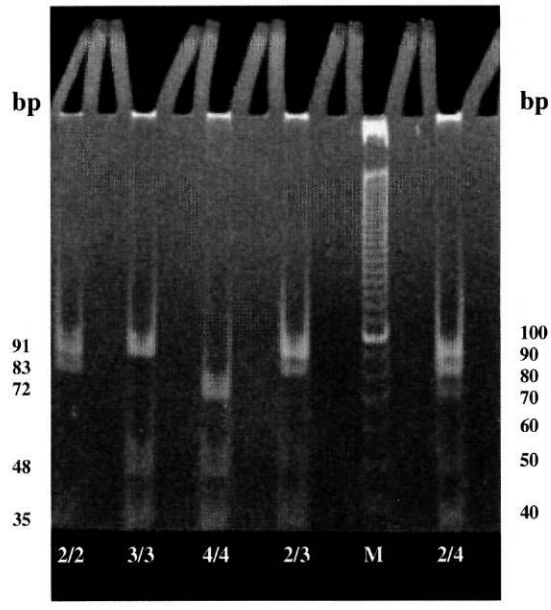


Figure 19–3. Detection of apo E genotype by PCR and digest with *Hha*I as described in Methods.
DNA fragments were separated by electrophoresis through a polyacrylamide gel (8%), and visualised by staining with ethidium bromide. Genotype is indicated below each track. E2/2 homozygote (lane marked 2/2), E3/3 homozygote (3/3), E4/4 homozygote (4/4), E2/3 heterozygote (2/3) [here, the band 48 bp is faint in this photo], and E2/4 heterozygote (2/4). Numerals on right side correspond to fragment sizes of a DNA standard (lane marked M). Numerals on left side correspond to expected fragment sizes.

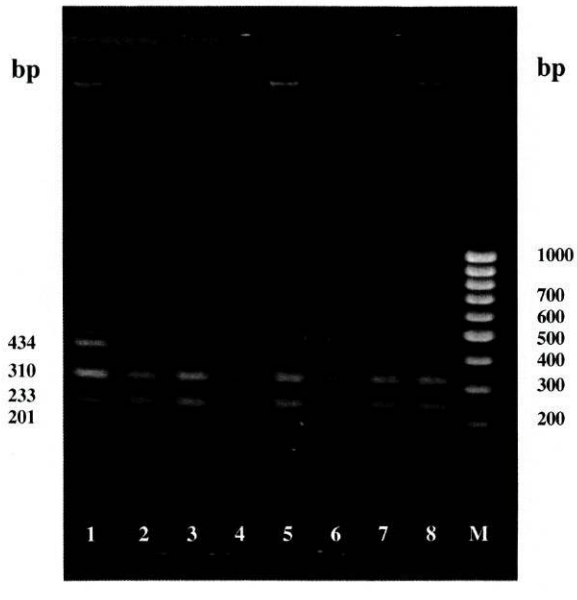


Figure 19–4. Detection of CRP 1059G/C polymorphism by PCR and digest with *Mae*III as described in Methods.
DNA fragments were separated by electrophoresis through a 2% agarose gel and visualised by staining with ethidium bromide. GC individuals (lanes 1 and 4) and GG individuals (lanes 2, 3, 5–8). Numerals on right side correspond to fragment sizes of a DNA standard (lane marked M). Numerals on left side correspond to expected fragment sizes.

Table 19–9. Frequencies of apo E and CRP genotypes in normal pregnant and pre-eclamptic (PEc) women

Polymorphism	Genotype	Normal (n = 67)	PEc (n = 51)	*P*
Apo E	**E 2/2**	1 (1.5%)	0 (0%)	NS
	E 2/3	5 (7.5%)	4 (7.8%)	
	E 2/4	1 (1.5%)	0 (0%)	
	E 3/3	46 (68.7%)	40 (78.4%)	
	E 3/4	13 (19.4%)	6 (11.8%)	
	E 4/4	1 (1.5%)	1 (2.0%)	
CRP	**GG**	61 (91.0%)	44 (86.3%)	NS
	GC	6 (9.0%)	6 (11.8%)	
	CC	0 (0%)	1 (1.9%)	

NS, not significant.

To assess associations between polymorphisms and some variables, both groups (normal and PEc) were considered together.

In the CRP polymorphism, GC individuals (n = 12) presented with lower CRP levels compared with GG individuals (n = 105), but statistical significance was not achieved [2.17 (1.20–4.99) *versus* 3.66 (2.35–6.77) mg/l, not significant].

To analyse the influence of apo E genotype on lipid and lipoprotein concentration as well as on LDL size, subjects were divided in three groups: E2 carriers (E2/2 and E2/3), E3/3 individuals and E4 carriers (E3/4 and E4/4). The E2/4 subject was not included in the analysis. Apo E genotype was noted to have significant effects on lipid and lipoprotein status (Table 19–10). The results obtained in E2 carriers (E2+) and E4 carriers (E4+), were mainly due to the contribution of E2/3 and E3/4 genotypes, respectively. E2+ presented with significantly lower values of LDLc compared to E4+ and E3/3 individuals and lower LDLc to apoB ratio compared to E4+ (Table 19–10). E2+ also presented with the highest TG level, although without statistical significance. On the other hand, HDLc and apo A-I levels were significantly reduced in E4+ compared with E3/3 (Table 19–10). Furthermore, E4+ presented with the highest Chol and LDLc levels and the LDLc to HDLc and apo B to apo A-I ratios were significantly higher in this group compared to the other two. No differences in LDL size (LDL-MPD) were found between the different apo E genotypes. All statistical significant

results remained significant after being adjusted for case/controls. After adjustment for weight, age and gestational age at sampling significance was lost for LDLc to HDLc ratio.

Table 19–10. Lipids and lipoproteins, according to apo E distribution, in pregnant women (normal and pre-eclamptic) in the third trimester of gestation

	Apo E		
	E 2+	**E 3/3**	**E 4+**
No. of cases	**10**	**86**	**21**
TG (mg/dL)	222.8 ± 94.9	207.6 ± 61.5	208.6 ± 83.1
Chol (mg/dL)	225.4 ± 70.5	281.2 ± 83.1	294.6 ± 46.9
Apo B (mg/dL)	101.2 ± 34.0	130.0 ± 42.9	137.1 ± 25.7
Apo A-I (mg/dL)	208.4 ± 64.8	206.8 ± 45.0	178.1 ± 42.5 †
HDLc (mg/dL)	54.5 ± 18.6	60.8 ± 13.5	52.2 ± 15.7 †
LDLc (mg/dL)	101.8 ± 41.4 ‡	145.1 ± 54.7	159.4 ± 32.7
LDLc/HDLc	2.10 ± 1.33	2.39 ± 0.99	3.05 ± 1.00 ‡
Apo B/ Apo A-I	0.52 ± 0.23	0.61 ± 0.20	0.76 ± 0.20 ‡
LDL-MPD (nm)	26.43 ± 0.61	26.18 ± 0.53	26.27 ± 0.69
LDLc/Apo B	0.99 ± 0.14 ¥	1.09 ± 0.16	1.17 ± 0.16

Values are mean ± SD; E2+ and E4+ represent carriers of alleles 2 (E2/2 and E2/3) and 4 (E3/4 and E4/4), respectively; the E2/4 subject was not included in the analysis; † $P<0.05$ *versus* E3/3; ‡ $P<0.05$ *versus* other two groups; ¥ $P<0.05$ *versus* E4+.

Final Correlations

In PE, the study of the associations between variables (of different areas) revealed the following:

- Uric acid, a marker of the severity of PE, did not correlate with blood pressure; uric acid correlated positively with proteinuria, although without statistical significance (data not shown).
- Diastolic blood pressure (DBP) correlated positively and significantly with RBC count, Hb concentration, and Ht values (Figures 19–5 A, B, and C, respectively). Systolic blood pressure (SBP) correlated inversely with platelet count but it did not reach statistical significance (data not shown).
- Elastase and lactoferrin correlated positively with uric acid (Figure 19–6) and inversely with apo A-I (Figure 19–7); all these correlations were statistically significant.
- The degree of proteinuria measured by dipstick showed a highly significant positive correlation with t-PA (Figure 19–8).
- D-dimer correlated significantly and positively with TG and inversely and significantly with apo A-I (Table 19–11). In the third trimester of normal gestation (n = 56), D-dimer also correlated inversely with apo A-I (r_s = –0.290, P<0.05).

Figure 19–5. Correlation of DBP with RBC count (A), Hb levels (B) and Ht values (C) in PE (n = 51). r, Pearson's correlation coefficient.

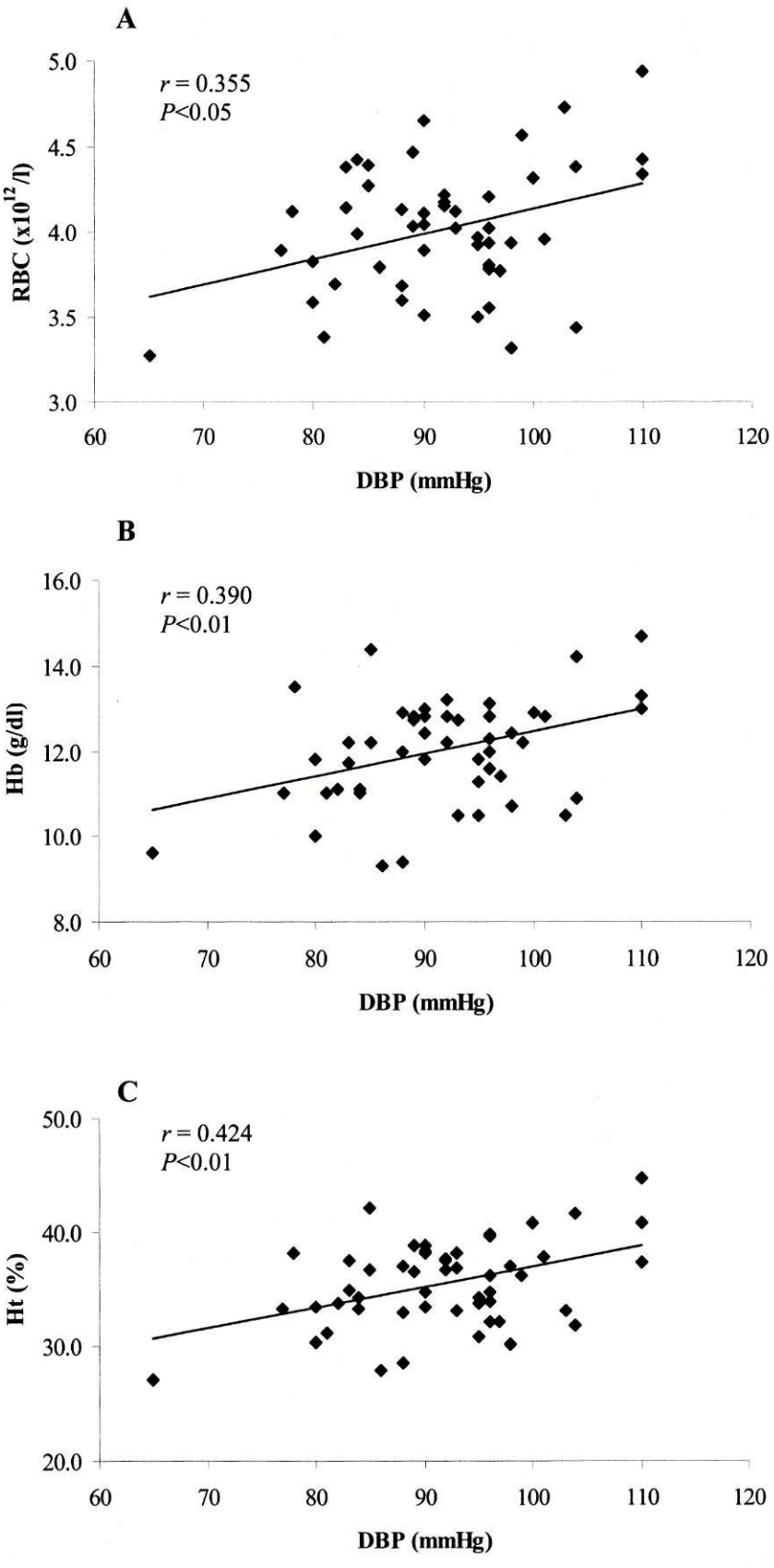


Figure 19–6. Correlation of uric acid with elastase and lactoferrin levels in PE (n = 51). r, Pearson's correlation coefficient.

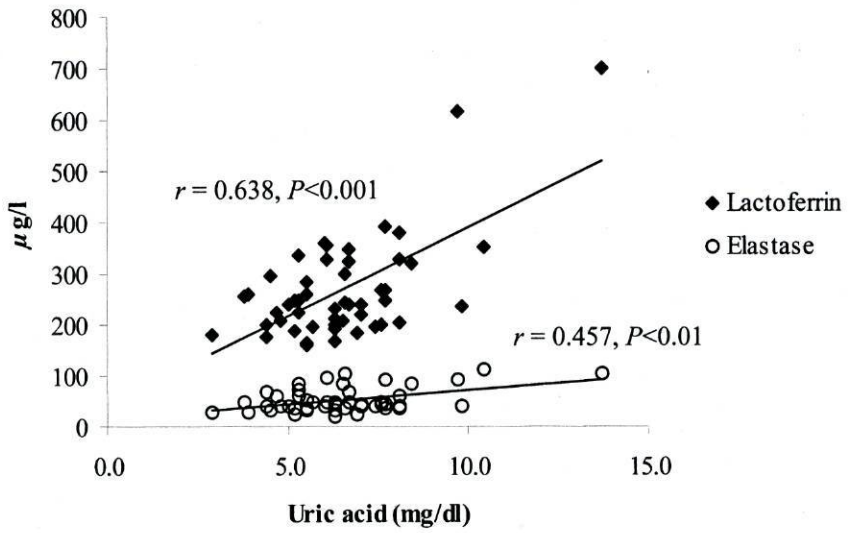


Figure 19–7. Correlation of apo A-I with elastase and lactoferrin levels in PE (n = 51). r, Pearson's correlation coefficient.

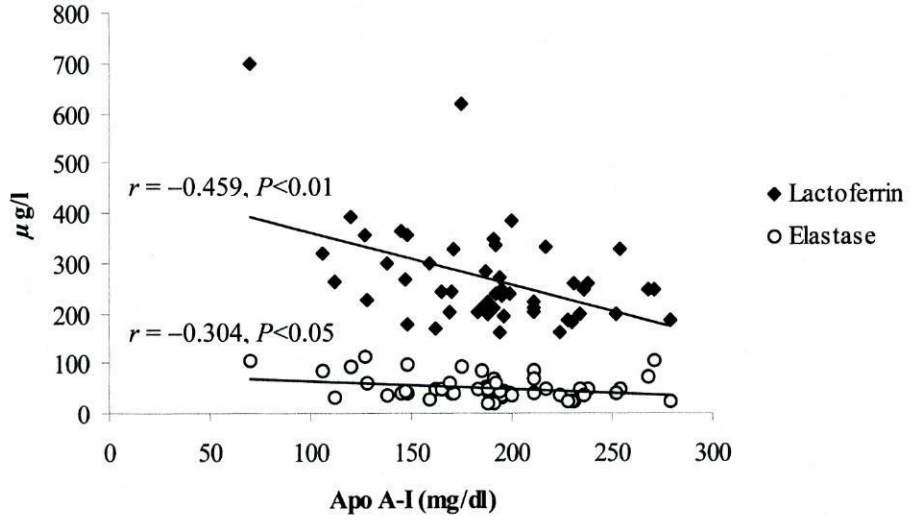


Figure 19–8. Correlation of proteinuria (dipstick test) and t-PA in PE with (r_S = 0.575, n = 37, P<0.001) and without (r_S = 0.454, n = 34, P<0.01) the three outliers of t-PA (white dots). r_S, Spearman's rank correlation coefficient.

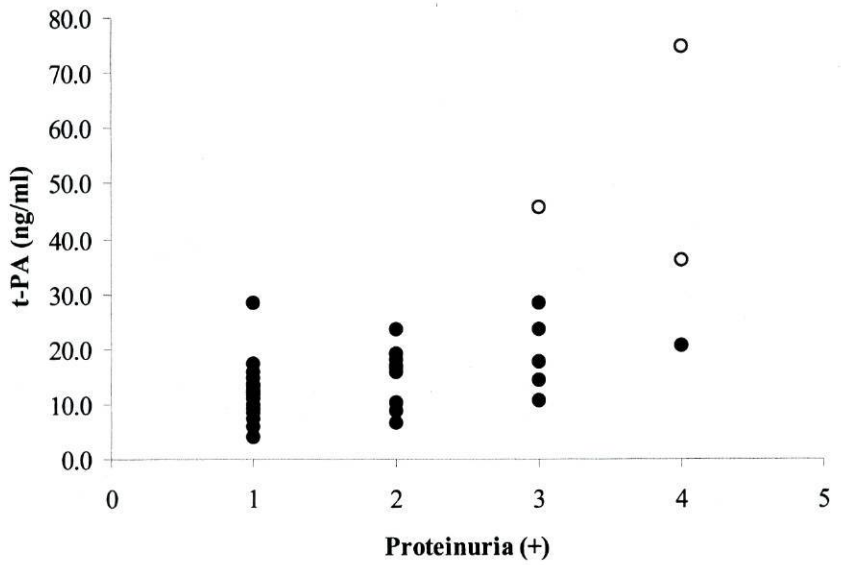


Table 19–11. Correlation of D-dimer with Apo A-I and TG in the third trimester of PE

		Apo A-I	TG
PE (n = 37)	D-dimer	r_s = –0.459, P<0.01	r_s = 0.356, P<0.05
PE (n = 34)*	D-dimer	r_s = –0.382, P<0.05	r_s = 0.443, P<0.01

r_S, Spearman's rank correlation coefficient; *, without outliers.

Section Four: Discussion and Conclusions

This work investigated systemic changes occurring in normal human pregnancy, with a particular interest on biochemical variables that might be altered as a consequence of the inflammatory process resulting from implantation. By evaluating the same variables, this work also compared normal pregnant women and women with established PE. Moreover, genetic polymorphisms were investigated in order to assess their association not only with PE but also with the levels of some of the referred variables.

The population studied was selected to present both a group of healthy human (pregnant and non-pregnant) and PEc women. To obtain uniformity, blood samples were collected on a non-fasting basis, as standardised blood sampling after a 12-h overnight fast was unfeasible in a study involving emergency cases. Moreover, the majority of the variables evaluated in this study are not significantly affected in the fasting state. Even serum lipid and lipoprotein levels have no substantial short-term effects, except for TG concentration (Franz and Wendler, 1992). In particular, fasting has no significant effect on LDL particle diameter (Hubel *et al.*, 1998a).

19. Normal Pregnancy

General Considerations

The investigation into normal pregnancy was comprised of studying the effect of pregnancy (by comparing healthy non-pregnant and normal pregnant women in a cross-sectional study) and changes occurring throughout the three trimesters of gestation (by studying normal pregnant women on a cross-sectional and longitudinal basis).

During normal pregnancy, similar results were found in both the cross-sectional and the longitudinal studies. However, some changes were detected earlier and others showed statistical significance only in the longitudinal study. Indeed, longitudinal studies seem to be more valuable in the detection of changes in variables with time, especially those variables presenting non-Gaussian distributions, such as Lp(a). The elimination of inter-individual variation is mainly responsible for this feature.

The control pregnancy group (non-pregnant subjects) was comprised of women free of medication, particularly hormonal, as it is already well established that such medication influences several variables, namely the lipid and lipoprotein profile (Tikkanen, 1999). In fact, human pregnancy is characterised by large increases in endogenous sex hormone production, namely 17β-oestradiol and progesterone (Desoye *et al.*, 1987).

APPs, Liver Function, and The Haemostatic System

The cross-sectional study demonstrated that human pregnancy is associated with an increase in circulating levels of CRP and haptoglobin, two APPs, without compromising liver cell integrity (Table 18–6). In actual fact, serum AST and ALT activities remained within the normal range of values.

However, no consistent changes were observed in CRP levels throughout gestation in both the cross-sectional (Table 18–6) and longitudinal (Table 18–7) studies.

As pregnancy progressed, the concentration of haptoglobin showed a falling trend in the cross-sectional study compared to an increasing tendency in the longitudinal study. In addition, haptoglobin levels did not change in the same manner in the different longitudinal cases studied. Interpretations of modifications in haptoglobin level become difficult because it increases during the inflammatory processes, but decreases during cases of intravascular haemolysis. The inter-individual combinations of the inflammatory process with a silent intravascular haemolysis occurring in pregnancy may account for this phenomenon.

In contrast to changes observed in those APPs, the other recognised APPs fibrinogen, t-PA, and PAI-1 increased markedly throughout gestation (longitudinal study; Table 18–14). These variables together with the evaluation of D-dimer levels demonstrated changes in the coagulant and fibrinolytic systems during gestation. Such changes are in agreement with studies indicating that fibrinogen (Stirling *et al.*, 1984), t-PA antigen (Halligan *et al.*, 1994; Sattar *et al.*, 1999) and D-dimer levels (Ballegeer *et al.*, 1987; Cadroy *et al.*, 1993; Sattar *et al.*, 1999), as well as PAI-1 activity (Estelles *et al.*, 1989), increase throughout normal pregnancy.

The rise in PAI-1 activity during pregnancy was higher than that observed for t-PA antigen (Table 18–14). Only a slight (but significant) rise in t-PA was shown when compared with the rise in PAI-1, which trebled its value throughout pregnancy. These relative changes in t-PA and PAI-1 may indicate inactivation of t-PA by PAI (formation of t-PA-PAI complexes) as PAI-1 is the primary inhibitor of t-PA in plasma (Jørgensen *et al.*, 1987). It

may also explain the reduced fibrinolytic potential during normal pregnancy reported elsewhere (Stirling *et al.*, 1984). The significant rise in the levels of D-dimer during normal pregnancy indicates a compensated state of low-grade intravascular coagulation. Moreover it shows that notwithstanding the marked impairment in fibrinolytic potential, the fibrinolytic system remains functionally active.

Lipids and Lipoproteins

In both the cross-sectional (Table 18–8) and longitudinal (Table 18–9) studies, TG, Chol, apo B and LDLc levels rose markedly and progressively as pregnancy advanced. HDLc and apo A-I concentrations also increased significantly, to reach a peak in the second trimester before decreasing thereafter. This increase in the number of HDL particles may help to protect the mother, counterbalancing the "atherogenic" modifications of the apo B containing lipoproteins and the hypercoagulable state during pregnancy. It may also be protective through an anti-inflammatory effect.

These observed changes in lipid and apolipoprotein levels during human gestation are in agreement with previous studies (Pocovi *et al.*, 1984; Piechota and Staszewski, 1992). As in previous reports (Potter and Nestel, 1979; Ordovas *et al.*, 1984; Piechota and Staszewski, 1992; Alvarez *et al.*, 1996), increments in TG levels during gestation were much more marked compared with those observed in Chol. The increment in TG level is primarily due to an increase in VLDL level (Sattar and Greer, 1999). In the late second trimester of normal human gestation, human placental lactogen and relative insulin resistance promote an increased flux of free fatty acids to the liver, where they are either oxidised or released to the circulation as VLDL (Sattar *et al.*, 1996). Oestrogen may inhibit hepatic lipid oxidation with a resulting increase in serum TG (Sattar and Greer, 1999).

In the present work, the evaluation of Lp(a) and LDL size as well as Ox-LDL were of particular interest.

The longitudinal study in normal pregnancy clearly showed that Lp(a) concentration rises markedly throughout gestation (Table 18–9; Figure 18–4B). This rise may contribute to the impaired fibrinolysis described during human gestation (Stirling *et al.*, 1984). The results presented here are in agreement with a previous longitudinal study performed in a smaller number (n = 10) of cases (Sattar *et al.*, 2000). In other work it was reported that Lp(a) levels reach a peak in the middle of pregnancy and decrease thereafter (Zechner *et al.*, 1986). In the present study from 21 longitudinal cases, only 4 (19.0%) showed a decrease in Lp(a) levels from the second to the third trimester, explaining the slight decrease of Lp(a) median value

between these two periods. However, the majority of longitudinal cases presented progressive increments between each trimester, justifying the steadily increase of 25th and 75th Lp(a) percentiles throughout gestation. Moreover, Lp(a) increments from the first to the third trimester, were quite different between individuals (ranging from 6 to almost 400%) and did not correlate with the increments in lipids. In the cross-sectional study (Table 18–8), despite no significant changes in median Lp(a) levels, a trend towards higher values was observed as pregnancy progressed. Moreover, Lp(a) medians were lower than those observed in the longitudinal analysis but similar to those found in other populations. The reason why median values were higher in the longitudinal analysis is possibly due to random factors, as Lp(a) presented a highly skewed distribution in all examined groups. The absence of significant differences in the cross-sectional study may also explain the poor correlation between Lp(a) levels and gestational age found in other reports (Cachia *et al.*, 1995; Wang *et al.*, 1998; Mori *et al.*, 2003).

Concerning the HDLc to apo A-I ratio, this decreased in early pregnancy and became progressively smaller as pregnancy advanced. This probably reflects the accumulation of TG in HDL particles, which is in agreement with the shift of HDL subclasses towards larger, TG-rich and more buoyant species during gestation (Alvarez *et al.*, 1996).

In contrast, as pregnancy advanced, there was a change in the LDL profile towards smaller, denser species. This reduction was already evident in the early stages of gestation and the elevated levels of TG seem to be responsible for the increase in small, dense LDL. Indeed, in the first trimester of gestation, TG levels were already significantly elevated and LDL size was significantly decreased compared with non-pregnant controls (Table 18–8). Moreover, a significant inverse correlation between serum TG and LDL size was observed during gestation (Figures 18–5A and 18–5B). The decrease in LDL size could reflect increased production and/or reduced clearance of small dense LDL (Silliman *et al.*, 1994). During pregnancy, concentrations of $VLDL_1$ progressively increase which promotes TG enrichment of LDL particles; the subsequent hydrolysis of TG in LDL even with lower gestational HL activity, may result in the remodelling of LDL subclasses into smaller, denser species (Sattar *et al.*, 1997b). Interestingly, in the present longitudinal study, only two out of twenty-three women showed no substantial change in LDL size throughout pregnancy. Moreover, in the 21 longitudinal cases demonstrating significant reductions in LDL size, the changes were more pronounced from the first to the second trimester, compared to the second *versus* the third trimester of gestation. This may be explained by a progressive decrease in HL activity as

pregnancy progresses (Alvarez *et al.*, 1996). Reduced activity of HL may also justify, at least in part, the TG enrichment of LDL particles (Alvarez *et al.*, 1996; Sattar *et al.*, 1997b), and particularly HDL particles (Alvarez *et al.*, 1996), with advancing gestation.

The curiosity was the analysis of the LDLc to apo B ratio. This was significantly reduced in the first trimester of gestation compared to the non-pregnant state but during gestation, a non-significant increment was observed (Tables 18–8 and 18–9). It must be borne in mind that apo B refers to serum apolipoprotein B concentration and thus the LDLc to apo B ratio is influenced by all apo B containing lipoproteins.

The literature usually reports LDL size as LDL-PPD (Silliman *et al.*, 1994; Hubel *et al.*, 1998b). However, it seems that LDL-MPD is a more consistent indicator of the size of LDL particles than LDL-PPD. While LDL-PPD only indicates the predominant size at the major LDL band, LDL-MPD describes the size of the entire LDL population (all bands taken into account). To address this problem, some authors use the "LDL particle score", a calculation that involves the contribution of the relative proportion of each of the LDL subfractions (Campos *et al.*, 1992). The higher the particle score, the smaller the particle size (the smaller the LDL-MPD). However, it makes more sense to use LDL-MPD, a real measure of particle size, to compare with LDL-PPD; as rather than giving an arbitrary number, the LDL-MPD value can be equated to LDL particle sizes that have been quoted in the literature over the last twenty years. Thus, it seems more adequate to measure LDL-PPD together with LDL-MPD.

Furthermore, *in vitro* studies have shown that small dense lipoproteins are more susceptible to oxidation (Graaf *et al.*, 1991) and the present work demonstrated, in an *in vivo* model (human pregnancy), that reductions in LDL size are clearly associated with increments in Ox-LDL levels (Tables 18–8 and 18–9). Also of importance, were the correlations obtained between non-fasting TG levels and Ox-LDL concentrations (Figures 18–5C and 18–5D). It may be possible that increments in TG are the primary driving force for the increases in Ox-LDL levels. Moreover, increments in Ox-LDL are likely to be the result of qualitative (formation of smaller, denser LDL species) and quantitative (increased number of LDL particles) changes.

It was reported that the resistance of LDL towards oxidation increases throughout gestation (Vriese *et al.*, 2001). On the other hand, the increased levels of conjugated dienes formed suggest that the amount of substrate available for lipid peroxidation in LDL also increases (Vriese *et al.*, 2001). Although LDL resistance to *in vitro* oxidation increases during gestation, it is quite possible that a continuous stimulus generated *in vivo* may consume

endogenous antioxidant defences in LDL, which coupled with an increased amount of substrate available for lipid peroxidation, may lead to an increment of oxidatively modified LDL particles in the circulation.

The increase in Ox-LDL throughout gestation (Tables 18–8 and 18–9) may contribute to the hypercoagulable state during pregnancy and the increased susceptibility of pregnant women to develop thrombotic disorders. Moreover, when uncontrolled, it may increase oxidative damage/inflammation and impair the pregnancy outcome. However, in normal pregnancies the Ox-LDL increment seems to be controlled.

A wide variety of epitopes may be presented by Ox-LDL (Hörkkö *et al.*, 2000). In this study, Ox-LDL was measured by a commercially available sandwich ELISA (Mercodia) which uses the same specific murine monoclonal antibody, mAb-4E6, that Holvoet *et al.* (1998) used in their assays. This monoclonal antibody is directed against a conformational epitope in the apo B-100 moiety of LDL that is generated as a consequence of substitution of lysine residues of apo B-100 with aldehydes (Holvoet *et al.*, 2001). However, it is possible that other oxidation-specific epitopes such as oxidised phospholipids, are generated in LDL during gestation. The content of oxidised Chol forms may also increase, as Ox-LDL and LDLc increased in parallel during gestation (Tables 18–8 and 18–9) and showed very strong correlations (Table 18–10).

This work also showed that changes in lipids and lipoproteins during pregnancy parallel changes occurring in the haemostatic system. Lipids and lipoproteins can contribute to either procoagulant or anticoagulant reactions. Procoagulant lipids/lipoproteins include Ox-LDL and TG-rich particles, which can accelerate the activation of prothrombin, factor X and factor VII (Griffin *et al.*, 2001). Potentially anticoagulant lipids and lipoproteins include phosphatidylethanolamine, cardiolipin, the neutral glycosphingolipids glucosylceramide and Gb3 ceramide, as well as HDL, each of which enhances the inactivation of factor Va by activated protein C (Griffin *et al.*, 2001). Lp(a) is another link between lipid metabolism and the haemostatic system. Lp(a) has anti-fibrinolytic activity (Hajjar *et al.*, 1989) and may be directly involved in the coagulation, by binding and inactivating tissue factor pathway inhibitor (Caplice *et al.*, 2001). Hyperlipidaemia (Griffin *et al.*, 2001), namely hypertriglyceridaemia (Simpson *et al.*, 1983) is associated with hypercoagulability and thus hyperlipidaemia may be, at least in part, responsible for the hypercoagulable state developed during pregnancy.

LDL Oxidation and Antioxidant Status

The oxidation of LDL is influenced by its content in antioxidants and some of these antioxidants correlate with their plasma concentration; high correlations were found between plasma and LDL content in γ-tocopherol and carotenoids, but not in α-tocopherol (Ziouzenkova *et al.*, 1996). Plasma or serum levels of α-tocopherol were reported to increase during pregnancy (Wang *et al.*, 1991a; Oostenbrug *et al.*, 1998; Vriese *et al.*, 2001) but given the lack of correlation between the α-tocopherol content of plasma and LDL, it is not clear how increases in this vitamin in plasma contribute to the *in vitro* oxidative resistance of LDL (Vriese *et al.*, 2001). Moreover, other lipid soluble antioxidants such as vitamin A (Oostenbrug *et al.*, 1998; Vriese *et al.*, 2001) and β-carotene (Oostenbrug *et al.*, 1998) decrease in plasma throughout pregnancy. Thus, it is possible that the total antioxidant content of plasma also decreases during pregnancy, creating a more favourable environment for LDL oxidation. In this longitudinal study, serum TAS decreased significantly from the first to the third trimester (Table 18–4) and the relative increments in Ox-LDL concentrations correlated inversely with the relative decreases in TAS levels between trimesters ($r = -0.347$, $P<0.05$). Interestingly, the increments in Ox-LDL concentrations and the decreases in TAS levels were less pronounced at the end of gestation.

The TAS kit that was used to assess total antioxidant capacity does not measure individual antioxidants but rather the overall effect of many antioxidants working in synergy. TAS may correlate with a wide range of components, such as uric acid, albumin, bilirubin, ascorbate, and glutathione reductase, the highest correlations being obtained with uric acid and albumin (Lamont *et al.*, 1997). Uric acid, which can act as a chain-breaking agent (by scavenging oxygen radicals) and as a preventive antioxidant (by forming a stable complex with iron ions), has been found to be a particularly strong antioxidant (Miller *et al.*, 1993; Koracevic *et al.*, 2001). Albumin, although having a lower antioxidant activity than uric acid, may even be of greater importance as an antioxidant due to its high concentration in plasma (Miller *et al.*, 1993). Other antioxidants, namely selenium, vitamin A, vitamin E, and bioflavonoids may also contribute to TAS (Lamont *et al.*, 1997). It is important to point out that in one study, although uric acid was both independently and significantly correlated with TAS, its contribution to r^2 was 0.31, suggesting that the major part of TAS was determined by factors other than uric acid (Rosell *et al.*, 1999). The same may be applied to the present study. Indeed, uric acid concentration increased significantly throughout gestation (Tables 18–3 and 18–4) and was positively and significantly correlated with TAS values (Table

18–5). However, TAS decreased as pregnancy progressed. It could be speculated that a decrease in serum levels of albumin as well as other antioxidants may be relevant to the decrease in serum TAS during pregnancy. Results obtained in this work contrast with those reported by Davidge *et al.* (1992), who reported a progressive increase in serum antioxidant activity throughout normal human gestation. However, to assess antioxidant activity in that study the authors used the brain homogenate auto-oxidation bioassay, which mainly detects the preventive antioxidants transferrin and caeruloplasmin, known to increase in pregnancy (Hubel, 1998).

Particular attention must be paid to caeruloplasmin. Indeed, although caeruloplasmin is generally considered an antioxidant, mainly due to its ferroxidase activity (Halliwell and Gutteridge, 1990), recent studies indicate that intact caeruloplasmin is prooxidant in nature, being able to oxidise LDL (Khovidhunkit *et al.*, 2000). Caeruloplasmin is an APP that carries about 95% of the plasma copper and is associated with HDL. Caeruloplasmin may enhance LDL modification by providing a source of transition metals for oxidative reactions (Van Lenten *et al.*, 2001). Thus, it is unclear as to whether caeruloplasmin is an antioxidant or prooxidant *in vivo*.

The Erythrocyte

This work clearly shows that pronounced changes in the erythrocyte profile occur during pregnancy (Tables 18–15 and 18–16). RBC count, Hb concentration and Ht decreased significantly in pregnant women. These findings express the "anaemic" state of pregnancy, caused by the physiological increase of plasma volume during pregnancy (Wintrobe *et al.*, 1993). Maximum haemodilution occurred in the second trimester of gestation, which is in close agreement with recent findings (Milman *et al.*, 2000). It must not be forgotten that in this study pregnant women received folic acid and iron supplements, inhibiting their deficiencies. Indeed, it was reported that placebo-treated women present significantly lower haematologic indices compared with iron-treated women (Milman *et al.*, 2000). Moreover, the present data also showed a rise in RBC count, Ht, and Hb concentration from the second to the third trimester of gestation.

Although serum TAS decreased significantly in the longitudinal study, the whole blood GPX activity increased significantly (Table 18–16). This probably reflects an increase of this antioxidant enzyme in erythrocytes during gestation. Indeed, although GPX activity was analysed in whole blood, the major contribution seems to be made by erythrocytes, since

plasma activity of this enzyme is low (Halliwell and Gutteridge, 1990). It was reported that a fraction of about 0.90 of the GPX in the blood is contained in the erythrocytes (Behne and Wolters, 1979).

This work also reports significant changes in the erythrocyte membrane band 3 profile in pregnancy. Pregnant women in the first trimester of pregnancy, when compared with non-pregnant cases, had significantly reduced HMWAg and increased Pfrag (Table 18–15). This suggests that pregnancy may have induced a rejuvenation of the RBC population. In fact, erythrocyte membranes from younger RBCs are poorer in HMWAg and richer in Pfrag (Santos-Silva *et al.*, 1998). On the other hand, pregnancy-induced changes in HMWAg and Pfrag may also suggest an enhanced proteolytic stress imposed on RBCs.

When the band 3 profile (found in the first trimester of pregnancy) was compared with that observed in the third trimester, a significant reduction in band 3 and a significant rise in Pfrag were found. This would suggest an increase in the population of younger RBCs. However, such a change would imply that the amount of HMWAg should also decrease, but this did not happen. In fact, when comparing the HMWAg to band 3 monomer and Pfrag to band 3 monomer ratios between the first and third trimesters, an increase in both was observed. Moreover, a trend towards an increase in Tbil was observed in the longitudinal study. In addition, the reduction in MCV and the rise in MBH, observed between the second and third trimesters, also suggests an accelerated RBC ageing or damaging process. Also indicative of RBC damage, Rebelo *et al.* (1995) found that RBCs of pregnant women are slightly more osmotically fragile than in the non-pregnant healthy population. In that study, levels of plasma haemoglobin were also higher in pregnant women, suggesting increased haemolysis of RBCs.

The present data suggest that pregnancy is actually associated with a higher RBC production and therefore, younger erythrocytes. However, the changes in band 3 profile also suggest a higher RBC removal which may result from an accelerated ageing or damaging process. If the rise in Pfrag reflects the youth of RBCs, it might be said that the increase in RBC production rate is higher than the increase in the RBC removal rate, thereby justifying the rejuvenation of the erythrocyte population in pregnancy. However, the possibility that the rise in Pfrag may also result from an enhanced proteolytic stress imposed on RBCs should not be excluded.

The band 3 profile of pregnancy is in agreement with previous reports indicating that human gestation is associated with an increase of younger RBCs (Tian *et al.*, 1998; Choi and Pai, 2001). The rise in RDW and particularly GPX activity, which was found throughout the

longitudinal study (Table 18–16), might also be explained by the appearance of a younger RBC population. This result is in agreement with a previous report that identified a significant increase in erythrocyte GPX activity during pregnancy (n = 20) (Uotila *et al.*, 1991). Another smaller study (n = 10) in which significant increases in GPX could not be detected, reported significant increases in erythrocyte superoxide dismutase activity (Carone *et al.*, 1993).

The data presented in this work show that the band 3 profile seems to be a marker of erythrocyte changes during pregnancy which is independent of the "physiological anaemia". The band 3 profile that was found in pregnancy may not only be due to an increase in damaged RBCs, but also to an increase in younger RBCs in the maternal circulation. This equilibrium in the erythrocyte production/removal rates may assure a younger and more functional RBC population throughout pregnancy, in order to face the increased oxygen demands of pregnancy and to prepare the mother for delivery.

Leukocytes

The number of leukocytes increased significantly in pregnancy and was mainly due to increments in neutrophil count (Table 18–11). Higher levels of cortisol in pregnant women trigger the mobilisation of the leukocyte marginal pool, increasing the number of circulating WBCs (Cleary and Duggan, 1991). In this cross-sectional study, GM-CSF levels were higher in pregnant women (Table 18–11), which may also account for the increase in WBC count, by stimulating granulo-monocytopoiesis.

Lactoferrin and elastase were used as markers of leukocyte activation. Although the concentration of both was higher in pregnant women than in non-pregnant controls, the difference between groups did not reach statistical significance (Table 18–11). Moreover, the ratios of these substances *per* neutrophil count were significantly reduced in pregnant women, and decreased as pregnancy advanced to reach a minimum value in the second trimester of gestation (Tables 18–11 and 18–12). These results are in agreement with previous findings on elastase, but do not confirm that the lactoferrin to neutrophil ratio is significantly higher in pregnant women compared with non-pregnant controls (Rebelo *et al.*, 1995). The differences in blood handling and methodology used may be responsible for the results obtained. Although the present work suggests that neutrophils are not activated during pregnancy, an increase in concentration of both lactoferrin and elastase in blood (especially when haemodilution is taken into account) is evident. This increase of neutrophil activation products in plasma can play a significant role in RBC injury (Santos-Silva *et al.*, 2002).

Moreover, the hypothesis that neutrophil activation may occur locally in a confined space must not be excluded, being expressed in the systemic circulation by small rises in the leukocyte activation products. The development of higher sensitivity assays may reveal different conclusions in the near future and clarify the controversy existing in the literature regarding neutrophil activation in pregnancy. Moreover, the present study did not assess the activation of other leukocytes, namely monocytes and lymphocytes. Sacks *et al.* (1998) showed a significant increase in basal intracellular reactive oxygen species values in monocytes and lymphocytes among normal pregnant women (third trimester of gestation) compared to the normal non-pregnant control women. In that study, monocytes from normal pregnant women also showed significantly higher surface expression of CD11b, CD14, and CD64. Further studies are required to substantiate these findings.

Final Conclusions

Human pregnancy is in summary, associated with profound changes in maternal blood. However, some of these changes are not well defined and the physiologic significance of some is still clearly not understood. The aim of the present work was to evaluate and/or clarify changes occurring in different organs and systems during gestation, with particular attention to those associated with the inflammatory process, by performing both a longitudinal and cross-sectional study in healthy pregnant women.

The follow up obtained in the longitudinal study, by reducing inter-individual variability, was particularly valuable in detecting changes throughout pregnancy in the examined variables especially in those presenting non-Gaussian distributions [i.e. Lp(a)]. Compared with the cross-sectional study, some modifications were detected earlier, while others showed statistical significance only in the longitudinal study.

The analysis of APPs and leukocytes confirmed that normal human gestation leads to an inflammatory response from the mother. Increments in CRP level and leukocyte count were pronounced early in gestation and high values of these variables were observed until term. The rise in leukocyte number was primarily due to an increase in the number of neutrophils. Despite the marked increase in neutrophils, the analysis of plasma levels of lactoferrin and elastase showed no evidence of neutrophil activation during gestation. However, it is important to note that plasma levels of both elastase and lactoferrin tended to be higher in pregnancy.

The inflammatory response in pregnancy was accompanied not only by an increase in younger erythrocytes, but also by an increase in damaged erythrocytes in the maternal

circulation. The analysis of the erythrocyte band 3 profile was of particular importance in detecting the changes observed in RBCs, by showing not only an increase in the Pfrag to monomer ratio but also an increase in the HMWAg to monomer ratio during gestation.

Other modifications, such as those observed in lipid and lipoprotein profile and the haemostatic system may also be at least in part, components of this inflammatory response. Human gestation was associated with changes in LDL profile towards smaller, denser species, with concomitant increments in the level of Ox-LDL. Moreover, there is evidence to suggest that TG are primarily involved in the pregnancy-induced modifications of LDL. It is also worth noting that changes in lipids and lipoproteins throughout gestation were accompanied by changes in the coagulation and fibrinolytic systems, with the development of a procoagulant state (progressive increments in fibrinogen and D-Dimer; increase in PAI-1 activity and in t-PA levels during gestation, with the increments in PAI-1 activity being much more pronounced than those observed for the t-PA antigen).

Although many of the observed changes in the maternal blood during pregnancy have an "atherogenic-like" feature, they seem to be controlled, thus being well tolerated by the mother. Moreover, the exact role of some of these changes remains to be clarified.

20. Pre-eclampsia (PE)

General Considerations

PE is a multisystem disorder, affecting several organs and systems, and it has been proposed that PE represents an excessive maternal inflammatory response to pregnancy (Redman *et al.*, 1999). However, despite intensive research in PE, contradictory results are found in the literature. In part, this may be explained by the lack of consistency in research articles regarding the definition of PE, making comparisons between studies difficult to interpret (Chappell *et al.*, 1999a). In addition, some studies have only investigated restricted areas of inflammation, overlooking an overall inflammatory condition.

In the present study, PEc women were selected following the ACOG definition of gestational hypertension accompanied by proteinuria and/or oedema (NHBPEP Working Group, 1990), as this was the definition adopted by obstetricians in the University Hospital of S. João, at the beginning of the study. However, it must be emphasised that, besides increased blood pressure, the majority of PEc women presented with proteinuria (Table 19–1), which is in close agreement with the generalised and accepted definition of PE (gestational hypertension plus proteinuria) (Sibai, 2002; Roberts *et al.*, 2003). Moreover, since proteinuria could be confounded with infection, routine bacteriological tests were performed.

The fact that PEc women were taking anti-hypertensive medication explains why blood pressure was not even higher. In some cases, blood samples were collected after the start of anti-hypertensive therapy. Although anti-hypertensives are known to modify some variables such as lipid and lipoprotein concentrations (Müller-Wieland and Krone, 1999), in pre-treated cases drugs were administered for only a few hours. Therefore, it was assumed that these drugs had no marked effect on the evaluated variables.

PEc women had significantly higher weight when compared with matched controls (Table 19–1). As maternal obesity is a risk factor for PE (Branch and Porter, 1999; Sibai, 2002), it is likely that in the present study a high number of PEc women were obese. However, this is just a hypothesis. Indeed, not only is it difficult to correlate maternal weight during gestation with measures of obesity, but also the increased maternal weight may simply be the result of oedema.

Diagnosis of cases with HELLP syndrome was made according to the Mississippi triple classification with a variant. To detect haemolysis, the determination of serum haptoglobin instead of lactic dehydrogenase was followed since it is a more specific and sensitive method

(Rath *et al.*, 2000). The determination of serum CRP complemented this diagnosis, since normal concentrations of haptoglobin are to be expected despite haemolysis, due to the presence of an inflammatory disease (Rath *et al.*, 2000). In the PEc group, as only three cases were diagnosed with HELLP syndrome and none of those was too severe (two cases with class 3 HELLP syndrome and another with partial HELLP syndrome) it was not attempted to create a subgroup. It should be referred however that, besides abnormal laboratory tests used in the diagnosis of HELLP (haemolysis, elevated liver enzymes and low platelet count), the most striking laboratory finding of these three cases was an extremely high level of neutrophil activation.

It is also important to refer that DBP correlated significantly and positively with RBC count, Hb concentration and Ht values (Figures 19–5 A, B, and C, respectively), suggesting that haemoconcentration is likely to occur in more severe forms of PE. However, mean Ht and mean Hb levels were not found to be statistically different between women with PE and control pregnant women (Table 19–8), indicating that significant differences between both groups in the biochemical variables were not a result of haemoconcentration in the PEc patients.

APPs, Liver Function, and The Haemostatic System

The present study showed that some APPs are elevated in women with PE. These women showed significantly higher levels of CRP compared with normal pregnant women, but this significance was lost after adjustment for maternal weight (Table 19–3). As CRP levels are associated with measures of obesity (Mendall *et al.*, 1996; Yudkin *et al.*, 1999; Folsom *et al.*, 2001), these results may reflect obesity-mediated inflammation. However this is just a hypothesis as already referred.

Increased levels of the pro-inflammatory cytokine IL-6 have been described in PE (Greer *et al.*, 1994; Teran *et al.*, 2001; Takacs *et al.*, 2003) and may account for the increased production of CRP. Previous reports have already described significantly higher levels of CRP in PE (Rebelo *et al.*, 1996; Teran *et al.*, 2001), although no reference was made to maternal weight or BMI. It remains uncertain as to whether higher CRP levels are present before the clinical manifestation of PE, as both positive (Wolf *et al.*, 2001) and negative (Savvidou *et al.*, 2002) data exist.

In contrast to CRP, no differences were found in mean serum haptoglobin levels (Table 19–3), another APP. As the haptoglobin concentration is greatly reduced in HELLP syndrome

(as a consequence of the microangiopathic haemolytic anaemia) the inclusion of cases with HELLP could be a major confounding factor in the analysis of the full PEc group. However by excluding these cases, the mean level of haptoglobin was not greatly affected, neither was the statistical comparison between control and PEc groups. This may indicate that the production of this APP is not increased in PE or, if it is, the haptoglobin value obtained may result from an enhanced intravascular haemolysis.

To assess hepatic function, serum levels of both AST and ALT were evaluated. Serum AST activity was significantly increased in PE (and this remained significant even after the removal of HELLP syndrome cases from the statistical analysis; Table 19–3). On the other hand, a rise in ALT activity, which is known to be a more specific indicator of hepatocellular damage, was not observed. These results clearly indicate that even if a hepatic involvement in PEc patients without HELLP syndrome exists, the hepatocyte is not severely affected.

Considering the HELLP syndrome cases, it is noteworthy that although both AST and ALT were dramatically increased, in one of these cases their values were particularly elevated and the AST activity was higher than that observed for ALT, indicating the existence of hepatic cell necrosis.

Variables related to haemostasis were also evaluated and when data was analysed, some outliers regarding D-dimer (both groups) and t-PA (PE) were detected. The presence of outliers in both groups may represent high variability in those substances between subjects. On the other hand, they may reflect an undiagnosed pathology in women presenting such high values. In fact they may even represent a technical error during the analytical determination. As any of these hypotheses can be rejected, the statistical analysis was performed with and without outliers. On completion, it was shown that such values had no substantial effect on the statistical results obtained, nor the subsequent conclusions drawn.

Women with PE had significantly higher levels of PAI-1 (Table 19–6), D-dimer and t-PA (Figure 19–2). Some studies have already reported similar results in PAI-1 activity (Estelles *et al.*, 1989; Estelles *et al.*, 1991; Shaarawy and Didy, 1996; Schjetlein *et al.*, 1997; Schjetlein *et al.*, 1999), t-PA antigen (Estelles *et al.*, 1991; Friedman *et al.*, 1995) and D-dimer (Bellart *et al.*, 1999) levels. As these substances were measured together in the present study (unlike previous studies) it was also possible to compare their relative changes.

PAI-1 and t-PA are both produced by endothelial cells and their greater levels in the third trimester in PE may suggest the development of endothelial dysfunction. The increased

concentrations of D-dimer indicate increased intravascular coagulation and activation of fibrinolysis.

As this study involved patients with established PE, the possibility of these variables working as PE markers cannot be concluded. Halligan *et al.* (1994) reported that elevated levels of PAI-1 antigen occur early in pregnancy, which subsequently develop PE (n = 4) suggesting that PAI-1 may have a predictive value. A larger investigation (Djurovic *et al.*, 2002) failed to confirm the predictive value of the PAI-1 antigen, although the difference in PAI-1 levels between women who later developed PE (n = 71) and matched controls (n = 71) was of borderline statistical significance (P = 0.06). Further larger studies are required to clarify this point.

No differences were found in platelet count between the PEc and the control groups (Table 19–6). Platelet count may be useful in predicting coagulation abnormalities in hypertensive disorders of pregnancy (Barron *et al.*, 1999). Women with PE seem to have a greater decrease in platelet count in pregnancy than normal women, although no difference was found in the platelet count at the end of gestation (Kobayashi *et al.*, 1999), in agreement with the present results. In the present study, there was a non-significant inverse correlation between platelet count and SBP, suggesting that in severe PE thrombocytopenia may occur.

Concerning associations between haemostatic variables, t-PA showed an inverse correlation with fibrinogen in PE (Table 19–7), suggesting that intravascular coagulation is occurring and that the fibrinolytic system is activated, consuming fibrinogen. A positive correlation of t-PA with D-dimer was therefore expected. However, an inverse correlation was observed, although without statistical significance (Table 19–7). This suggests that there is an increased production of t-PA by endothelial cells, the activator of plasminogen to degrade fibrinogen and fibrin. The overproduction of t-PA is therefore likely to be related to endothelial disturbance and could explain the positive correlation of t-PA with the degree of proteinuria (Figure 19–8), a known marker of the severity of PE. An overproduction of t-PA in PE by endothelial cells to accomplish some other functions could be hypothesised. For instance, it has been reported that t-PA has a mitogenic role in SMCs, suggesting that its overproduction may contribute to vascular repair, although it may also favour atherogenesis (Raghunath *et al.*, 1995).

It is important to point out that certain leukocyte proteases, notably elastase and cathepsin G, degrade fibrin (Bithell, 1993). In this report, higher values of PMN elastase were

found in PEc women (Table 19–5) and thus may contribute to increases in fibrin degradation products.

The increase in some APPs and the activation of the clotting system suggest a higher degree of inflammation in PE compared with normal pregnancy. Previous work has claimed that endothelial dysfunction in PE may be part of a more generalised intravascular inflammatory reaction involving the complement and clotting systems as well as leukocytes (Redman *et al.*, 1999).

Leukocytes

Leukocyte count is a marker of inflammation and the present study showed a trend to higher values in PE, resulting from a higher neutrophils value (Table 19–5). Moreover, the trend to higher values in GM-CSF and the significantly higher number of PEc cases presenting a shift to the left in the differential leukocyte count (Table 19–5), suggests an increased demand in the inflammatory leukocytes. Furthermore, the significantly higher plasma elastase levels in the PEc group suggest neutrophil activation. Moreover, unlike previous reports, also indicating significantly higher plasma elastase levels in PE (Greer *et al.*, 1989; Halim *et al.*, 1996; Kobayashi *et al.*, 1998), it was found that the elastase to neutrophil ratio is increased in this pathologic syndrome. On the other hand, lactoferrin did not differ between groups and moreover, its trend was towards lower values in PE (Table 19–5), in agreement with other authors (Prieto *et al.*, 1997; Crocker *et al.*, 1999).

Considering the results of the present study, it could be hypothesised that the response of the activated leukocytes in this disorder is selective, enhancing the production of elastase, but not lactoferrin. However, this is unlikely to occur as neutrophilic degranulation of azurophilic (primary) granules (containing elastase) requires a stronger stimulus than degranulation of specific (secondary) granules (containing lactoferrin) (Prieto *et al.*, 1997). A more plausible explanation for the present results is that, although the major circulating lactoferrin is neutrophil derived, it may also be produced by other cells (Levay and Viljoen, 1995). Contrary to the evaluation of elastase, the ELISA kit that was used is not specific for the evaluation of PMN lactoferrin levels. Thus in this study, elastase is a more specific marker of neutrophil activation. The lack of correlation between elastase and lactoferrin in the third trimester of normal pregnancy supports the fact that lactoferrin may have other sources apart from the neutrophil.

The results are in agreement with the hypothesis that neuthrophil activation is implicated in the pathophysiology of PE (Clark *et al.*, 1998a). Besides elastase, activated neutrophils release other proteases and toxic substances capable of injuring endothelial cells (Harlan, 1985). For instance, neutrophils generate reactive oxygen species that can have dramatic effects on endothelial function, by affecting the lipid integrity of the membranes and inactivating the endothelium-derived relaxing factor. Endothelial cell damage and dysfunction are common to all pathological features of PE (Clark *et al.*, 1998a). Furthermore, the positive and significant correlations of lactoferrin and elastase with uric acid levels (Figure 19–6) demonstrate that neutrophil activation is correlated with the severity of the disorder. In agreement with this, the three cases with the highest lactoferrin and elastase levels presented with HELLP syndrome or partial HELLP syndrome. In a reported study of patients with HELLP syndrome, immunohistochemical analysis of liver biopsies revealed high amounts of leukocyte elastase and TNF-α protein in the areas of liver cell necrosis (Wetzka *et al.*, 1999). This suggests that the liver injury is cytokine and neutrophil mediated. Moreover, WBC counts (Terrone *et al.*, 2000) and serum TNF-α levels (Visser *et al.*, 1994) were reported to be higher in HELLP syndrome than in PE and a correlation between leukocytosis and severity of HELLP syndrome has been described (Terrone *et al.*, 2000). In the present study, WBC was not particularly elevated in patients with HELLP syndrome. However, the number of cases was small and none was too severe according to the Mississippi triple classification. However, cases with class 3 or partial HELLP syndrome had the highest values for lactoferrin and elastase. Moreover, GM-CSF levels were also high in both cases with class 3 HELLP syndrome. These results support the hypothesis that HELLP syndrome can be an inflammatory process (Terrone *et al.*, 2000).

The exact mechanism of neutrophil activation in PE is unknown. Increased cytokine levels may account for neutrophil priming and moreover, increased concentrations of cytokines and dyslipidaemia may provide a mechanism of increased neutrophil adhesion to the endothelium (Clark *et al.*, 1998a). This may occur by direct action on neutrophils or indirectly through endothelial activation and consequent increased cellular adhesion molecule expression. Up-regulation of the membrane-bound cellular adhesion molecule is accompanied by the shedding of soluble isoforms from these molecules (Clark *et al.*, 1998a) and concentrations of the soluble adhesion molecules E-selectin and ICAM-1 were reported to be elevated in women with PE (Austgulen *et al.*, 1997; Krauss *et al.*, 1997). Since cell adhesion molecules mediate the adherence of leukocytes to the endothelium, such results suggest a role

for the stimulated endothelium in contributing to the vascular damage/dysfunction of PE. Thus, there are complex interrelationships between activated neutrophils and the endothelium in PE. Although neutrophil activation is likely to be responsible for endothelial dysfunction, it is unclear what comes first: neutrophil activation or endothelial cell dysfunction (Clark *et al.*, 1998a).

Antioxidant Status

A major antioxidant action of human plasma is to bind transition metal ions such as iron and copper, in forms that will not stimulate free radical reactions. This is achieved by antioxidants such as lactoferrin, transferrin, caeruloplasmin, albumin, uric acid and haemopexin (Halliwell and Gutteridge, 1990). The trend to reduced levels of lactoferrin observed in PE may increase the availability of prooxidant iron and thus oxidative events. Indeed, a disturbed iron metabolism seems to be present in PE (Hubel, 1998). Relative to normal pregnancy, higher serum iron levels (Hubel *et al.*, 1996a; Vitoratos *et al.*, 1999) and decreased total transferrin concentration (Hubel *et al.*, 1996a) are described in PE, resulting in an approximate doubling of transferrin saturation (Hubel *et al.*, 1996a). It is true that uric acid, which can act as an antioxidant, is increased in PE and in the present study this was correlated with TAS. However, as mean TAS levels were similar between subjects and controls (Table 19–2), other antioxidants are likely to be reduced in PE.

The Erythrocyte

Despite the fact that neutrophils seemed to be activated in PE, no marked evidence was found for erythrocyte damage in PE. Significantly higher serum Tbil levels (Table 19–8) would suggest an enhanced destruction of RBCs, secondary to the accelerated ageing or damaging process. However, the statistical significance of Tbil was lost after removal of HELLP syndrome cases. In PE, the significantly higher RBC count and RDW values (Table 19–8) may express increased RBC turnover and a younger RBC population. In agreement with this, HMWAg and MBH were somewhat reduced in PE. GPX activity was similar in both groups (Table 19–8). Some authors have found a higher erythrocyte GPX activity in PE suggesting a protective response against increased oxidative stress (Uotila *et al.*, 1993; Diedrich *et al.*, 2001).

Lipids and Lipoproteins

The present work found differences in the lipid and lipopoprotein profile in PE (Table 19–4). The higher mean weight in women with PE compared with matched controls could be a major confounding factor in all lipid results. However, statistical differences were still present after adjustment for weight.

The significantly higher mean TG level in PE agrees with several previous reports (Rosing *et al.*, 1989; Hubel *et al.*, 1996b; Murai *et al.*, 1997; Sattar *et al.*, 1997a; Hubel *et al.*, 1998a; Ware-Jauregui *et al.*, 1999; Wetzka *et al.*, 1999; Wakatsuki *et al.*, 2000). The reason why TG concentrations are elevated in PE remains unclear, but it may involve enhanced insulin resistance, reduced hepatic β-oxidation, or reduced catabolism of TG (ie, reduced LPL activity secondary to cytokine inhibition) (Sattar *et al.*, 1996). Cytokines may also induce both lipolysis and *de novo* fatty acid synthesis, thus providing fatty acids for increased TG production (Sattar *et al.*, 1996).

Hypertriglyceridaemia in PE is proposed by some authors to include oxidant stress by promoting changes in the composition of LDL and consequently enhancing the formation of small dense LDL (Sattar *et al.*, 1997a; Hubel *et al.*, 1998a). However, others found that in PE the LDL profile is dominated by the most buoyant LDL and that most dense LDL particles are significantly decreased (Winkler *et al.*, 2003). In the present study, the evaluation of the relative proportion of LDL subfractions by gradient gel electrophoresis, comparing PEc with normal pregnancies, revealed no differences in the relative percentage of LDL-III, but LDL-II was significantly higher in PE (Table 19–4). Moreover, LDL-MPD was significantly reduced in the pathologic group, but not LDL-PPD. The significantly higher proportion of LDL II associated with a more homogeneous LDL population in the PEc group was responsible for this. In fact, although LDL-PPD did not differ between groups, the LDL population seems to be more disperse in the control group, explaining the significantly higher LDL-MPD.

In addition, the PEc group (Table 19–4) was found to have significantly lower levels of HDLc and apo A-I, the major protein constituent of HDL. Again, this remained true after correction for weight. Some studies have already reported similar results in HDLc (Rosing *et al.*, 1989; Sattar *et al.*, 1997a) and apo A-I (Rosing *et al.*, 1989) levels but others have failed to detect differences in HDLc (Hubel *et al.*, 1996b; Wetzka *et al.*, 1999; Wakatsuki *et al.*, 2000). The lack of differences in the latter studies could be explained by the small number of subjects studied. In the present report, results on HDLc levels are in agreement with two large

studies, one involving 125 PEc cases and 179 normotensive control subjects (Ware-Jauregui *et al.*, 1999) and the other involving 200 PEc cases and 97 controls (Schjetlein *et al.*, 1999).

In PE, the reduced levels of HDLc and apo A-I seem to indicate an uncompensated protective state. This may reveal a failure to increase the level of HDL during gestation, or simply naturally occurring lower levels of HDL in these women. In fact, HDLc and apo A-I concentrations were still significantly reduced in the puerperium of PEc women and were also lower than the values observed in the first trimester of normal pregnancy (Belo *et al.*, 2002). Consistent with this are the significantly reduced levels of HDLc in PEc women at six weeks *postpartum* reported by others (Barden *et al.*, 2001).

Concerning the analysis of the LDLc to apo B ratio, although the reduced LDLc to apo B ratio in the PEc group (Table 19–4) is in agreement with the reduction of LDL diameter, the reduced ratio may also be due to an increase in other apo B containing lipoproteins. VLDL for instance, is likely to be increased in PE as TG levels are much higher in this group. Sattar *et al.* (1997a) found that both $VLDL_1$ and $VLDL_2$ are significantly increased in PE compared to normal pregnancies and VLDL has been suggested as one of the factors contributing to the disturbance of endothelial function in PE (Arbogast *et al.*, 1994; Dekker and Sibai, 1998). VLDL is known to injure endothelial cells (Arbogast *et al.*, 1994) and promote synthesis and release of PAI-1 (Stewart and Monge, 1993). Large VLDL subfractions were reported to be principally responsible for these effects. In addition, decreases in the concentrations of pI (isoelectric point) 5.6 albumin, which protects against VLDL-induced injury, occur in PE. Excessive conversion of pI 5.6 albumin to the non-protective (pI 4.8) form may be caused by increased free fatty acids in women with PE (Arbogast *et al.*, 1994; Dekker and Sibai, 1998).

Although Lp(a) may also be potentially involved in the pathogenesis of PE, no agreement exists in the literature regarding its association with this disorder (Leerink *et al.*, 1997; Wang *et al.*, 1998; Wetzka *et al.*, 1999; Bar *et al.*, 2002; Mori *et al.*, 2003). In the present study, higher levels of Lp(a) were not observed in PE (Table 19–4). Moreover, serum Lp(a) levels were not associated with the severity of PE, since they did not correlate with known markers of this syndrome such as blood pressure, proteinuria and/or uric acid levels (data not shown). Thus, the present results do not suggest an important role of Lp(a) in the development of PE.

Regarding associations between variables involving lipids or lipoproteins, it is noteworthy that D-Dimer levels correlated positively with TG (in PE) and inversely with apo

A-I (both in PE and in normal pregnancy) concentrations (Table 19–11). These correlations may indicate a potentially anticoagulant effect of HDL particles and a procoagulant behaviour of TG-rich lipoproteins. Moreover in PE, apo A-I also correlated significantly and inversely with lactoferrin and elastase (Figure 19–7), suggesting an anti-inflammatory role of HDL.

Genetic Polymorphisms

The abnormal measures of circulating parameters in PE may have a genetic explanation and/or contribution. Indeed, it is true that PE runs at higher rates in families with a history of such a disorder (Chesley *et al.*, 1968; Sutherland *et al.*, 1981; Cincotta and Brennecke, 1998). Most data suggest that maternal genotype is the major factor responsible for the susceptibility, but some studies have ascribed an influence of the foetus, and thus the contribution of the father (Cooper *et al.*, 1988; Lie *et al.*, 1998).

By analysing maternal DNA, it was shown that none of the candidate polymorphisms presented in this study (on apo E and CRP genes) seem to be associated with this disorder. It is true that the number of subjects involved in the study is relatively small for a genetic analysis of this nature. However, since no indication of a trend towards a difference was observed (Table 19–9), it seems reasonable to assume that expanding the study to a larger sample population would not alter the conclusions.

Some authors have noticed a higher frequency of the E2 and E4 alleles in women with PE and suggested that these alleles may be risk factors for PE (Williams *et al.*, 1996; Nagy *et al.*, 1998). The present results differ from those but are in agreement with recent studies in South African (Chikosi *et al.*, 2000) and Finnish populations (Makkonen *et al.*, 2001). Given the lack of consistency from the conclusions of different studies, it appears unlikely that apo E plays a pivotal role in the development of PE.

This is the first study investigating any association between CRP 1059G/C polymorphism and PE. In a recent study involving non-pregnant individuals, carriers of the *Mae*III CRP polymorphism (GC or CC) were reported to have significantly lower CRP concentrations when compared with non-carriers (GG) (Zee and Ridker, 2002). In the present study, a higher frequency of GG individuals in the PEc group could not be found, so this polymorphism is not the sole explanation for the higher CRP concentrations observed. Moreover, although a trend to lower serum CRP values was observed in subjects carrying this polymorphism (cases and controls), this trend was not statistically significant.

The present study does not confirm a preliminary report demonstrating that genetic variation in apo E gene *locus* influences serum CRP level (Mänttäri *et al.*, 2001). However, apo E genotype was associated with changes in lipid and lipoprotein profiles in pregnant women (Table 19–10). The results observed in carriers of E2 (E2+) and E4 (E4+) alleles were mainly due to the contribution of E2/3 and E3/4 subjects respectively. E2+ presented with the lowest LDLc levels compared to the E3/3 genotype and E4+, in agreement with previous reports in non-pregnant (Larson *et al.*, 2000) and pregnant (McGladdery and Frohlich, 2001) individuals. E2+ also presented with the highest TG levels, though without any statistical significance. On the other hand, HDLc and apo A-I levels were significantly reduced in E4+, compared with the E3/3 genotype. A previous study in pregnant women found no differences in HDLc between women with different apo E genotypes (McGladdery and Frohlich, 2001). However, a meta-analysis reported HDLc to be lower in E3/4 than in E3/3 non-pregnant subjects (Dallongeville *et al.*, 1992). Furthermore in this study, E4+ presented with the highest Chol and LDLc levels and therefore LDLc to HDLc and apo B to apo A-I ratios were significantly higher in this group compared to the other two.

The association of the apo E genetic variants with lipids and lipoproteins was obtained in the third trimester of the combined groups (cases and controls) but since both groups differ statistically in the lipid profile, that could be a major confounding factor in the association found. However, all results remained significant after being adjusted for case/controls. Moreover, after adjustment for environmental factors, such as weight, age and gestational age at sampling, significance was lost only for the LDLc to HDLc ratio.

The relatively low number of subjects involved may explain in part, the lack of association of CRP genotypes with serum CRP levels, and the lack of significance of some parameters with apo E isoforms. In addition, the strength of the relation between polymorphisms in the apo E and CRP genes and serum variables may be affected by unadjusted environmental factors. For example, the effect of the apo E polymorphic variants in determining LDLc concentration is affected by alcohol consumption (Corella *et al.*, 2001). Also, factors such as obesity, smoking, and diet are involved in the expression of lipids and/or CRP concentrations. At the same time, the *Mae*III CRP polymorphism is silent at the amino acid level (CTG→CTC, Leu→Leu) (Cao and Hegele, 2000). Thus, it may be in linkage disequilibrium with a yet-to-be-identified, functional mutation in the CRP gene or a nearby gene that is responsible for the expressed levels of CRP.

Final Conclusions

In summary, although the pathogenesis of PE is unknown, a generalised inflammatory process has recently been implicated in its pathogenesis (Redman *et al.*, 1999). PE is a multifactorial disorder and, thus, a combination of different risk factors, even in women presenting only signs of low-grade inflammation, may have an important role in the pathogenesis and clinical complications of PE.

The present study, undertaken in women with established PE, confirms that PE is associated with abnormal laboratory findings in maternal blood and supports the hypothesis that PE is associated with a greater degree of inflammation compared with normal pregnancies. Components of the inflammatory response in PE include: a higher concentration of CRP; the existence of endothelial dysfunction (lack of positive correlation of t-PA with fibrinogen and D-dimer levels; positive correlation of t-PA with the degree of proteinuria); activation of the haemostatic system (higher levels of t-PA and D-dimer; higher PAI-1 activity); the existence of neutrophil activation (higher levels of elastase); correlation of neutrophil activation with the severity of this disorder (particularly high concentrations of plasma lactoferrin and elastase in HELLP syndrome cases; correlation of lactoferrin and elastase with uric acid levels); and the existence of a lipid profile with enhanced atherogenic and/or pro-inflammatory potential (higher TG levels, lower LDL-MPD, and lower apo A-I and HDLc concentrations).

Curiously enough, the erythrocyte band 3 profile observed in PE was not significantly different from that observed in normal pregnancy. Assuming that the enhanced inflammatory state in PE, particularly the marked neutrophil activation, injures the RBC, an altered band 3 profile in PE was expected. However, it is noteworthy that a significantly higher RBC count and RDW with a trend to lower values of HMWAg and MBH was observed, suggesting an increased RBC turnover. If this were the case, it might mask the evidence for greater RBC damage associated with the inflammatory process.

None of the candidate polymorphisms (associated with the CRP and apo E genes) showed any association with PE. However, in pregnant women, genetic variants in apo E were associated with changes in lipid and lipoprotein profiles similar to that seen in the general population.

Whether the enhanced systemic inflammation observed in the PEc mother is a cause or a consequence of the pathologic process remains to be clarified. However, the statistically significant differences and/or correlations obtained with TG, t-PA, and elastase, suggest that these variables may work as risk factors in PE.

Appendix. Abbreviations

ACOG	American College of Obstetricians and Gynaecologists
AIDS	acquired immunodeficiency syndrome
ALT	alanine aminotransferase
ANOVA	analysis of variance
APC	activated protein C
APC:SR	activated protein C sensitivity ratio
Apo	apolipoprotein
APP	acute phase protein
APR	acute phase response
AST	aspartate aminotransferase
BMI	body mass index
bp	base pairs
CE	cholesterol esterase
ChOD	cholesterol oxidase
Chol	cholesterol
CRP	C-reactive protein
CVD	cardiovascular disease
dATP	2'-deoxyadenosine 5'-triphosphate
DBP	diastolic blood pressure
dCTP	2'-deoxycytidine 5'-triphosphate
D-dimer	fibrin fragment D-dimer
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunoassay
GFR	glomerular filtration rate
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPX	glutathione peroxidase
Hb	haemoglobin

HDL	high-density lipoprotein
HDLc	high-density lipoprotein cholesterol
HELLP	haemolysis, elevated liver enzymes, and low platelet count
HL	hepatic lipase
HLA	human leukocyte antigen
HMWAg	high molecular weight aggregates
HRP	horseradish peroxidase
Ht	haematocrit
ICAM	intercellular adhesion molecule
IDL	intermediate-density lipoprotein
IDLc	intermediate-density lipoprotein cholesterol
Ig	immunoglobulin
IL	interleukin
IQR	interquartile range
ISSHP	International Society for the Study of Hypertension in Pregnancy
IUGR	intrauterine growth restriction
kDa	kilodalton
LCAT	lecithin:cholesterol acyltransferase
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LDLc	low-density lipoprotein cholesterol
LDL-MPD	low-density lipoprotein mean particle diameter
LDL-PPD	low-density lipoprotein peak particle diameter
Lp(a)	lipoprotein (a)
LPL	lipoprotein lipase
MBH	membrane bound haemoglobin
MCH	mean cell haemoglobin
MCHC	mean cell haemoglobin concentration
MCV	mean cell volume
MDA-LDL	malondialdehyde-conjugated low-density lipoprotein
NAD^+/NADH	nicotinamide adenine dinucleotide (oxidized/reduced)
$NADP^+$/NADPH	nicotinamide adenine dinucleotide phosphate (Oxidized/reduced)
ND	normally distributed
NHBPEP	National High Blood Pressure Education Program

NND	not-normally distributed
OPD	*o*-phenylenediamine
Ox-LDL	oxidised low-density lipoprotein
PAF	platelet-activating factor
PAGE	polyacrylamide gel electrophoresis
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism
PE	pre-eclampsia
PEc	pre-eclamptic
Pfrag	proteolytic fragments
pI	isoelectric point
PMN	polymorphonuclear leukocyte
PMSF	phenylmethylsulfonyl fluoride
PUFA	polyunsaturated fatty acid
r	Pearson's correlation coefficient
RAS	renin-angiotensin system
RBC	red blood cell
RDW	red cell distribution width
r_s	Spearman's rank correlation coefficient
SBP	systolic blood pressure
SD	standard deviation
SDS	sodium dodecyl sulphate
SMC	smooth muscle cell
sPLA2	secretory phospholipase A2
TAS	total antioxidant status
Tbil	total bilirubin
TG	triglycerides
TNF	tumour necrosis factor
t-PA	tissue type plasminogen activator
VCAM	vascular cell adhesion molecule
VLDL	very-low-density lipoprotein
vWf	von Willebrand factor
WBC	white blood cell

References

Adams MR, Kinlay S, Blake GJ, Orford JL, Ganz P, and Selwyn AP. (2000). Atherogenic lipids and endothelial dysfunction: mechanisms in the genesis of ischemic syndromes. *Annu Rev Med* 51: 149–167.

Alvarez JJ, Montelongo A, Iglesias A, Lasuncion MA, and Herrera E. (1996). Longitudinal study on lipoprotein profile, high density lipoprotein subclass, and postheparin lipases during gestation in women. *J Lipid Res* 37: 299–308.

Arbogast BW, Leeper SC, Merrick RD, Olive KE, and Taylor RN. (1994). Which plasma factors bring about disturbance of endothelial function in pre-eclampsia? *Lancet* 343: 340–341.

Arici M, and Walls J. (2001). End-stage renal disease, atherosclerosis, and cardiovascular mortality: is C-reactive protein the missing link? *Kidney Int* 59: 407–414.

Armstrong VW, Wieland E, Diedrich F, Renner A, Rath W, Kreuzer H, Kuhn W, *et al.* (1994). Serum antibodies to oxidised low-density lipoprotein in pre-eclampsia and coronary heart disease. *Lancet* 343: 1570.

Assali NS, Rauramo L, and Peltonen T. (1960). Measurement of uterine blood flow and uterine metabolism. *Am J Obstet Gynecol* 79: 86–98.

Åstedt B. (1993). The placenta and haemostatic balance, pp. 291–309. *In* Redman CWG, Sargent IL and Starkey PM [eds.], The human placenta. Blackwell, Oxford.

Aurell M, and Cramér K. (1966). Serum lipids and lipoproteins in human pregnancy. *Clin Chim Acta* 13: 278–284.

Austgulen R, Lien E, Vince G, and Redman CW. (1997). Increased maternal plasma levels of soluble adhesion molecules (ICAM-1, VCAM-1, E-selectin) in preeclampsia. *Eur J Obstet Gynecol Reprod Biol* 71: 53–58.

Ballegeer V, Mombaerts P, Declerck PJ, Spitz B, Assche FAV, and Collen D. (1987). Fibrinolytic response to venous occlusion and fibrin fragment D-dimer levels in normal and complicated pregnancy. *Thromb Haemost* 58: 1030–1032.

Bar J, Harell D, Bardin R, Pardo J, Chen R, Hod M, and Sullivan M. (2002). The elevated plasma lipoprotein(a) concentrations in preeclampsia do not precede the development of the disorder. *Thromb Res* 105: 19–23.

Barden A, Graham D, Beilin LJ, Ritchie J, Baker R, Walters BN, and Michael CA. (1997). Neutrophil CD11B expression and neutrophil activation in pre-eclampsia. *Clin Sci (Lond)* 92: 37–44.

Barden A, Ritchie J, Walters B, Michael C, Rivera J, Mori T, Croft K, *et al.* (2001). Study of plasma factors associated with neutrophil activation and lipid peroxidation in preeclampsia. *Hypertension* 38: 803–808.

Barron WM, Heckerling P, Hibbard JU, and Fisher S. (1999). Reducing unnecessary coagulation testing in hypertensive disorders of pregnancy. *Obstet Gynecol* 94: 364–370.

Behne D, and Wolters W. (1979). Selenium content and glutathione peroxidase activity in the plasma and erythrocytes of non-pregnant and pregnant women. *J Clin Chem Clin Biochem* 17: 133–135.

Bellart J, Gilabert R, Angles A, Piera V, Miralles RM, Monasterio J, and Cabero L. (1999). Tissue factor levels and high ratio of fibrinopeptide A:D-dimer as a measure of endothelial procoagulant disorder in pre-eclampsia. *Br J Obstet Gynaecol* 106: 594–597.

Belo L, Caslake M, Gaffney D, Santos-Silva A, Pereira-Leite L, Quintanilha A, and Rebelo I. (2002). Changes in LDL size and HDL concentration in normal and preeclamptic pregnancies. *Atherosclerosis* 162: 425–432.

Bhakdi S, Torzewski M, Klouche M, and Hemmes M. (1999). Complement and atherogenesis: binding of CRP to degraded, nonoxidized LDL enhances complement activation. *Arterioscler Thromb Vasc Biol* 19: 2348–2354.

Bithell TC. (1993). Blood coagulation, pp. 566–615. *In* Lee GR, Bithell TC, Foerster J, Athens JW and Lukens JN [eds.], Wintrobe's clinical hematology, 9th ed. Lea & Febiger, Philadelphia.

Blake GJ, and Ridker PM. (2001). Novel clinical markers of vascular wall inflammation. *Circ Res* 89: 763–771.

Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254.

Branch DW, Mitchell MD, Miller E, Palinski W, and Witztum JL. (1994). Pre-eclampsia and serum antibodies to oxidised low-density lipoprotein. *Lancet* 343: 645–646.

Branch DW, and Porter TF. (1999). Hypertensive disorders of pregnancy, pp. 309–326. *In* Scott JR, Saia PJD, Hammond CB and Spellacy WN [eds.], Danforth's obstetrics & gynecology, 8th ed. Lippincott Williams &Wilkins, Philadelphia.

Brovelli A, Castellana MA, Minetti G, Piccinini G, Seppi C, De-Renzis MR, and Balduini C. (1991). Conformational changes and oxidation of membrane proteins in senescent human erythrocytes. *Adv Exp Med Biol* 307: 59–73.

Bussen SS, Sutterlin MW, and Steck T. (1998). Plasma renin activity and aldosterone serum concentration are decreased in severe preeclampsia but not in the HELLP-syndrome. *Acta Obstet Gynecol Scand* 77: 609–613.

Buster JE, and Carson SA. (2002). Endocrinology and diagnosis of pregnancy, pp. 3–36. *In* Gabbe SG, Niebyl JR and Simpson JL [eds.], Obstetrics: normal and problem pregnancies, 4th ed. Churchill Livingstone, Philadelphia.

Byrne CD. (1999). Triglyceride-rich lipoproteins: are links with atherosclerosis mediated by a procoagulant and proinflammatory phenotype? *Atherosclerosis* 145: 1–15.

Cachia O, Leger CL, Boulot P, Vernet MH, Michel F, Paulet ACd, and Descomps B. (1995). Red blood cell vitamin E concentrations in fetuses are related to but lower than those in mothers during gestation. A possible association with maternal lipoprotein (a) plasma levels. *Am J Obstet Gynecol* 173: 42–51.

Cadroy Y, Grandjean H, Pichon J, Desprats R, Berrebi A, Fournie A, and Boneu B. (1993). Evaluation of six markers of haemostatic system in normal pregnancy and pregnancy complicated by hypertension or pre-eclampsia. *Br J Obstet Gynaecol* 100: 416–420.

Campos H, Genest JJ, Blijlevens E, McNamara JR, Jenner JL, Ordovas JM, Wilson PW, *et al.* (1992). Low density lipoprotein particle size and coronary artery disease. *Arterioscler Thromb* 12: 187–195.

Cao H, and Hegele RA. (2000). Human C-reactive protein (CRP) 1059G/C polymorphism. *J Hum Genet* 45: 100–101.

Caplice NM, Panetta C, Peterson TE, Kleppe LS, Mueske CS, Kostner GM, Broze GJ, *et al.* (2001). Lipoprotein (a) binds and inactivates tissue factor pathway inhibitor: a novel link between lipoproteins and thrombosis. *Blood* 98: 2980–2987.

Carone D, Loverro G, Greco P, Capuano F, and Selvaggi L. (1993). Lipid peroxidation products and antioxidant enzymes in red blood cells during normal and diabetic pregnancy. *Eur J Obstet Gynecol Reprod Biol* 51: 103–109.

Carpentier YA, and Scruel O. (2002). Changes in the concentration and composition of plasma lipoproteins during the acute phase response. *Curr Opin Clin Nutr Metab Care* 5: 153–158.

Ceciliani F, Giordano A, and Spagnolo V. (2002). The systemic reaction during inflammation: the acute-phase proteins. *Curr Pharm Des* 9: 211–223.

Cermak J, Key NS, Bach RR, Balla J, Jacob HS, and Vercellotti GM. (1993). C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. *Blood* 82: 513–520.

Chappell L, Poulton L, Halligan A, and Shennan AH. (1999a). Lack of consistency in research papers over the definition of pre-eclampsia. *Br J Obstet Gynaecol* 106: 983–985.

Chappell LC, Seed PT, Briley AL, Kelly FJ, Lee R, Hunt BJ, Parmar K, *et al.* (1999b). Effect of antioxidants on the occurrence of pre-eclampsia in women at increased risk: a randomised trial. *Lancet* 354: 810–816.

Chesley LC, Annitto JE, and Cosgrove RA. (1968). The familial factor in toxemia of pregnancy. *Obstet Gynecol* 32: 303–311.

Chikosi AB, Moodley J, and Pegoraro RJ. (2000). Apolipoprotein E polymorphism in preeclampsia. *S Afr Med J* 90: 128–129.

Chin JH, Azhar S, and Hoffman BB. (1992). Inactivation of endothelial derived relaxing factor by oxidized lipoproteins. *J Clin Invest* 89: 10–18.

Choi JW, and Pai SH. (2001). Change in erythropoiesis with gestational age during pregnancy. *Ann Hematol* 80: 26–31.

Cicala C, and Cirino G. (1998). Linkage between inflammation and coagulation: an update on the molecular basis of the crosstalk. *Life Sci* 62: 1817–1824.

Cincotta RB, and Brennecke SP. (1998). Family history of pre-eclampsia as a predictor for pre-eclampsia in primigravidas. *Int J Gynaecol Obstet* 60: 23–27.

Clark P, Boswell F, and Greer IA. (1998a). The neutrophil and preeclampsia. *Semin Reprod Endocrinol* 16: 57–64.

Clark P, Brennand J, Conkie JA, McCall F, Greer IA, and Walker ID. (1998b). Activated protein C sensitivity, protein C, protein S and coagulation in normal pregnancy. *Thromb Haemost* 79: 1166–1170.

Cleary LM, and Duggan DB. (1991). Hematologic abnormalities complicating medical disorders, pp. 1585–1591. *In* Williams WJ, Beutler E, Erslev AJ and Lichtman MA [eds.], Hematology, International, 4th ed. McGraw-Hill, New York.

Cockerill GW, Huehns TY, Weerasinghe A, Stocker C, Lerch PG, Miller NE, and Haskard DO. (2001). Elevation of plasma high-density lipoprotein concentration reduces interleukin-1-induced expression of E-selectin in an in vivo model of acute inflammation. *Circulation* 103: 108–112.

Cooper DW, Hill JA, Chesley LC, and Bryans CI. (1988). Genetic control of susceptibility to eclampsia and miscarriage. *Br J Obstet Gynaecol* 95: 644–653.

Corella D, Guillen M, Saiz C, Portoles O, Sabater A, Cortina S, Folch J, *et al.* (2001). Environmental factors modulate the effect of the APOE genetic polymorphism on plasma lipid concentrations: ecogenetic studies in a Mediterranean Spanish population. *Metabolism* 50: 936–944.

Crocker IP, Wellings RP, Fletcher J, and Baker PN. (1999). Neutrophil function in women with pre-eclampsia. *Br J Obstet Gynaecol* 106: 822–828.

Crouch SP, Crocker IP, and Fletcher J. (1995). The effect of pregnancy on polymorphonuclear leukocyte function. *J Immunol* 155: 5436–5443.

Curtiss LK, and Boisvert WA. (2000). Apolipoprotein E and atherosclerosis. *Curr Opin Lipidol* 11: 243–251.

Czerwinski M, Wasniowska K, Steuden I, Duk M, Wiedlocha A, and Lisowska E. (1988). Degradation of the human erythrocyte membrane band 3 studied with monoclonal antibody directed against an epitope on the cytoplasmic fragment of band 3. *Eur J Biochem* 174: 647–654.

Dacie JV, and Lewis SM. (1991). Preparation and staining methods for blood and bone-marrow films, pp. 75–85. *In* Dacie JV and Lewis SM [eds.], Practical haematology, 7th ed. Churchill Livingstone, Edinburgh.

Dahlen GH. (1994). Lp(a) lipoprotein in cardiovascular disease. *Atherosclerosis* 108: 111–126.

Dallongeville J, Lussier-Cacan S, and Davignon J. (1992). Modulation of plasma triglyceride levels by apoE phenotype: a meta-analysis. *J Lipid Res* 33: 447–454.

Danesh J, Collins R, Appleby P, and Peto R. (1998). Association of fibrinogen, C-reactive protein, albumin, or leukocyte count with coronary heart disease: meta-analyses of prospective studies. *JAMA* 279: 1477–1482.

Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, Gallimore JR, *et al.* (2000). Low grade inflammation and coronary heart disease: prospective study and updated meta-analyses. *BMJ* 321: 199–204.

Davey DA, and MacGillivray I. (1988). The classification and definition of the hypertensive disorders of pregnancy. *Am J Obstet Gynecol* 158: 892–898.

Davidge ST, Hubel CA, Brayden RD, Capeless EC, and McLaughlin MK. (1992). Sera antioxidant activity in uncomplicated and preeclamptic pregnancies. *Obstet Gynecol* 79: 897–901.

Declerck PJ, Alessi MC, Verstreken M, Kruithof EKO, Juhan-Vague I, and Collen D. (1988). Measurement of plasminogen activator inhibitor 1 in biologic fluids with a murine monoclonal antibody-based enzyme-linked immunosorbent assay. *Blood* 71: 220–225.

Dekker GA, and Sibai BM. (1998). Etiology and pathogenesis of preeclampsia: current concepts. *Am J Obstet Gynecol* 179: 1359–1375.

Desoye G, Schweditsch MO, Pfeiffer KP, Zechner R, and Kostner GM. (1987). Correlation of hormones with lipid and lipoprotein levels during normal pregnancy and postpartum. *J Clin Endocrinol Metab* 64: 704–712.

Diedrich F, Renner A, Rath W, Kuhn W, and Wieland E. (2001). Lipid hydroperoxides and free radical scavenging enzyme activities in preeclampsia and HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome: no evidence for circulating primary products of lipid peroxidation. *Am J Obstet Gynecol* 185: 166–172.

Djurovic S, Clausen T, Wergeland R, Brosstad F, Berg K, and Henriksen T. (2002). Absence of enhanced systemic inflammatory response at 18 weeks of gestation in women with subsequent pre-eclampsia. *BJOG* 109: 759–764.

Dodge JT, Mitchell C, and Hanahan DJ. (1963). The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch Biochem Biophys* 100: 119–130.

Dunlop W. (1981). Serial changes in renal haemodynamics during normal human pregnancy. *Br J Obstet Gynaecol* 88: 1–9.

Edwards SW. (1994a). The development and structure of mature neutrophils, pp. 33–76. *In* Edwards SW [ed.], Biochemistry and physiology of the neutrophil. Cambridge University Press, Cambridge.

Edwards SW. (1994b). Neutrophil priming: regulation of neutrophil function during inflammatory activation, pp. 235–261. *In* Edwards SW [ed.], Biochemistry and physiology of the neutrophil. Cambridge University Press, Cambridge.

El-Salahy EM, Ahmed MI, El-Gharieb A, and Tawfik H. (2001). New scope in angiogenesis: role of vascular endothelial growth factor (VEGF), NO, lipid peroxidation, and vitamin E in the pathophysiology of pre-eclampsia among Egyptian females. *Clin Biochem* 34: 323–329.

Ernst E, Hammerschmidt DE, Bagge U, Matrai A, and Dormandy JA. (1987). Leukocytes and the risk of ischemic diseases. *JAMA* 257: 2318–2324.

Esmon CT, Fukudome K, Mather T, Bode W, Regan LM, Stearns-Kurosawa DJ, and Kurosawa S. (1999). Inflammation, sepsis, and coagulation. *Haematologica* 84: 254–259.

Esmon CT. (2000). The anticoagulant and anti-inflammatory roles of the protein C anticoagulant pathway. *J Autoimmun* 15: 113–116.

Estelles A, Gilabert J, Aznar J, Loskutoff DJ, and Schleef RR. (1989). Changes in the plasma levels of type 1 and type 2 plasminogen activator inhibitors in normal pregnancy and in patients with severe preeclampsia. *Blood* 74: 1332–1338.

Estelles A, Gilabert J, España F, Aznar J, and Galbis M. (1991). Fibrinolytic parameters in normotensive pregnancy with intrauterine fetal growth retardation and in severe preeclampsia. *Am J Obstet Gynecol* 165: 138–142.

Fåhraeus L, Larsson-Cohn U, and Wallentin L. (1985). Plasma lipoproteins including high density lipoprotein subfractions during normal pregnancy. *Obstet Gynecol* 66: 468–472.

Feinberg BB, and Gonik B. (1991). General precepts of the immunology of pregnancy. *Clin Obstet Gynecol* 34: 3–16.

Folsom AR, Pankow JS, Tracy RP, Arnett DK, Peacock JM, Hong Y, Djousse L, *et al.* (2001). Association of C-reactive protein with markers of prevalent atherosclerotic disease. *Am J Cardiol* 88: 112–117.

Franz H, and Wendler D. (1992). A controlled study of maternal serum concentrations of lipoproteins in pregnancy-induced hypertension. *Arch Gynecol Obstet* 252: 81–86.

Freund G, and Arvan DA. (1990). Clinical biochemistry of preeclampsia and related liver diseases of pregnancy: a review. *Clin Chim Acta* 191: 123–151.

Friedman SA, Schiff E, Emeis JJ, Dekker GA, and Sibai BM. (1995). Biochemical corroboration of endothelial involvement in severe preeclampsia. *Am J Obstet Gynecol* 172: 202–203.

Gaffney D, and Campbell RA. (1994). A PCR based method to determine the kalow allele of the cholinesterase gene: the E_1^k allele frequency and its significance in the normal population. *J Med Genet* 31: 248–250.

Gibbs CP. (1981). Maternal physiology. *Clin Obstet Gynecol* 24: 525–543.

Gordon DJ, and Rifkind BM. (1989). High-density lipoprotein-The clinical implications of recent studies. *N Engl J Med* 321: 1311–1316.

Gordon MC. (2002). Maternal physiology in pregnancy, pp. 63–91. *In* Gabbe SG, Niebyl JR and Simpson JL [eds.], Obstetrics: normal and problem pregnancies, 4th ed. Churchill Livingstone, Philadelphia.

Graaf J, Hak-Lemmers HLM, Hectors MPC, Demacker PNM, Hendriks JCM, and Stalenhoef AFH. (1991). Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. *Arterioscler Thromb* 11: 298–306.

Granger JP, Alexander BT, Llinas MT, Bennett WA, and Khalil RA. (2001). Pathophysiology of hypertension during preeclampsia linking placental ischemia with endothelial dysfunction. *Hypertension* 38: 718–722.

Gratacós E, Casals E, Deulofeu R, Cararach V, Alonso PL, and Fortuny A. (1998a). Lipid peroxide and vitamin E patterns in pregnant women with different types of hypertension in pregnancy. *Am J Obstet Gynecol* 178: 1072–1076.

Gratacós E, Filella X, Palacio M, Cararach V, Alonso PL, and Fortuny A. (1998b). Interleukin-4, interleukin-10, and granulocyte-macrophage colony stimulating factor in second-trimester serum from women with preeclampsia. *Obstet Gynecol* 92: 849–853.

Greer IA, Haddad NG, Dawes J, Johnstone FD, and Calder AA. (1989). Neutrophil activation in pregnancy-induced hypertension. *Br J Obstet Gynaecol* 96: 978–982.

Greer IA, Lyall F, Perera T, Boswell F, and Macara LM. (1994). Increased concentrations of cytokines interleukin-6 and interleukin-1 receptor antagonist in plasma of women with preeclampsia: a mechanism for endothelial dysfunction? *Obstet Gynecol* 84: 937–940.

Griffin JH, Fernandez JA, and Deguchi H. (2001). Plasma lipoproteins, hemostasis and thrombosis. *Thromb Haemost* 86: 386–394.

Hajjar KA, Gavish D, Breslow JL, and Nachman RL. (1989). Lipoprotein(a) modulation of endothelial cell surface fibrinolysis and its potential role in atherosclerosis. *Nature* 339: 303–305.

Halim A, Kanayama N, Maradny EE, Maehara K, Bhuiyan AB, and Terao T. (1996). Correlated plasma elastase and sera cytotoxicity in eclampsia. A possible role of endothelin-1 induced neutrophil activation in preeclampsia-eclampsia. *Am J Hypertens* 9: 33–38.

Halligan A, Bonnar J, Sheppard B, Darling M, and Walshe J. (1994). Haemostatic, fibrinolytic and endothelial variables in normal pregnancies and pre-eclampsia. *Br J Obstet Gynaecol* 101: 488–492.

Halliwell B, and Gutteridge JMC. (1990). The antioxidants of human extracellular fluids. *Arch Biochem Biophys* 280: 1–8.

Haram K, Augensen K, and Elsayed S. (1983). Serum protein pattern in normal pregnancy with special reference to acute-phase reactants. *Br J Obstet Gynaecol* 90: 139–145.

Harlan JM. (1985). Leukocyte-endothelial interactions. *Blood* 65: 513–525.

Havel RJ, Eder HA, and Bragdon JH. (1955). The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* 34: 1345–1353.

Hawrylyshyn P, Bernstein P, Milligan JE, Soldin S, Pollard A, and Papsin FR. (1983). Premature rupture of membranes: the role of C-reactive protein in the prediction of chorioamnionitis. *Am J Obstet Gynecol* 147: 240–246.

Higgins JR, Walshe JJ, Darling MRN, Norris L, and Bonnar J. (1998). Hemostasis in the uteroplacental and peripheral circulations in normotensive and pre-eclamptic pregnancies. *Am J Obstet Gynecol* 179: 520–526.

Highton J, and Hessian P. (1984). A solid-phase enzyme immunoassay for C-reactive protein: clinical value and the effect of rheumatoid factor. *J Immunol Methods* 68: 185–192.

Hill LM. (1978). Metabolism of uric acid in normal and toxemic pregnancy. *Mayo Clin Proc* 53: 743–751.

Hixson JE, and Vernier DT. (1990). Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with Hha I. *J Lipid Res* 31: 545–548.

Hixson JE, and Group PR. (1991). Apolipoprotein E polymorphisms affect atherosclerosis in young males. *Arterioscler Thromb* 11: 1237–1244.

Holvoet P, Vanhaecke J, Janssens S, Van-de-Werf F, and Collen D. (1998). Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation* 98: 1487–1494.

Holvoet P, Mertens A, Verhamme P, Bogaerts K, Beyens G, Verhaeghe R, Collen D, *et al.* (2001). Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* 21: 844–848.

Hörkkö S, Binder CJ, Shaw PX, Chang MK, Silverman G, Palinski W, and Witztum JL. (2000). Immunological responses to oxidized LDL. *Free Radic Biol Med* 28: 1771–1779.

Hubel CA, Kozlov AV, Kagan VE, Evans RW, Davidge ST, McLaughlin MK, and Roberts JM. (1996a). Decreased transferrin and increased transferrin saturation in sera of women with preeclampsia: implications for oxidative stress. *Am J Obstet Gynecol* 175: 692–700.

Hubel CA, McLaughlin MK, Evans RW, Hauth BA, Sims CJ, and Roberts JM. (1996b). Fasting serum triglycerides, free fatty acids, and malondialdehyde are increased in preeclampsia, are positively correlated, and decrease within 48 hours post partum. *Am J Obstet Gynecol* 174: 975–982.

Hubel CA. (1997). Plasma vitamin E in preeclampsia. *Am J Obstet Gynecol* 177: 484–485.

Hubel CA. (1998). Dyslipidemia, iron, and oxidative stress in preeclampsia: assessment of maternal and feto-placental interactions. *Semin Reprod Endocrinol* 16: 75–92.

Hubel CA, Lyall F, Weissfeld L, Gandley RE, and Roberts JM. (1998a). Small low-density lipoproteins and vascular cell adhesion molecule-1 are increased in association with hyperlipidemia in Preeclampsia. *Metabolism* 47: 1281–1288.

Hubel CA, Shakir Y, Gallaher MJ, McLaughlin MK, and Roberts JM. (1998b). Low-density lipoprotein particle size decreases during normal pregnancy in association with triglyceride increases. *J Soc Gynecol Investig* 5: 244–250.

Hulthe J, and Fagerberg B. (2002). Circulating oxidized LDL is associated with subclinical atherosclerosis development and inflammatory cytokines (AIR Study). *Arterioscler Thromb Vasc Biol* 22: 1162–1167.

Hyka N, Dayer JM, Modoux C, Kohno T, Edwards CKr, Roux-Lombard P, and Burger D. (2001). Apolipoprotein A-I inhibits the production of interleukin-1beta and tumor

necrosis factor-alpha by blocking contact-mediated activation of monocytes by T lymphocytes. *Blood* 97: 2381–2389.

Iglesias A, Montelongo A, Herrera E, and Lasuncion MA. (1994). Changes in cholesteryl ester transfer protein activity during normal gestation and postpartum. *Clin Biochem* 27: 63–68.

Imanishi H, Nakai T, Abe T, and Takino T. (1986). Glutathione-linked enzyme activities in red cell aging. *Clin Chim Acta* 159: 73–76.

Ishihara N, Matsuo H, Murakoshi H, Laoag-Fernandez JB, Samoto T, and Maruo T. (2002). Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. *Am J Obstet Gynecol* 186: 158–166.

Islami D, Shoukir Y, Dupont P, Campana A, and Bischof P. (2001). Is cellular fibronectin a biological marker for pre-eclampsia? *Eur J Obstet Gynecol Reprod Biol* 97: 40–45.

Jamelle RN. (1997). Eclampsia - a taxing situation in the Third World. *Int J Gynaecol Obstet* 58: 311–312.

Jørgensen M, Philips M, Thorsen S, Selmer J, and Zeuthen J. (1987). Plasminogen activator inhibitor-1 is the primary inhibitor of tissue-type plasminogen activator in pregnancy plasma. *Thromb Haemost* 58: 872–878.

Kay MMB, Goodman SR, Sorensen K, Whitfield CF, Wong P, Zaki L, and Rudloff V. (1983). Senescent cell antigen is immunologically related to band 3. *Proc Natl Acad Sci U S A* 80: 1631–1635.

Kay MMB. (1984). Localization of senescent cell antigen on band 3. *Proc Natl Acad Sci U S A* 81: 5753–5757.

Kay MMB, Marchalonis JJ, Schluter SF, and Bosman G. (1991). Human erythrocyte aging: cellular and molecular biology. *Transfus Med Rev* 5: 173–195.

Kelley MA. (1999). Triage and management of the pregnant hypertensive patient. *J Nurse Midwifery* 44: 558–571.

Khoo JC, Miller E, McLoughlin P, and Steinberg D. (1990). Prevention of low density lipoprotein aggregation by high density lipoprotein or apolipoprotein A-I. *J Lipid Res* 31: 645–652.

Khovidhunkit W, Memon RA, Feingold KR, and Grunfeld C. (2000). Infection and inflammation-induced proatherogenic changes of lipoproteins. *J Infect Dis* 181(Suppl 3): S462–472.

Knapen MFCM, Zusterzeel PLM, Peters WHM, and Steegers EAP. (1999). Glutathione and glutathione-related enzymes in reproduction. A review. *Eur J Obstet Gynecol Reprod Biol* 82: 171–184.

Kobayashi H, Sadakata H, Suzuki K, She MY, Shibata S, and Terao T. (1998). Thrombomodulin release from umbilical endothelial cells initiated by preeclampsia plasma-induced neutrophil activation. *Obstet Gynecol* 92: 425–430.

Kobayashi T, Tokunaga N, Sugimura M, Suzuki K, Kanayama N, Nishiguchi T, and Terao T. (1999). Coagulation/fibrinolysis disorder in patients with severe preeclampsia. *Semin Thromb Hemost* 25: 451–454.

Kokia E, Barkai G, Reichman B, Segal P, Goldman B, and Mashiach S. (1990). Maternal serum lipid profile in pregnancies complicated by hypertensive disorders. *J Perinat Med* 18: 473–478.

Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, and Cosic V. (2001). Method for the measurement of antioxidant activity in human fluids. *J Clin Pathol* 54: 356–361.

Krauss T, Kuhn W, Lakoma C, and Augustin HG. (1997). Circulating endothelial cell adhesion molecules as diagnostic markers for the early identification of pregnant women at risk for development of preeclampsia. *Am J Obstet Gynecol* 177: 443–449.

Kushner I, and Rzewnicki D. (1999). Acute phase response, pp. 317–329. *In* Gallin JI, Snyderman R, Fearon DT, Haynes BF and Nathan C [eds.], Inflammation: basic principles and clinical correlates, 3rd ed. Lippincott Williams & Wilkins, Philadelphia.

Lachmeijer AMA, Dekker GA, Pals G, Aarnoudse JG, ten-Kate LP, and Arngrímsson R. (2002). Searching for preeclampsia genes: the current position. *Eur J Obstet Gynecol Reprod Biol* 105: 94–113.

Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Lagrand WK, Visser CA, Hermens WT, Niessen HWM, Verheugt FWA, Wolbink GJ, and Hack CE. (1999). C-reactive protein as a cardiovascular risk factor: more than an epiphenomenon? *Circulation* 100: 96–102.

Lamont J, Campbell J, and FitzGerald P. (1997). Measurement of individual vs total antioxidants. *Clin Chem* 43: 852–854.

Larson IA, Ordovas JM, DeLuca C, Barnard JR, Feussner G, and Schaefer EJ. (2000). Association of apolipoprotein (Apo)E genotype with plasma apo E levels. *Atherosclerosis* 148: 327–335.

Leerink CB, de-Vries CV, and van-der-Klis FR. (1997). Elevated levels of serum lipoprotein(a) and apolipoprotein(a) phenotype are not related to pre-eclampsia. *Acta Obstet Gynecol Scand* 76: 625–628.

Letsky EA. (1999). Haemostatic problems associated with pregnancy, pp. 210–237. *In* Edmonds DK [ed.], Dewhurst's textbook of obstetrics and gynaecology for postgraduates, 6th ed. Blackwell, Oxford.

Levay PF, and Viljoen M. (1995). Lactoferrin: a general review. *Haematologica* 80: 252–267.

Li DK, and Wi S. (2000). Changing paternity and the risk of preeclampsia/eclampsia in the subsequent pregnancy. *Am J Epidemiol* 151: 57–62.

Lie RT, Rasmussen S, Brunborg H, Gjessing HK, Lie-Nielsen E, and Irgens LM. (1998). Fetal and maternal contributions to risk of pre-eclampsia: population based study. *BMJ* 316: 1343–1347.

Little RE, and Gladen BC. (1999). Levels of lipid peroxides in uncomplicated pregnancy: a review of the literature. *Reprod Toxicol* 13: 347–352.

Low PS, Waugh SM, Zinke K, and Drenckhahn D. (1985). The role of hemoglobin denaturation and band 3 clustering in red blood cell aging. *Science* 227: 531–533.

Lusis AJ. (2000). Atherosclerosis. *Nature* 407: 233–241.

Lutz HU, Bussolino F, Flepp R, Fasler S, Stammler P, Kazatchkine MD, and Arese P. (1987). Naturally occurring anti-band-3 antibodies and complement together mediate phagocytosis of oxidatively stressed human erythrocytes. *Proc Natl Acad Sci U S A* 84: 7368–7372.

Lutz HU. (1992). Naturally occurring anti-band 3 antibodies. *Transfus Med Rev* 6: 201–211.

Lux SE, and Palek J. (1995). Disorders of the red cell membrane, pp. 1701–1818. *In* Handin RI, Lux SE and Stossel TP [eds.], Blood: principles and practice of hematology. Lippincott Williams &Wilkins, Philadelphia.

Magann EF, and Martin JN. (1999). Twelve steps to optimal management of HELLP syndrome. *Clin Obstet Gynecol* 42: 532–550.

Makkonen N, Heinonen S, Hiltunen M, Helisalmi S, Mannermaa A, and Kirkinen P. (2001). Apolipoprotein E alleles in women with pre-eclampsia. *J Clin Pathol* 54: 652–654.

Mänttäri M, Manninen V, Palosuo T, and Ehnholm C. (2001). Apolipoprotein E polymorphism and C-reactive protein in dyslipidemic middle-aged men. *Atherosclerosis* 156: 237–238.

Many A, Hubel CA, and Roberts JM. (1996). Hyperuricemia and xanthine oxidase in preeclampsia, revisited. *Am J Obstet Gynecol* 174: 288–291.

Marathe GK, Prescott SM, Zimmerman GA, and McIntyre TM. (2001). Oxidized LDL contains inflammatory PAF-like phospholipids. *Trends Cardiovasc Med* 11: 139–142.

McGladdery SH, and Frohlich JJ. (2001). Lipoprotein lipase and apoE polymorphisms: relationship to hypertriglyceridemia during pregnancy. *J Lipid Res* 42: 1905–1912.

Meekins JW, Pijnenborg R, Hanssens M, Assche AV, and McFadyen IR. (1994). Immunohistochemical detection of lipoprotein(a) in the wall of placental bed spiral arteries in normal and severe preeclamptic pregnancies. *Placenta* 15: 511–524.

Memon RA, Staprans I, Noor M, Holleran WM, Uchida Y, Moser AH, Feingold KR, *et al.* (2000). Infection and inflammation induce LDL oxidation in vivo. *Arterioscler Thromb Vasc Biol* 20: 1536–1542.

Mendall MA, Patel P, Ballam L, Strachan D, and Northfield TC. (1996). C reactive protein and its relation to cardiovascular risk factors: a population based cross sectional study. *BMJ* 312: 1061–1065.

Mertens A, and Holvoet P. (2001). Oxidized LDL and HDL: antagonists in atherothrombosis. *FASEB J* 15: 2073–2084.

Metcalfe J, Romney SL, Ramsey LH, Reid DE, and Burwell CS. (1955). Estimation of uterine blood flow in normal human pregnancy at term. *J Clin Invest* 34: 1632–1638.

Miles LA, Fless GM, Levin EG, Scanu AM, and Plow EF. (1989). A potential basis for the thrombotic risks associated with lipoprotein(a). *Nature* 339: 301–303.

Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, and Milner A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin Sci* 84: 407–412.

Milman N, Byg KE, and Agger AO. (2000). Hemoglobin and erythrocyte indices during normal pregnancy and postpartum in 206 women with and without iron supplementation. *Acta Obstet Gynecol Scand* 79: 89–98.

Mori M, Mori A, Saburi Y, Sida M, and Ohta H. (2003). Levels of lipoprotein(a) in normal and compromised pregnancy. *J Perinat Med* 31: 23–28.

Morris JM, Gopaul NK, Endresen MJR, Knight M, Linton EA, Dhir S, Änggård EE, *et al.* (1998). Circulating markers of oxidative stress are raised in normal pregnancy and pre-eclampsia. *Br J Obstet Gynaecol* 105: 1195–1199.

Muller WA. (1999). Leukocyte-endothelial cell adhesion molecules in transendothelial migration, pp. 585–592. *In* Gallin JI, Snyderman R, Fearon DT, Haynes BF and Nathan C [eds.], Inflammation: basic principles and clinical correlates, 3rd ed. Lippincott Williams & Wilkins, Philadelphia.

Müller-Wieland D, and Krone W. (1999). Drug-induced effects, pp. 1037–1048. *In* Betteridge DJ, Illingworth DR and Shepherd J [eds.], Lipoproteins in health and disease. Arnold, London.

Murai JT, Muzykanskiy E, and Taylor RN. (1997). Maternal and fetal modulators of lipid metabolism correlate with the development of preeclampsia. *Metabolism* 46: 963–967.

Myatt L, and Miodovnik M. (1999). Prediction of preeclampsia. *Semin Perinatol* 23: 45–57.

Nagy B, Rigo J, Fintor L, Karadi I, and Toth T. (1998). Apolipoprotein E alleles in women with severe pre-eclampsia. *J Clin Pathol* 51: 324–325.

Navab M, Berliner JA, Subbanagounder G, Hama S, Lusis AJ, Castellani LW, Reddy S, *et al.* (2001). HDL and the inflammatory response induced by LDL-derived oxidized phospholipids. *Arterioscler Thromb Vasc Biol* 21: 481–488.

Newnham J. (1998). Consequences of fetal growth restriction. *Curr Opin Obstet Gynecol* 10: 145–149.

NHBPEP Working Group. (1990). National High Blood Pressure Education Program Working Group Report on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol* 163: 1691–1712.

NHBPEP Working Group. (2000). Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol* 183: S1–S22.

Nichols AV, Krauss RM, and Musliner TA. (1986). Nondenaturing polyacrylamide gradient gel electrophoresis. *Methods Enzymol* 128: 417–431.

Nigon F, Lesnik P, Rouis M, and Chapman MJ. (1991). Discrete subspecies of human low density lipoproteins are heterogeneous in their interaction with the cellular LDL receptor. *J Lipid Res* 32: 1741–1753.

Nofer JR, Kehrel B, Fobker M, Levkau B, Assmann G, and von-Eckardstein A. (2002). HDL and arteriosclerosis: beyond reverse cholesterol transport. *Atherosclerosis* 161: 1–16.

Nova A, Sibai BM, Barton JR, Mercer BM, and Mitchell MD. (1991). Maternal plasma level of endothelin is increased in preeclampsia. *Am J Obstet Gynecol* 165: 724–727.

O'Brien JM, Milligan DA, and Barton JR. (2000). Impact of high-dose corticosteroid therapy for patients with HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome. *Am J Obstet Gynecol* 183: 921–924.

Olerup O, and Zetterquist H. (1992). HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: An alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue antigens* 39: 225–235.

Oostenbrug GS, Mensink RP, Al MDM, Houwelingen AC, and Hornstra G. (1998). Maternal and neonatal plasma antioxidant levels in normal pregnancy, and the relationship with fatty acid unsaturation. *Br J Nutr* 80: 67–73.

Ordovas JM, Pocovi M, and Grande F. (1984). Plasma lipids and cholesterol esterification rate during pregnancy. *Obstet Gynecol* 63: 20–25.

Packard C, Caslake M, and Shepherd J. (2000). The role of small, dense low density lipoprotein (LDL): a new look. *Int J Cardiol* 74: S17–S22.

Packard CJ, and Shepherd J. (1997). Lipoprotein heterogeneity and apolipoprotein B metabolism. *Arterioscler Thromb Vasc Biol* 17: 3542–3556.

Pankow JS, Folsom AR, Cushman M, Borecki IB, Hopkins PN, Eckfeldt JH, and Tracy RP. (2001). Familial and genetic determinants of systemic markers of inflammation: the NHLBI family heart study. *Atherosclerosis* 154: 681–689.

Parthasarathy S, Barnett J, and Fong LG. (1990). High-density lipoprotein inhibits the oxidative modification of low-density lipoprotein. *Biochim Biophys Acta* 1044: 275–283.

Pasceri V, Willerson JT, and Yeh ETH. (2000). Direct proinflammatory effect of C-reactive protein on human endothelial cells. *Circulation* 102: 2165–2168.

Pepys MB, and Berger A. (2001). The renaissance of C reactive protein. *BMJ* 322: 4–5.

Perry KG, and Martin JN. (1992). Abnormal hemostasis and coagulopathy in preeclampsia and eclampsia. *Clin Obstet Gynecol* 35: 338–350.

Piechota W, and Staszewski A. (1992). Reference ranges of lipids and apolipoproteins in pregnancy. *Eur J Obstet Gynecol Reprod Biol* 45: 27–35.

Pocovi M, Ordovas JM, and Grande-Covian F. (1984). Plasma lipids and apolipoproteins A and B in human pregnancy. *Rev Esp Fisiol* 40: 183–190.

Poranen AK, Ekblad U, Uotila P, and Ahotupa M. (1996). Lipid peroxidation and antioxidants in normal and pre-eclamptic pregnancies. *Placenta* 17: 401–405.

Potter JM, and Nestel PJ. (1979). The hyperlipidemia of pregnancy in normal and complicated pregnancies. *Am J Obstet Gynecol* 133: 165–170.

Powers RW, Evans RW, Majors AK, Ojimba J, Ness RB, Crombleholme WR, and Roberts JM. (1998). Plasma homocysteine concentration is increased in preeclampsia and is associated with evidence of endothelial activation. *Am J Obstet Gynecol* 179: 1605–1611.

Prieto JA, Panyutich AV, and Heine RP. (1997). Neutrophil activation in preeclampsia. Are defensins and lactoferrin elevated in preeclamptic patients? *J Reprod Med* 42: 29–32.

Qiu C, Williams MA, Leisenring WM, Sorensen TK, Frederick IO, Dempsey JC, and Luthy DA. (2003). Family history of hypertension and type 2 diabetes in relation to preeclampsia risk. *Hypertension* 41: 408–413.

Raghunath PN, Tomaszewski JE, Brady ST, Caron RJ, Okada SS, and Barnathan ES. (1995). Plasminogen activator system in human coronary atherosclerosis. *Arterioscler Thromb Vasc Biol* 15: 1432–1443.

Raijmakers MTM, Roes EM, te-Morsche RHM, Steegers EAP, and Peters WHM. (2003). Haptoglobin and its association with the HELLP syndrome. *J Med Genet* 40: 214–216.

Rath W, Faridi A, and Dudenhausen JW. (2000). HELLP syndrome. *J Perinat Med* 28: 249–260.

Rebelo I, Carvalho-Guerra F, Pereira-Leite L, and Quintanilha A. (1995). Lactoferrin as a sensitive blood marker of neutrophil activation in normal pregnancies. *Eur J Obstet Gynecol Reprod Biol* 62: 189–194.

Rebelo I, Carvalho-Guerra F, Pereira-Leite L, and Quintanilha A. (1996). Comparative study of lactoferrin and other blood markers of inflammatory stress between preeclamptic and normal pregnancies. *Eur J Obstet Gynecol Reprod Biol* 64: 167–173.

Redman CWG. (1993). The placenta, pre-eclampsia and chronic villitis, pp. 433–467. *In* Redman CWG, Sargent IL and Starkey PM [eds.], The human placenta. Blackwell, Oxford.

Redman CWG, Sacks GP, and Sargent IL. (1999). Preeclampsia: an excessive maternal inflammatory response to pregnancy. *Am J Obstet Gynecol* 180: 499–506.

Regnault TRH, Galan HL, Parker TA, and Anthony RV. (2002). Placental development in normal and compromised pregnancies- a review. *Placenta* 23: S119–S129.

Ridker PM. (2001). High-sensitivity C-reactive protein: potential adjunct for global risk assessment in the primary prevention of cardiovascular disease. *Circulation* 103: 1813–1818.

Roberts JM. (1984). Pregnancy-related hypertension, pp. 703–752. *In* Creasy RK and Resnik R [eds.], Maternal-fetal medicine: principles and practice. W.B. Saunders, Philadelphia.

Roberts JM, and Redman CW. (1993). Pre-eclampsia: more than pregnancy-induced hypertension. *Lancet* 341: 1447–1451.

Roberts JM. (1998). Endothelial dysfunction in preeclampsia. *Semin Reprod Endocrinol* 16: 5–15.

Roberts JM, and Hubel CA. (1999). Is oxidative stress the link in the two-stage model of pre-eclampsia? *Lancet* 354: 788–789.

Roberts JM, and Lain KY. (2002). Recent insights into the pathogenesis of pre-eclampsia. *Placenta* 23: 359–372.

Roberts JM, Pearson G, Cutler J, and Lindheimer M. (2003). Summary of the NHLBI Working Group on Research on Hypertension During Pregnancy. *Hypertension* 41: 437–445.

Robillard PY, Dekker GA, and Hulsey TC. (1999). Revisiting the epidemiological standard of preeclampsia: primigravidity or primipaternity? *Eur J Obstet Gynecol Reprod Biol* 84: 37–41.

Robson SC. (1999). Hypertension and renal disease in pregnancy, pp. 166–185. *In* Edmonds DK [ed.], Dewhurst's textbook of obstetrics and gynaecology for postgraduates, 6th ed. Blackwell, Oxford.

Rodgers GM. (1988). Hemostatic properties of normal and perturbed vascular cells. *FASEB J* 2: 116–123.

Rodgers GM, Taylor RN, and Roberts JM. (1988). Preeclampsia is associated with a serum factor cytotoxic to human endothelial cells. *Am J Obstet Gynecol* 159: 908–914.

Rogers RG, and Thorp JM. (1997). Pregnancy-induced hypertension: genesis of and response to endothelial injury and the role of endothelin 1. *Obstet Gynecol Surv* 52: 723–727.

Romem Y, and Artal R. (1985). C-reactive protein in pregnancy and in the postpartum period. *Am J Obstet Gynecol* 151: 380–383.

Rosell M, Regnstrom J, Kallner A, and Hellenius ML. (1999). Serum urate determines antioxidant capacity in middle-aged men - a controlled, randomized diet and exercise intervention study. *J Intern Med* 246: 219–226.

Rosing U, Samsioe G, Ölund A, Johansson B, and Kallner A. (1989). Serum levels of apolipoprotein A-I, A-II and HDL-cholesterol in second half of normal pregnancy and in pregnancy complicated by pre-eclampsia. *Horm Metab Res* 21: 376–382.

Ross R. (1999). Atherosclerosis - an inflammatory disease. *N Engl J Med* 340: 115–126.

Rudel LL, and Kesäniemi YA. (2000). Low-density lipoprotein particle composition: what is the contribution to atherogenicity? *Curr Opin Lipidol* 11: 227–228.

Sacks GP, Studena K, Sargent K, and Redman CW. (1998). Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. *Am J Obstet Gynecol* 179: 80–86.

Santos-Silva A, Castro EMB, Teixeira NA, Guerra FC, and Quintanilha A. (1995). Altered erythrocyte membrane band 3 profile as a marker in patients at risk for cardiovascular disease. *Atherosclerosis* 116: 199–209.

Santos-Silva A, Castro EMB, Teixeira NA, Guerra FC, and Quintanilha A. (1998). Erythrocyte membrane band 3 profile imposed by cellular aging, by activated neutrophils and by neutrophilic elastase. *Clin Chim Acta* 275: 185–196.

Santos-Silva A, Rebelo I, Castro EMB, Belo L, Catarino C, Monteiro I, Almeida MD, *et al.* (2002). Erythrocyte damage and leukocyte activation in ischemic stroke. *Clin Chim Acta* In press.

Saphier CJ, and Repke JT. (1998). Hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome: a review of diagnosis and management. *Semin Perinatol* 22: 118–133.

Sattar N, Gaw A, Packard CJ, and Greer IA. (1996). Potential pathogenic roles of aberrant lipoprotein and fatty acid metabolism in pre-eclampsia. *Br J Obstet Gynaecol* 103: 614–620.

Sattar N, Bendomir A, Berry C, Shepherd J, Greer IA, and Packard CJ. (1997a). Lipoprotein subfraction concentrations in preeclampsia: pathogenic parallels to atherosclerosis. *Obstet Gynecol* 89: 403–408.

Sattar N, Greer IA, Louden J, Lindsay G, McConnell M, Shepherd J, and Packard CJ. (1997b). Lipoprotein subfraction changes in normal pregnancy: threshold effect of plasma triglyceride on appearance of small, dense low density lipoprotein. *J Clin Endocrinol Metab* 82: 2483–2491.

Sattar N, and Greer IA. (1999). Lipids and the pathogenesis of pre-eclampsia. *Curr Obstet Gynaecol* 9: 190–195.

Sattar N, Greer IA, Rumley A, Stewart G, Shepherd J, Packard CJ, and Lowe GDO. (1999). A longitudinal study of the relationships between haemostatic, lipid, and oestradiol changes during normal human pregnancy. *Thromb Haemost* 81: 71–75.

Sattar N, Clark P, Greer IA, Shepherd J, and Packard CJ. (2000). Lipoprotein (a) levels in normal pregnancy and in pregnancy complicated with pre-eclampsia. *Atherosclerosis* 148: 407–411.

Savvidou MD, Lees CC, Parra M, Hingorani AD, and Nicolaides KH. (2002). Levels of C-reactive protein in pregnant women who subsequently develop pre-eclampsia. *BJOG* 109: 297–301.

Scalera F. (2003). Intracellular glutathione and lipid peroxide availability and the secretion of vasoactive substances by human umbilical vein endothelial cells after incubation with TNF-alpha. *Eur J Clin Invest* 33: 176–182.

Schauf B, Lang U, Stute P, Schneider S, Dietz K, Aydeniz B, and Wallwiener D. (2002). Reduced red blood cell deformability, an indicator for high fetal or maternal risk, is found in preeclampsia and IUGR. *Hypertens Pregnancy* 21: 147–160.

Schiff E, Friedman SA, Stampfer M, Kao L, Barrett PH, and Sibai BM. (1996). Dietary consumption and plasma concentrations of vitamin E in pregnancies complicated by preeclampsia. *Am J Obstet Gynecol* 175: 1024–1028.

Schjetlein R, Haugen G, and Wisløff F. (1997). Markers of intravascular coagulation and fibrinolysis in preeclampsia: association with intrauterine growth retardation. *Acta Obstet Gynecol Scand* 76: 541–546.

Schjetlein R, Abdelnoor M, Haugen G, Husby H, Sandset PM, and Wisløff F. (1999). Hemostatic variables as independent predictors for fetal growth retardation in preeclampsia. *Acta Obstet Gynecol Scand* 78: 191–197.

Scott JR. (2003). Magnesium sulfate for mild preeclampsia. *Obstet Gynecol* 101: 213.

Serin YS, Ozcelik B, Bapbuo M, Kylyc H, Okur D, and Erez R. (2002). Predictive value of tumor necrosis factor alpha (TNF-alpha) in preeclampsia. *Eur J Obstet Gynecol Reprod Biol* 100: 143–145.

Shaarawy M, and Didy HE. (1996). Thrombomodulin, plasminogen activator inhibitor type 1 (PAI-1) and fibronectin as biomarkers of endothelial damage in preeclampsia and eclampsia. *Int J Gynaecol Obstet* 55: 135–139.

Shaarawy M, Aref A, Salem ME, and Sheiba M. (1998). Radical-scavenging antioxidants in pre-eclampsia and eclampsia. *Int J Gynaecol Obstet* 60: 123–128.

Sibai BM. (2002). Hypertension, pp. 945–1004. *In* Gabbe SG, Niebyl JR and Simpson JL [eds.], Obstetrics: normal and problem pregnancies, 4th ed. Churchill Livingstone, Philadelphia.

Silliman K, Tall AR, Kretchmer N, and Forte TM. (1993). Unusual high-density lipoprotein subclass distribution during late pregnancy. *Metabolism* 42: 1592–1599.

Silliman K, Shore V, and Forte TM. (1994). Hypertriglyceridemia during late pregnancy is associated with the formation of small dense low-density lipoproteins and the presence of large buoyant high-density lipoproteins. *Metabolism* 43: 1035–1041.

Simpson HCR, Mann JI, Meade TW, Chakrabarti R, Stirling Y, and Woolf L. (1983). Hypertriglyceridaemia and hypercoagulability. *Lancet* 1: 786–790.

Singh RB, Mengi SA, Xu Y-J, Arneja AS, and Dhalla NS. (2002). Pathogenesis of atherosclerosis: a multifactorial process. *Exp Clin Cardiol* 7: 40–53.

Southorn PA, and Powis G. (1988). Free radicals in medicine. II. Involvement in human disease. *Mayo Clin Proc* 63: 390–408.

Spickett CM, Reglinski J, Smith WE, Wilson R, Walker JJ, and McKillop J. (1998). Erythrocyte glutathione balance and membrane stability during preeclampsia. *Free Radic Biol Med* 24: 1049–1055.

Starkey PM. (1993). The decidua and factors controlling placentation, pp. 362–413. *In* Redman CWG, Sargent IL and Starkey PM [eds.], The human placenta. Blackwell, Oxford.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC, and Witztum JL. (1989). Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 320: 915–924.

Stewart DJ, and Monge JC. (1993). Hyperlipidemia and endothelial dysfunction. *Curr Opin Lipidol* 4: 319–324.

Stirling Y, Woolf L, North WRS, Seghatchian MJ, and Meade TW. (1984). Haemostasis in normal pregnancy. *Thromb Haemost* 52: 176–182.

Sullivan GW, Sarembock IJ, and Linden J. (2000). The role of inflammation in vascular diseases. *J Leukoc Biol* 67: 591–602.

Sutherland A, Cooper DW, Howie PW, Liston WA, and MacGillivray I. (1981). The indicence of severe pre-eclampsia amongst mothers and mothers-in-law of pre-eclamptics and controls. *Br J Obstet Gynaecol* 88: 785–791.

Takacs P, Green KL, Nikaeo A, and Kauma SW. (2003). Increased vascular endothelial cell production of interleukin-6 in severe preeclampsia. *Am J Obstet Gynecol* 188: 740–744.

Takei A, Huang Y, and Lopes-Virella MF. (2001). Expression of adhesion molecules by human endothelial cells exposed to oxidized low density lipoprotein. Influences of degree of oxidation and location of oxidized LDL. *Atherosclerosis* 154: 79–86.

Taylor RN. (1997). Review: immunobiology of preeclampsia. *Am J Reprod Immunol* 37: 79–86.

Teran E, Escudero C, Moya W, Flores M, Vallance P, and Lopez-Jaramillo P. (2001). Elevated C-reactive protein and pro-inflammatory cytokines in Andean women with pre-eclampsia. *Int J Gynaecol Obstet* 75: 243–249.

Terrone DA, Rinehart BK, May WL, Moore A, Magann EF, and Martin JN. (2000). Leukocytosis is proportional to HELLP syndrome severity: evidence for an inflammatory form of preeclampsia. *South Med J* 93: 768–771.

The Magpie Trial Collaborative Group. (2002). Do women with pre-eclampsia, and their babies, benefit from magnesium sulphate? The Magpie Trial: a randomised placebo-controlled trial. *Lancet* 359: 1877–1890.

Tian LP, Nelson EAS, Senok AC, Yu LM, Oppenheimer SJ, and Li K. (1998). Red cell age and susceptibility to malaria during pregnancy. *Acta Obstet Gynecol Scand* 77: 717–721.

Tikkanen MJ. (1999). Sex hormones, pp. 967–984. *In* Betteridge DJ, Illingworth DR and Shepherd J [eds.], Lipoproteins in health and disease. Arnold, London.

Torzewski J, Torzewski M, Bowyer DE, Fröhlich M, Koenig W, Waltenberger J, Fitzsimmons C, *et al.* (1998). C-reactive protein frequently colocalizes with the terminal complement complex in the intima of early atherosclerotic lesions of human coronary arteries. *Arterioscler Thromb Vasc Biol* 18: 1386–1392.

Towbin H, Staehelin T, and Gordon J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76: 4350–4354.

Tsatsaris V, Goffin F, Munaut C, Brichant JF, Frankenne F, and Foidart JM. (2002). The VEGF family in pregnancies with preeclampsia or growth retardation. *Hypertens Pregnancy* 21: 20.

Tsukimori K, Maeda H, Ishida K, Nagata H, Koyanagi T, and Nakano H. (1993). The superoxide generation of neutrophils in normal and preeclamptic pregnancies. *Obstet Gynecol* 81: 536–540.

Uotila J, Tuimala R, Aarnio T, Pyykkö K, and Ahotupa M. (1991). Lipid peroxidation products, selenium-dependent glutathione peroxidase and vitamin E in normal pregnancy. *Eur J Obstet Gynecol Reprod Biol* 42: 95–100.

Uotila J, Solakivi T, Jaakkola O, Tuimala R, and Lehtimaki T. (1998). Antibodies against copper-oxidised and malondialdehyde-modified low density lipoproteins in pre-eclampsia pregnancies. *Br J Obstet Gynaecol* 105: 1113–1117.

Uotila JT, Tuimala RJ, Aarnio TM, Pyykkö KA, and Ahotupa MO. (1993). Findings on lipid peroxidation and antioxidant function in hypertensive complications of pregnancy. *Br J Obstet Gynaecol* 100: 270–276.

van Hinsbergh VWM. (2001). The endothelium: vascular control of haemostasis. *Eur J Obstet Gynecol Reprod Biol* 95: 198–201.

Van Lenten BJ, Navab M, Shih D, Fogelman AM, and Lusis AJ. (2001). The role of high-density lipoproteins in oxidation and inflammation. *Trends Cardiovasc Med* 11: 155–161.

Vapaatalo H, and Mervaala E. (2001). Clinically important factors influencing endothelial function. *Med Sci Monit* 7: 1075–1085.

Vickers MA, Green FR, Terry C, Mayosi BM, Julier C, Lathrop M, Ratcliffe PJ, *et al.* (2002). Genotype at a promoter polymorphism of the interleukin-6 gene is associated with baseline levels of plasma C-reactive protein. *Cardiovasc Res* 53: 1029–1034.

Vigil-De Gracia P. (2001a). Pregnancy complicated by pre-eclampsia-eclampsia with HELLP syndrome. *Int J Gynaecol Obstet* 72: 17–23.

Vigil-De Gracia P. (2001b). Acute fatty liver and HELLP syndrome: two distinct pregnancy disorders. *Int J Gynaecol Obstet* 73: 215–220.

Visser W, Beckmann I, Bremer HA, Lim HL, and Wallenburg HC. (1994). Bioactive tumour necrosis factor alpha in pre-eclamptic patients with and without the HELLP syndrome. *Br J Obstet Gynaecol* 101: 1081–1082.

Vitoratos N, Salamalekis E, Dalamaga N, Kassanos D, and Creatsas G. (1999). Defective antioxidant mechanisms via changes in serum ceruloplasmin and total iron binding capacity of serum in women with pre-eclampsia. *Eur J Obstet Gynecol Reprod Biol* 84: 63–67.

Vriese SR, Dhont M, and Christophe AB. (2001). Oxidative stability of low density lipoproteins and vitamin E levels increase in maternal blood during normal pregnancy. *Lipids* 36: 361–366.

Wakatsuki A, Ikenoue N, Okatani Y, Shinohara K, and Fukaya T. (2000). Lipoprotein particles in preeclampsia: susceptibility to oxidative modification. *Obstet Gynecol* 96: 55–59.

Walsh SW. (1998). Maternal-placental interactions of oxidative stress and antioxidants in preeclampsia. *Semin Reprod Endocrinol* 16: 93–104.

Wang DN. (1994). Band 3 protein: structure, flexibility and function. *FEBS Lett* 346: 26–31.

Wang J, Mimuro S, Lahoud R, Trudinger B, and Wang XL. (1998). Elevated levels of lipoprotein(a) in women with preeclampsia. *Am J Obstet Gynecol* 178: 146–149.

Wang Y, Walsh SW, Guo J, and Zhang J. (1991a). Maternal levels of prostacyclin, thromboxane, vitamin E, and lipid peroxides throughout normal pregnancy. *Am J Obstet Gynecol* 165: 1690–1694.

Wang YP, Walsh SW, Guo JD, and Zhang JY. (1991b). The imbalance between thromboxane and prostacyclin in preeclampsia is associated with an imbalance between lipid peroxides and vitamin E in maternal blood. *Am J Obstet Gynecol* 165: 1695–1700.

Ware-Jauregui S, Sanchez SE, Zhang C, Laraburre G, King IB, and Williams MA. (1999). Plasma lipid concentrations in pre-eclamptic and normotensive Peruvian women. *Int J Gynaecol Obstet* 67: 147–155.

Watts DH, Krohn MA, Wener MH, and Eschenbach DA. (1991). C-reactive protein in normal pregnancy. *Obstet Gynecol* 77: 176–180.

Weintraub WS, and Harrison DG. (2000). C-reactive protein, inflammation and atherosclerosis: do we really understand it yet? *Eur Heart J* 21: 958–960.

Wetzka B, Winkler K, Kinner M, Friedrich I, Marz W, and Zahradnik HP. (1999). Altered lipid metabolism in preeclampsia and HELLP syndrome: links to enhanced platelet reactivity and fetal growth. *Semin Thromb Hemost* 25: 455–462.

Williams MA, Zingheim RW, King IB, Sorensen TK, Marcovina SM, and Luthy DA. (1996). Apolipoprotein E (apo E) phenotypes and risk of preeclampsia. *Am J Obstet Gynecol* 174: 447.

Wilson ML, Goodwin TM, Pan VL, and Ingles SA. (2003). Molecular epidemiology of preeclampsia. *Obstet Gynecol Surv* 58: 39–66.

Winkler K, Wetzka B, Hoffmann MM, Friedrich I, Kinner M, Baumstark MW, Wieland H, *et al.* (2000). Low density lipoprotein (LDL) subfractions during pregnancy: accumulation of buoyant LDL with advancing gestation. *J Clin Endocrinol Metab* 85: 4543–4550.

Winkler K, Wetzka B, Hoffmann MM, Friedrich I, Kinner M, Baumstark MW, Zahradnik HP, *et al.* (2003). Triglyceride-rich lipoproteins are associated with hypertension in preeclampsia. *J Clin Endocrinol Metab* 88: 1162–1166.

Wintrobe MM, Lukens JN, and Lee GR. (1993). The approach to the patient with anemia, pp. 715–744. *In* Lee GR, Bithell TC, Foerster J, Athens JW and Lukens JN [eds.], Wintrobe's clinical hematology, 9th ed. Lea & Febiger, Philadelphia.

Witztum JL. (1993). Susceptibility of low-density lipoprotein to oxidative modification. *Am J Med* 94: 347–349.

Wolf M, Kettyle E, Sandler L, Ecker JL, Roberts J, and Thadhani R. (2001). Obesity and preeclampsia: the potential role of inflammation. *Obstet Gynecol* 98: 757–762.

Woolf N. (1999). Pathology of atherosclerosis, pp. 533–539. *In* Betteridge DJ, Illingworth DR and Shepherd J [eds.], Lipoproteins in health and disease. Arnold, London.

Wu JJ. (1996). Lipid peroxidation in preeclamptic and eclamptic pregnancies. *Eur J Obstet Gynecol Reprod Biol* 64: 51–54.

Wu LH, Wu JT, and Hopkins PN. (1997). Apolipoprotein E: laboratory determinations and clinical significance, pp. 329–355. *In* Rifai N, Warnick GR and Dominiczak MH [eds.], Handbook of lipoprotein testing. AACC Press, Washington, DC.

Yu H, and Rifai N. (2000). High-sensitivity C-reactive protein and atherosclerosis: from theory to therapy. *Clin Biochem* 33: 601–610.

Yudkin JS, Stehouwer CDA, Emeis JJ, and Coppack SW. (1999). C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? *Arterioscler Thromb Vasc Biol* 19: 972–978.

Zachara BA, Wardak C, Didkowski W, Maciag A, and Marchaluk E. (1993). Changes in blood selenium and glutathione concentrations and glutathione peroxidase activity in human pregnancy. *Gynecol Obstet Invest* 35: 12–17.

Zechner R, Desoye G, Schweditsch MO, Pfeiffer KP, and Kostner GM. (1986). Fluctuations of plasma lipoprotein-A concentrations during pregnancy and post partum. *Metabolism* 35: 333–336.

Zee RYL, and Ridker PM. (2002). Polymorphism in the human C-reactive protein (CRP) gene, plasma concentrations of CRP, and the risk of future arterial thrombosis. *Atherosclerosis* 162: 217–219.

Zeek PM, and Assali NS. (1950). Vascular changes in the decidua associated with eclamptogenic toxemia of pregnancy. *Am J Clin Pathol* 20: 1099–1109.

Zhang J, Zeisler J, Hatch MC, and Berkowitz G. (1997). Epidemiology of pregnancy-induced hypertension. *Epidemiol Rev* 19: 218–232.

Zhang YX, Cliff WJ, Schoefl GI, and Higgins G. (1999). Coronary C-reactive protein distribution: its relation to development of atherosclerosis. *Atherosclerosis* 145: 375–379.

Zhou Y, Damsky CH, and Fisher SJ. (1997a). Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? *J Clin Invest* 99: 2152–2164.

Zhou Y, Fisher SJ, Janatpour M, Genbacev O, Dejana E, Wheelock M, and Damsky CH. (1997b). Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J Clin Invest* 99: 2139–2151.

Ziouzenkova O, Winklhofer-Roob BM, Puhl H, Roob JM, and Esterbauer H. (1996). Lack of correlation between the alpha-tocopherol content of plasma and LDL, but high correlations for gamma-tocopherol and carotenoids. *J Lipid Res* 37: 1936–1946.

Zusterzeel PL, Wanten GJ, Peters WH, Merkus HM, and Steegers EA. (2001). Neutrophil oxygen radical production in pre-eclampsia with HELLP syndrome. *Eur J Obstet Gynecol Reprod Biol* 99: 213–218.

Zwaka TP, Hombach V, and Torzewski J. (2001). C-reactive protein-mediated low density lipoprotein uptake by macrophages: implications for atherosclerosis. *Circulation* 103: 1194–1197.