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FREE PAPP-A: A NOVEL MARKER IN ACUTE CORONARY SYNDROME PATIENTS

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To my family

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LIST OF ORIGINAL PUBLICATIONS

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I Qiu-Ping Qin, Saara Kokkala, Juha Lund, Natalia Tamm, Liisa-Maria Voipio-Pulkki, and Kim Pettersson (2005) Molecular distinction of circulating pregnancy-associated plasma protein A in myocardial infarction and pregnancy. *Clin Chem* **51**:75-83.

II Saara Wittfooth, Qiu-Ping Qin, Juha Lund, Ilkka Tierala, Kari Pulkki, Harri Takalo, and Kim Pettersson (2006) Immunofluorometric point-of-care assays for the detection of acute coronary syndrome-related noncomplexed pregnancy-associated plasma protein A. *Clin Chem* **52**:1794-1801.

III Saara Wittfooth, Qiu-Ping Qin, and Kim Pettersson (2008) Performance of immunofluorometric point-of-care assays for free pregnancy-associated plasma protein A detection in whole blood samples. *Clin Chem Lab Med* **46**:18-20.

IV Juha Lund, Saara Wittfooth, Qiu-Ping Qin, Tuomo Ilva, Pekka Porela, Kari Pulkki, Kim Pettersson, and Liisa-Maria Voipio-Pulkki (2009) Free vs. total pregnancy-associated plasma protein A (PAPP-A) as a predictor of 12 months outcome in patients presenting with non-ST-elevation acute coronary syndrome. *Submitted manuscript*

V Risto Tertti, Saara Wittfooth, Pekka Porela, K. E. Juhani Airaksinen, Kaj Metsärinne, and Kim Pettersson (2009) Intravenous administration of low molecular weight and unfractionated heparin elicits a rapid increase in serum pregnancy-associated plasma protein A. *Clin Chem* **55**:1214-7.

In addition, some unpublished data are included.

ABBREVIATIONS

ACS	acute coronary syndrome
ATIII	antithrombin III
βhCG	free β-form of human chorionic gonadotropin
BNP	brain-type natriuretic peptide
BMP	bone morphogenetic protein
β-PDD	β-phorbol 12,13-didecanoate
BSA	bovine serum albumin
CAD	coronary artery disease
CCP	complement control protein
CI	confidence interval
CK-MB	MB isoform of creatine kinase
CRP	C-reactive protein
cTnI	cardiac troponin I
cTnT	cardiac troponin T
dB-cAMP	dibutyryl cyclic adenosine monophosphate
DNA	deoxyribonucleic acid
DS	Down's syndrome
EC	endothelial cells
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
FSH	follicle stimulating hormone
GAG	glycosaminoglycan
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGF-R1	type I IGF receptor
IL	interleukin
KO	knocked-out, the gene for the specified protein inactivated
LDL	low-density lipoprotein
LMWH	low molecular weight heparin
LNR	Lin-Notch repeat
MBP	major basic protein of eosinophils
MI	myocardial infarction
MoM	multiples of the median
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
NODAT	new-onset diabetes mellitus after transplantation
NSTE-ACS	ACS without ST-segment elevation in ECG
NSTEMI	MI without elevation of the ST-segment in ECG
PAPP-A	pregnancy-associated plasma protein A
PAPP-A/proMBP	a heterotetrameric protein complex consisting of two PAPP-A subunits and two proMBP subunits
PCI	percutaneous coronary intervention
PMA	phorbol 12-myristate-13-acetate
proMBP	the proform of eosinophil major basic protein
RT-PCR	reverse transcription polymerase chain reaction
SA	stable angina
SCR	short consensus repeat
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
STEMI	myocardial infarction with elevation of the ST-segment in ECG
TNFα	tumor necrosis factor-α
UA	unstable angina
UFH	unfractionated heparin
VSMC	vascular smooth muscle cells

ABSTRACT

In recent years, one important objective of cardiovascular research has been to find new markers that would improve the risk stratification and diagnosis of patients presenting with symptoms of acute coronary syndrome (ACS). Pregnancy-associated plasma protein A (PAPP-A) is a large metalloproteinase involved in insulin-like growth factor signalling. It is expressed in various tissues and seems to be involved in many physiological and pathological processes, such as folliculogenesis, bone formation, wound healing, pregnancy and atherosclerosis. The aim of this thesis was to investigate PAPP-A in ACS patients.

Circulating concentrations of PAPP-A had been previously shown to be elevated in ACS. In this study it was revealed that the form of PAPP-A causing this elevation was the free noncomplexed PAPP-A. Thus, the form of PAPP-A in the circulation of ACS patients differed from the complexed PAPP-A form abundantly present in the circulation during pregnancy. A point-of-care method based on time-resolved immunofluorometric assays was developed, which enabled the rapid detection of free PAPP-A. The method was found to perform well with serum and heparin plasma samples as well as with heparinized whole blood samples. With this method the concentrations of free PAPP-A in healthy individuals were shown to be negligible. When the clinical performance of the method was evaluated with serum samples from ACS patients, it was shown that the free PAPP-A concentration in the admission sample was an independent predictor of myocardial infarction and death. Moreover, as a prognostic marker, free PAPP-A was revealed to be superior to total PAPP-A, i.e. the combination of free and complexed PAPP-A, which has been measured by the other groups in this field.

As heparin products are widely used as medication in ACS patients, the effect of heparin products on free PAPP-A molecule and circulating concentrations were also investigated in this study. It was shown that intravenous administration of low molecular weight or unfractionated heparin elicits a rapid release of free PAPP-A into the circulation in haemodialysis patients and patients undergoing angiography. Moreover, the interaction between PAPP-A and heparin was confirmed in gel filtration studies. Importantly, the patients included in the clinical evaluation of the free PAPP-A detection method developed had not received any heparin product medication before the admission sample and thus the results were not affected by the heparin effect.

In conclusion, free PAPP-A was identified as a novel marker associated with ACS. The point-of-care methods developed enable rapid detection of this molecule which predicts adverse outcome when measured in the admission sample of ACS patients. However, the effect revealed of heparin products on circulating PAPP-A concentrations should be acknowledged when further studies are conducted related to free or total PAPP-A in ACS.

1 INTRODUCTION

Acute coronary syndromes (ACS) are a major cause of morbidity and mortality in the world today. Early diagnosis has a key role in limiting myocardial damage in ACS patients. Prompt risk stratification and diagnosis enable rapid initiation of therapy with the aggressiveness of the therapy in concordance with the risk of the patient for adverse outcome. Circulating markers of myocardial necrosis, most commonly cardiac troponins I and T (cTnI, cTnT) due to their superior sensitivity and tissue-specificity, are widely used in the diagnosis of ACS patients along with the electrocardiogram (ECG). (For reviews, see Maisel *et al.*, 2006; Morrow *et al.*, 2007; Bonaca and Morrow, 2008.)

The limitation of cardiac troponins has been that as makers of myocardial necrosis they are, by definition, released into the circulation only after damage of the heart muscle tissue. Moreover, with the methods previously available, it took several hours after the onset of symptoms for the elevations to become detectable (Donnelly and Millar-Craig, 1998). However, the tests for cardiac troponins have lately evolved significantly and become more and more sensitive. This has enabled the detection of even lower elevations earlier in the course of ACS (Reichlin *et al.*, 2009). On the other hand, this has also created problems in patient management (Fye, 2006; Jaffe *et al.*, 2006; Bonaca and Morrow, 2008). With the sensitive assays cardiac troponin elevations are being detected in significantly larger patient populations with some of the patients not necessarily in need of aggressive therapy. Moreover, mild troponin elevations, although still indicating myocardial damage, can also be associated with other conditions without high imminent risk for irreversible adverse cardiac outcome (Panteghini, 2004).

In recent years a major objective in cardiovascular disease research has been to find new biomarkers that would appear in the circulation before significant myocardial necrosis develops. Such markers are anticipated to enable earlier diagnosis or better risk stratification of the patients arriving at the emergency unit with symptoms of ACS such as chest discomfort and shortness of breath. It is hoped that the new markers will provide better risk prediction especially in patients without cardiac troponin elevations on admission. Moreover, the new markers should aid in the management of patients who have only mild elevations in cardiac troponin measured by a sensitive assay but who do not necessarily need aggressive treatment with higher risk. Numerous candidate molecules have appeared which are linked to the various pathological processes leading to or associated with ACS. These include markers presumably related to inflammation, plaque destabilization, plaque rupture, thrombosis, ischemia and myocardial dysfunction (for reviews, see Apple *et al.*, 2005; Jaffe *et al.*, 2006; Maisel *et al.*, 2006; See and de Lemos, 2006; Bonaca and Morrow, 2008; O'Donoghue and Morrow, 2008). Lately, pregnancy-associated plasma protein A (PAPP-A) has emerged as an interesting candidate marker in this context.

2 REVIEW OF THE LITERATURE

The aim of this literature review is to give a comprehensive overview of what is currently known about the PAPP-A molecule, the association of PAPP-A with physiological and pathological processes and the use of PAPP-A as a circulating biomarker in different conditions.

2.1 Structure of PAPP-A

2.1.1 Molecular structure

PAPP-A was initially found in the plasma of pregnant women among three other highly abundant proteins by Lin and colleagues in 1974. The molecules were detected with anti-third trimester pregnancy serum polyclonal antibodies not reactive with normal human serum and were alphabetically named (PAPP-A, PAPP-B, PAPP-C and PAPP-D) according to their precipitation order in the immunodiffusion method used (Lin *et al.*, 1974). Later it was revealed that the molecule detectable in pregnancy plasma that had been named PAPP-A was actually a covalent complex of PAPP-A and the proform of eosinophil major basic protein (proMBP) in equimolar amounts (Oxvig *et al.*, 1993). More specifically, the molecule is a heterodimer consisting of two PAPP-A subunits and two proMBP subunits connected by disulfide bridges (PAPP-A/proMBP complex) (Oxvig *et al.*, 1994b). However, PAPP-A produced in cell culture conditions is a homodimer of two PAPP-A subunits (Lawrence *et al.*, 1999; Overgaard *et al.*, 2000).

In non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) PAPP-A/proMBP complex from pregnancy plasma migrates as a 500 kDa entity. Upon reduction PAPP-A subunit is seen at 200 kDa. However, proMBP forms a smear at 50-90 kDa most likely because of high and variable glycosylation of this subunit. (Oxvig *et al.*, 1994a.) The calculated size of PAPP-A subunit is 199 kDa, when the determined protein and carbohydrate contents are taken into account. The calculated size of the PAPP-A/proMBP complex is 474 kDa. (Oxvig *et al.*, 1994b.)

The gene of PAPP-A is found in human chromosome region 9q33.1 (Sinosich, 1990). PAPP-A is encoded by 22 exons (Overgaard *et al.*, 2003) and the sequence of PAPP-A seems to be highly conserved between mammals (Mazerbourg *et al.*, 2001; Hourvitz *et al.*, 2002). For example, between mouse and human the sequence identity is 91.1% (Soe *et al.*, 2002). Homology has also been noticed between human and zebrafish PAPP-A (Overgaard *et al.*, 2003).

PAPP-A is transcribed as an mRNA of 8400 nucleotides of which 4881 nucleotides are translated into preproPAPP-A of 1627 amino acids (Haaning *et al.*, 1996). Thus the PAPP-A transcript has an unusually long 5' untranslated region. The preproform includes a putative signal peptide of 22 residues and a highly basic (calculated pI 12.9) propeptide of 58 residues (Haaning *et al.*, 1996). The PAPP-A propeptide is not required for folding or secretion of the protein and the proform is most likely cleaved by a protease recognizing Arg-X-X-Arg motif, possibly furin, prior to secretion (Kristensen *et al.*, 1994; Overgaard *et al.*, 2000). The mature PAPP-A monomer polypeptide contains 1547 amino acid residues representing the C-terminal part of the proform of PAPP-A (Kristensen *et al.*, 1994). The structure of the mature PAPP-A polypeptide is shown in Figure 1.

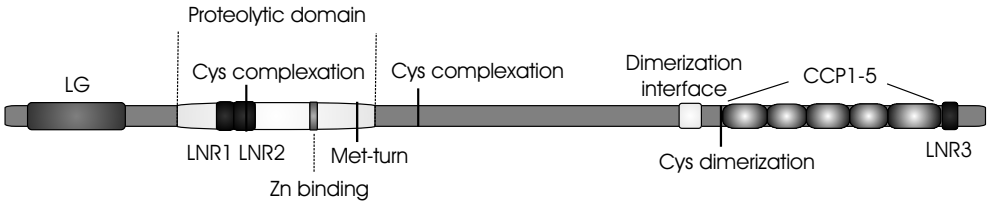

Figure 1. The structure of PAPP-A polypeptide with the functional modules and important amino acids. (CCP1-5, the complement control protein modules 1-5 involved in surface binding; LNR1-3, Lin-Notch repeats important in substrate recognition and activity; LG, laminin G-like module of unknown function)

In the amino acid sequence of the PAPP-A monomer there are 13 putative sites for N-glycosylation and 7 putative sites for attachment of glycosaminoglycan groups (Kristensen *et al.*, 1994). Of the 13 potential sites for N-linked carbohydrate 11 are substituted. However, PAPP-A is most likely not O-glycosylated (Overgaard *et al.*, 2003). In pregnancy serum there are 44 N-acetylglucosamine monosaccharides for each PAPP-A polypeptide chain (Overgaard *et al.*, 2000). In recombinantly produced PAPP-A, however, the degree of glycosylation has been reported to be lower (Overgaard *et al.*, 2000).

There are 82 Cys residues in the amino acid sequence of the mature PAPP-A monomer (Kristensen *et al.*, 1994). Cys-1130 is responsible for the covalent dimerization of PAPP-A monomers while Cys-381 and Cys-652 are involved in the complexation with proMBP and are free in PAPP-A dimer (Overgaard *et al.*, 2003; Glerup *et al.*, 2005). All other cysteines seem to form intrachain bridges within structural domains except Cys-563, for which the status is unknown (Overgaard *et al.*, 2003).

Recently, some proteins with sequence homology with PAPP-A have been identified. A previously unknown protein found in 2001 in a search of public DNA sequence databases showed 46% homology with PAPP-A and was thus named PAPP-A2 (Overgaard *et al.*, 2001). A splicing variant of PAPP-A with a highly basic 29-residue insert at an exon junction was detected in mice in 2002 and was accordingly named PAPP-Ai (Soe *et al.*, 2002). Furthermore, an archaeal protein resembling PAPP-A, ulilysin of *Methanosarcina acetivorans,* was recently found in a bioinformatic search in which structure prediction was utilized (Tallant *et al.*, 2006). With a molecular size of 29-kDa this protein seems to consist only of a catalytic domain similar to that of PAPP-A. Although the sequence homology is not high, the key residues important for catalysis, substrate binding and structural stability are fully conserved between PAPP-A and ulilysin (Tallant *et al.*, 2006).

Apart from these proteins, no homology has been detected with other proteins apart from certain structural module similarities which will be discussed in the following section. It is noteworthy that the sequence of PAPP-A is not related to α-2-macroglobulin, although in some early reports published in the 1980s these proteins were assumed to be alike. This faulty assumption arose from certain similar physical properties including molecular weight, amino acid composition, isoelectric point and electrophoretic mobility (Sutcliffe *et al.*, 1980; Rosen, 1986).

2.1.2 Functional modules

Proteolytic domain

A putative Zn^{2+} binding site similar to that found in the metalloproteinases belonging to the metcinzin superfamily (astacins, reprolysins/adamalysins, serralysins, matrix metalloproteinases) has been identified in the amino acid sequence of PAPP-A (Kristensen *et al.*, 1994). PAPP-A has the elongated zinc-binding motif conserved between metzincins, HEXXHXXGXXH (from His-482 to His-492, HEIGHSLGLYH in the sequence of PAPP-A), which coordinates the catalytic zinc ion with three His residues (Boldt *et al.*, 2001). The importance of this sequence for the activity of PAPP-A has been confirmed in mutation studies as the substitution of Glu-483 with Ala (E483A) renders PAPP-A inactive (Boldt *et al.*, 2001).

In addition to the elongated zinc-binding motif, PAPP-A also contains another metzincin defining structure: a Met-turn presumed to interact directly with the residues of the active zinc-binding site (Boldt *et al.*, 2001). The Met-turn of PAPP-A is located 63 residues apart from the zinc-binding motif (Met-556) the distance being, however, unusually long for metzincins (Boldt *et al.*, 2001). In addition, contrary to other metzincins, the family-specific residue following the third His residue of the zinc-binding motif is Val, and Asn is found two residues before the Met of the Met-turn (Boldt *et al.*, 2001). With secondary structure prediction, PAPP-A was found to have a topology similar to metzincins with three α-helices and five β-strands common to metzincins within a 312-residue sequence stretch from Val-272 to Tyr-583 that defines the proteolytic domain of PAPP-A (Boldt *et al.*, 2001). However, PAPP-A contains more intradomain disulfide bonds within the proteolytic domain than the other known members of metzincins (Overgaard *et al.*, 2003). Due to these differences, PAPP-A was suggested to be the founder member of a new family within the metzincin superfamily, pappalysins, and was named pappalysin-1 (Boldt *et al.*, 2001). The three-dimensional structure of ulilysin has been revealed which gives a suggestive model of the structure of the catalytic site of PAPP-A (Tallant *et al.*, 2006).

Complement control protein modules

In the C-terminal part of PAPP-A there are five motifs of ~60 residues that are related to the short consensus repeat (SCR) modules found in complement proteins and selectins (Kristensen *et al.*, 1994). In complement proteins these motifs, which are also known as complement control protein (CCP) modules, contain four cysteines. However, the CCP modules of PAPP-A contain 6 Cys residues as do the CCP modules of selectins (Overgaard *et al.*, 2003).

Laursen and colleagues (2002b) revealed that the CCP modules of PAPP-A, more specifically modules CCP3 and CCP4, are involved in the binding of PAPP-A to cell surfaces. Preincubation of PAPP-A with glycosaminoglycans (GAG) heparin or heparan sulphate but not chondroitin sulphate or dermatan sulphate reduced the binding remarkably. A similar effect was seen with pre-treatment of the cells with heparinase. Thus, the cell surface binding of PAPP-A is most likely mediated through heparan sulphate-like GAGs that are commonly found on cell surfaces. The binding is reversible as in the experiments of Laursen and colleagues incubation of the cells with heparin after PAPP-A attachment completely abrogated binding. Moreover, it was shown that PAPP-A does not have to be synthesized in the cell on which it binds.

Weyer and colleagues noticed that three clusters of basic residues consisting of positively charged Arg and Lys residues are especially important in the interaction between PAPP-A and GAGs (Weyer *et al.*, 2004). In the predicted three-dimensional structure of CCP3 and CCP4, these clusters form a circular patch of approximately 15 Å of basic residues on the surface of CCP3. With two basic residues of CCP4 in the vicinity, the positively charged patch mediates the binding to the negatively charged GAGs. (Weyer *et al.*, 2004)

Evidence of the physiological importance of the surface binding of PAPP-A has been obtained in a study by Conover and colleagues (Conover *et al.*, 2007). Experiments were performed with human peripheral monocytes, which were *in vitro* differentiated and activated to macrophages, and with active macrophages, which were isolated from atherosclerotic lesions of a rabbit model of atherosclerosis. It was shown that activated macrophages bind PAPP-A via GAGs on the cell surface which leads to internalization of PAPP-A by these cells. PAPP-A molecules with a mutated CCP3 domain, however, did not bind to or become internalized by the macrophages. In another study PAPP-A was found to be associated with the membranes of solubilized human trophoblasts of the placenta (Sun *et al.*, 2002). Interestingly, the affinity of the GAG binding site of PAPP-A seems to be higher for heparin than for the GAG on cell surfaces (Weyer *et al.*, 2004).

Lin-Notch repeat modules

In the amino acid sequence of PAPP-A three motifs have been identified which are related to Lin-Notch repeats (LNR) (Kristensen *et al.*, 1994). These motifs had previously been seen in Notch receptors, a group of presumed membrane proteins involved in early tissue differentiation. In Notch receptors three LNR motifs are arranged in tandem in the putative extracellular part. However, in PAPP-A LNR modules 1 and 2 are found within the proteolytic domain (residues 334-360 and 361-393) and LNR 3 is separated from LNR2 by more than 1000 amino acids (residues 1478-1503) (Kristensen *et al.*, 1994). The amino acid residues conserved between the LNR modules of PAPP-A and Notch receptors have been predicted to be involved in calcium coordination based on studies with isolated the Notch 1 LNR1 module (Boldt *et al.*, 2004)

The LNR modules of PAPP-A seem to control the substrate specificity of the proteolytic activity of PAPP-A, which will be discussed in detail in section 2.2. All LNR modules are required for successful PAPP-A mediated cleavage of insulin-like growth factor binding protein (IGFBP)-4, but LNR1 and LNR2 are enough for the cleavage of IGFBP-5 (Boldt *et al.*, 2004). Recent data from Weyer and colleagues (2007) shows that the LNR3 of one PAPP-A subunit interacts with the LNR1 and LNR2 modules of the other PAPP-A subunit in the dimer of PAPP-A subunits. This was confirmed by showning that a C-terminal truncated variant of PAPP-A lacking the LNR3 module and a PAPP-A variant mutated at the active site can form a functional protease able to cleave IGFBP-4 when co-expressed in cells. Moreover, in the structure of the LNR3 module certain charged residues (Asp-1521, Arg-1529, Asp-1530) were found to be especially important for the activity (Weyer *et al.*, 2007).

Laminin G-like module

Using secondary structure prediction and sequence alignment, a protein domain was identified within PAPP-A by Boldt and colleagues (2006) that resembles the laminin G-like (LG) modules. These modules are found, for example, in the α-chain of laminin. This module is

located in the PAPP-A sequence within the first N-terminal 243 residues and before the proteolytic domain. Although the sequence homology with other LG modules is weak, 14 characteristic β-chains were found in the predicted secondary structure which was supported by the existence of the disulfide bond between Cys-64 and Cys-155. The LG module of PAPP-A was not obvious from the exon structure as the large exon 2 of PAPP-A spans most of the LG module and also the first half of the proteolytic domain. However, although the module was shown to fold autonomously, it is likely to be required for the correct folding of the proteolytic domain. The function of the module is yet unknown but, based on the results of Boldt and colleagues (2006), it does not seem to be involved in substrate recognition, cell surface binding or complex formation with proMBP.

Dimerization interface

As previously mentioned, Cys-1130 is responsible for the covalent dimerization of PAPP-A monomers (Overgaard *et al.*, 2003). However, amino acids 1064-1098 seem to possibly create a non-covalent dimerization interface that enables the activity against IGFBP-4 without covalent dimerization (Weyer *et al.*, 2007).

2.1.3 Complexation with proMBP

Eosinophils, important cells of the immune system, produce a highly cationic protein, major basic protein (MBP), which is stored in the granules of eosinophils with other basic proteins. These proteins are highly toxic and are released from eosinophils as a defence against invading microorganisms and parasites. Moreover, MBP also enhances the immune response by activating mast cells and basophils. (Reviewed by Janeway *et al.*, 2005.)

Popken-Harris and colleagues (1998) showed that in developing eosinophils MBP is produced as a precursor form, proMBP. The propiece of 90 amino acid residues is highly acidic and possibly protects developing eosinophils from damage by the toxic MBP during the processing of the uncondensed granules to form mature condensed granules. In mature eosinophils only the mature MBP of 117 amino acid residues was found in the condensed granules.

In another study by Popken-Harris and colleagues (1995) it was noticed that unlike mature MBP, proMBP is not cytotoxic and not able to stimulate basophil histamine release. Moreover, proMBP inhibited these effects of MBP dose-dependently. The cytotoxic actions of MBP may be due to the cationic charge of the protein as it may react with the acidic lipids and cause disordering of the lipid cell membranes of the micro-organisms and parasites. ProMBP inhibits cytotoxicity probably by neutralizing the basic charge of MBP.

The group of Popken-Harris (1995) reported that the size of the proMBP polypeptide predicted from the cDNA sequence is 23.76 kDa. However, they also revealed that the propiece of proMBP is highly and variably glycosylated. Thus, proMBP is electrophoretically extremely heterogeneous with an apparent molecular mass of 30-100 kDa. Overgaard and colleagues (1994a) showed that the total carbobydrate content of proMBP is 38.6%. They also revealed that proMBP contains one N-bound carbohydrate and several O-bound carbohydrate groups as well as at least one O-bound GAG. It is noteworthy that the covalent GAG substitution of proMBP possibly by heparan sulphate at Ser-62 is an unusual modification of soluble proteins.

Glerup and colleagues (Glerup *et al.*, 2005) reported that upon the formation of the hetero-tetrameric complex of PAPP-A and proMBP, the status of six cysteines of proMBP subunits and two cysteines of PAPP-A subunits change. More specifically, in the process three intramolecular disulfide bridges break within proMBP subunits (Cys51/Cys-169, Cys-89/Cys-189, Cys-104/Cys-107). This leads to the formation of two disulphide bridges between the proMBP subunits (Cys-104/Cys-104, Cys-107/Cys-107) and two disulphide bridges between proMBP and PAPP-A subunits (Cys-169 of proMBP / Cys-652 of PAPP-A, Cys-51 or proMBP / Cys-381 of PAPP-A). The total redox potential is maintained because a new bond is formed for every breaking disulfide bond.

It has been noticed in *in vitro* studies that the complexation occurs much more efficiently in the presence rather than in the absence of cells (Sivanandam *et al.*, 2004; Glerup *et al.*, 2007). Co-overexpression of PAPP-A and proMBP in cells or co-culture of cells expressing PAPP-A or proMBP together leads to rapid production of covalent PAPP-A/proMBP complex. On the other hand, incubation of culture medium from PAPP-A producing cells and proMBP producing cells together in the absence of the cells results in very slow complexation (Sivanandam *et al.*, 2004; Glerup *et al.*, 2007).

PAPP-A/proMBP complex shows only very weak binding to the cell surface (Laursen *et al.*, 2002b). Moreover, the process of complexation leads to surface detachment of PAPP-A (Glerup *et al.*, 2007). Heparinase treated PAPP-A/proMBP binds to cells in a similar manner to PAPP-A dimer implying that the GAG of proMBP probably interacts with the GAG binding site of PAPP-A replacing the GAG of the cell surface from the site (Laursen *et al.*, 2002b). It is noteworthy that both the GAG of proMBP and the formation of covalent bonds with PAPP-A are required for the surface detachment of PAPP-A (Glerup *et al.*, 2007).

It has been shown that in *in vitro* conditions in a culture medium without cells and with physiological concentrations of PAPP-A (20 nM) and proMBP (200 nM) the complex formation is greatly enhanced by the addition of micromolar concentrations of reductants such as glutathione, dithiothreitol or β-mercaptoethanol (Glerup *et al.*, 2005). On the other hand, increasing concentrations of NaCl inhibit PAPP-A/proMBP complex formation suggesting that ionic interactions are probably important in the complexation process (Glerup *et al.*, 2007).

2.2 Activity of PAPP-A

2.2.1 IGFBP Substrates

In 1999, PAPP-A as a dimer of PAPP-A subunits was identified as the previously unknown insulin-like growth factor (IGF)-dependent IGFBP-4 protease produced by human fibroblasts in culture medium (international enzyme code EC 3.4.24.79) (Lawrence *et al.*, 1999). Later PAPP-A was also confirmed to be responsible for similar activity in other systems such as follicular fluid of various mammals (Mazerbourg *et al.*, 2001), in human pregnancy serum (Byun *et al.*, 2001), and in culture medium conditioned by human and porcine coronary artery vascular smooth muscle cells (VSMC) (Bayes-Genis *et al.*, 2001b), luteinizing human granulosa cells (Iwashita *et al.*, 1998), cells of mouse osteoblast cell line MC3T3-E1 (Bunn *et al.*, 2004), bovine mammary fibroblast cells (Fleming *et al.*, 2005), human trophoblast and decidualized endometrial stromal cells (Giudice *et al.*, 2002) and human osteoblasts (Qin *et al.*, 2000). In addition to IGFBP-4, PAPP-A has also been shown to

degrade IGFBP-5 (Laursen *et al.*, 2001; Rivera and Fortune, 2003b) and IGFBP-2 (Monget *et al.*, 2003; Gerard *et al.*, 2004; Kumar *et al.*, 2005).

IGFs and IGFBPs

IGF-I and -II are single-chain polypeptides (~7.5 kDa) that function as peptide hormones. They share homology with both themselves and insulin. IGFs are important regulators of various cell functions as they, for example, stimulate protein synthesis, promote migration, enhance proliferation and inhibit apoptosis. Circulating IGFs are primarily produced by the liver. However, IGF expression has also been found locally in most tissues all over the body. IGFs interact with their cell membrane-associated receptors, namely type I and type II IGF receptors. Type I IGF receptor (IGF-R1) is homologous to the insulin receptor and has tyrosine kinase activity. Importantly, IGF-R1 is responsible for IGF-I and IGF-II induced intracellular signalling. Type II IGF receptor, also known as mannose-6-phosphate receptor, is not structurally related to IGF-R1, has no known intracellular signalling actions and possibly just mediates the clearance of IGF-II. (For reviews, see Donnelly and Holly, 1996; Le Roith, 1997; Bayes-Genis *et al.*, 2000; Delafontaine *et al.*, 2004; Holly and Perks, 2006; Beattie *et al.*, 2008.)

The interaction of IGFs with the receptors is regulated by six IGFBPs (IGFBP1-6) of the size of 22-30 kDa, which bind IGFs with high affinity, even higher than that of IGF I receptor. The affinity constants for this interaction are shown in Table 1, a lower K_D value reflecting higher affinity. The expression of different IGFBPs is tissue-specific and developmental stage-specific. Thus, the concentrations of IGFBPs vary in different body compartments. The IGFBPs function as extra-cellular storage pools for IGFs by increasing the half-life of IGFs and modulating the availability of IGFs in tissues. In the circulation more than 95% of the IGFs is bound with IGFBP-3 in a complex that also includes an additional large protein, acid-labile subunit. When IGF leaves this complex, it may enter tissues in other complexes with other IGFBPs. In addition to IGF binding, the IGFBPs may also have IGF-independent functions such as stimulation of the VSMC migration by IGFBP-5. (For reviews, see Donnelly and Holly, 1996; Le Roith, 1997; Bayes-Genis *et al.*, 2000; Delafontaine *et al.*, 2004; Holly and Perks, 2006; Beattie *et al.*, 2008.)

Table 1. Equilibrium affinity constants of the interactions of the receptor, binding proteins and binding protein fragments with IGF-I and IGF-II (K_D / 10^{-9} M).

	IGF-I	**IGF-II**	**Reference**
IGF-R1	4.45	23	Beattie *et al.* 2008
IGFBP-1	0.8	0.33	Laursen *et al.* 2007
IGFBP-2	0.36	0.11	Laursen *et al.* 2007
IGFBP-3	0.32	0.16	Laursen *et al.* 2007
IGFBP-4	0.27	0.18	Laursen *et al.* 2007
IGFBP-5	0.48	0.23	Laursen *et al.* 2007
IGFBP-6	2.6	0.14	Laursen *et al.* 2007
IGFBP-4 fragments			
N-terminal	4.5	0.2	Beattie *et al.* 2008
C-terminal	ND[a]	216	Beattie *et al.* 2008
IGFBP-5 fragments			
N-terminal	49	7	Beattie *et al.* 2008
C-terminal	21	183	Beattie *et al.* 2008

[a] K_D not determined because of too slow reaction

The IGF binding of IGFBPs is mediated by conserved amino acid residues of the N-terminal part and the C-terminal part of the binding protein (Fig. 2). For example, an N-terminal sequence spanning the area between Lys-67 and Cys-79 in IGFBP-4 seems imperative for IGF binding. In the C-terminal part of IGFBP-4 the sequence located between the residues Cys-205 and Val-214 including the conserved sequence of Cys-Tyr-Cys-Val-Asp is also important by facilitating IGF binding (Qin *et al.*, 1998; Beattie *et al.*, 2008).

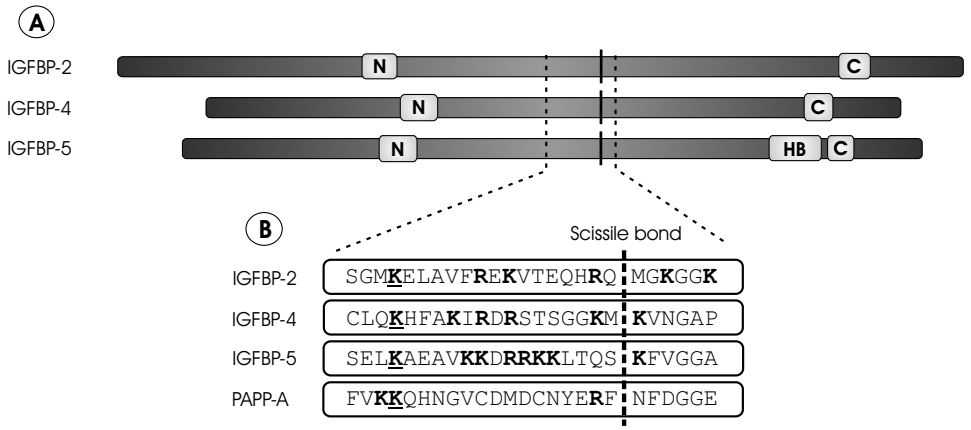

Figure 2. A) The general structure of IGFBP-2, -4 and -5 with the areas involved in the IGF binding indicated (N, N-terminal IGF-binding sequence; C, C-terminal IGF-binding sequence; HB, highly basic heparin binding sequence). **B)** The amino acid sequences of the sites where PAPP-A cleaves IGFBP-2, -4 and -5 and the autocleavage site of PAPP-A. The basic residues are in bold and the lysine located 16 residues N-terminal from the scissile site of bond is underlined.

Beattie and colleagues (2008) have studied the interaction of IGFBP-5 with heparin. IGFBP-5 is able to interact with cell surface and extracellular matrix proteoglycans by binding to heparan sulphate moieties although IGFBP-2 and IGFBP-4 do not bind heparan sulphate. The amino acid residues mediating this interaction are located in the C-terminal part of the protein at the highly basic heparin binding sequence between Arg-201 and Arg-218 (Fig. 2). Interestingly, this sequence also appears to be important for high affinity binding of IGFs with IGFBP-5 even though it is not found in IGFBP-4. Moreover, in *in vitro* studies by Beattie and colleagues (Beattie *et al.*, 2008), IGFs were shown to mediate the detachment of IGFBP-5 from heparin, which is in line with the IGFBP-5 release from the cell surface by IGF.

Proteolysis of IGFBPs by PAPP-A

Signalling through the IGF axis is regulated by proteases which degrade IGFBPs. In all systems studied PAPP-A seems be the predominant if not the only IGFBP-4 protease (Overgaard *et al.*, 2000; Rivera and Fortune, 2003a). However, of the substrates of PAPP-A, there are other proteases at least for IGFBP-5 (Bayes-Genis *et al.*, 2001b). As previously stated, the PAPP-A dimer binds to cell surfaces through a GAG found on the cell surface. Cell surface bound PAPP-A is active and hence the GAG binding directs the PAPP-A mediated release of free IGFs to the vicinity of the IGF receptors present on the cell surface (Fig. 3) (Laursen *et al.*, 2002a).

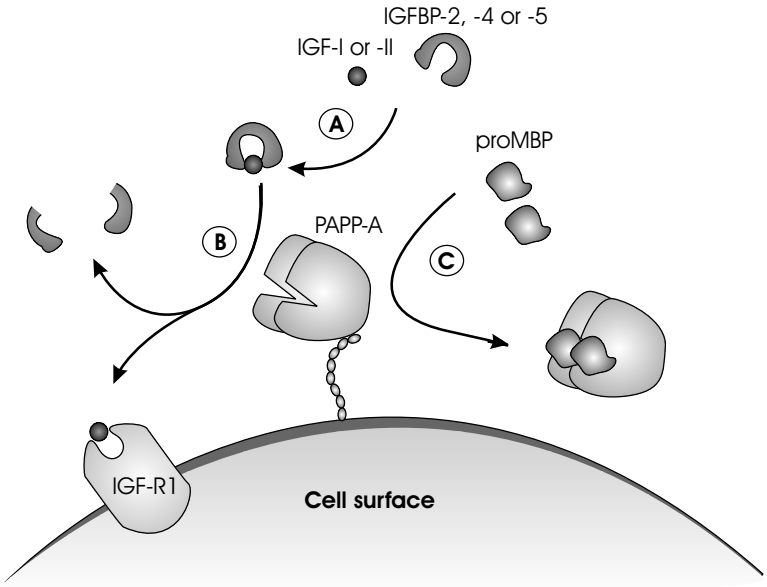

Figure 3. The role of PAPP-A in the IGF signalling axis. **A)** The free IGF-I and -II molecules are bound by IGFBP-2, -4 and -5. **B)** PAPP-A dimer attached to a cell surface glycosaminoglycan cleaves the IGFBP and thus releases IGF to interact with type I IGF receptor (IGF-R1). **C)** ProMBP subunits form a covalent complex with the PAPP-A dimer which leads to inactivation of PAPP-A and detachment from the surface.

The fragments of cleaved IGFBP-4 and -5 also have some affinity for IGFs that limits, to some extent, the interaction of IGFs with their receptors (Laursen *et al.*, 2007). The first findings on this matter considered the affinity of IGFBP-4 fragments, which seemed to be higher for IGF-II than IGF-I (Smith *et al.*, 2001). Later more detailed studies followed reporting the affinity values between IGF-I and IGF-II and the cleavage products of both IGFBP-4 and IGFBP-5 and confirmed these findings (Table 1) (Laursen *et al.*, 2007). Interestingly, unlike what is seen with IGFBP-4, the degradation fragments of IGFBP-5 appear to interact cooperatively with IGFs. This leads to more pronounced inhibition of receptor activation with the combination of N-terminal and C-terminal fragments than with one of the fragments alone (Laursen *et al.*, 2007).

PAPP-A cleaves IGFBP-4 within the variable mid-region between Met-135 and Lys-136 (Fig. 2) (Chelius *et al.*, 2000; Qin *et al.*, 2000; Laursen *et al.*, 2002a). This cleavage site seems to be conserved between mammals (Chelius *et al.*, 2000). Mutations of basic residues at or near the cleavage site of IGFBP-4 (Lys-134, Lys-136) inhibit degradation (Bunn *et al.*, 2004). However, basic residues distant from the scissile bond of IGFBP-4 (Lys-120, Arg-126, Arg-128) are also important for cleavage (Laursen *et al.*, 2002a). The efficient degradation of an IGFBP-4 derived peptide requires at least 16-17 residues on the N-terminal side of the cleavage site (Laursen *et al.*, 2002a). Interaction between IGF (IGF-I or -II) and IGFBP-4 is essential for degradation activity (Qin *et al.*, 2000; Gyrup and Oxvig, 2007). Very slow IGFBP-4 degradation can be seen in the absence of IGFs but the activity of the protease is significantly enhanced by the presence of IGF-I or IGF-II (Overgaard *et al.*, 2000; Boldt *et al.*, 2001; Byun *et al.*, 2001; Laursen *et al.*, 2001; Bunn *et al.*, 2004; Gyrup and Oxvig, 2007). Importantly, it has been shown that IGF does not function as a cofactor of PAPP-A but rather binds to IGFBP-4 making it a better substrate for PAPP-A (Laursen *et al.*, 2002a).

IGFBP-5 is cleaved by PAPP-A between Ser-143 and Lys-144 (Fig. 2) (Laursen *et al.*, 2001; Laursen *et al.*, 2002a). In IGFBP-5, basic residues are found in similar positions relative to the scissile bond as in IGFBP-4 (Laursen *et al.*, 2002a). However, only Lys-128 located 16 residues from the scissile bond is important for cleavage (Laursen *et al.*, 2002a). The cleavage of IGFBP-5 is not dependent on IGFs but is slightly inhibited by them (Gyrup and Oxvig, 2007). In the presence of IGF-II the cleavage rates of IGFBP-4 and IGFBP-5 by PAPP-A are similar, while IGFBP-5 without IGFs seems to be the best substrate for PAPP-A (Laursen *et al.*, 2001; Gyrup and Oxvig, 2007).

PAPP-A cleaves IGFBP-2 between Gln-165 and Met-166 (Fig. 2) (Monget *et al.*, 2003; Gerard *et al.*, 2004). The degradation is dependent on IGF dose and more IGFs are needed for efficient cleavage than with IGFBP-4 (Monget *et al.*, 2003; Gyrup and Oxvig, 2007). Thus, similarly as IGFBP-4, IGFBP-2 probably undergoes conformational change upon IGF binding which makes it a better substrate for PAPP-A. However, IGFBP-2 is less sensitive to proteolysis by PAPP-A than IGFBP-4 and the cleavage reaction is notably slower with IGFBP-2 (Monget *et al.*, 2003; Gyrup and Oxvig, 2007). The effect of the basic residues of IGFBP-2 on cleavage efficiency of PAPP-A has not been studied. However, Lys located 16 residues from the scissile bond important in the degradation of IGFBP-4 and IGFBP-5 is also found in IGFBP-2 (Fig. 2).

2.2.2 Other substrates

In addition to degradation of IGFBP-2, -4 and -5, PAPP-A has been shown to be capable of autocleavage. Boldt and colleagues (2001) noticed that cultivation of cells producing recombinant wild-type PAPP-A in serum-free medium resulted in pronounced (>90%) autocleavage of PAPP-A. The extent of autocleavage was associated with the length of the incubation. However, in serum-containing medium the autolysis was only limited (< 5%). It was shown that due to autocleavage, the PAPP-A monomer of 200 kDa was turned into a 50 kDa N-terminal fragment and a 150 kDa C-terminal fragment. The cleavage site was found between Phe-386 and Asn-387 at the C-terminal end of the LNR2 within the proteolytic domain. Thus, the cleavage site is in close vicinity to the Cys-381 involved in the complexation with proMBP. Near the cleavage sites of IGFBP-4 and -5 and the autocleavage site of PAPP-A a basic residue is found in all only at position -16 which seems to be important for cleavage activity (Fig. 2) (Laursen *et al.*, 2002a).

After PAPP-A finding of 1974, other activities for this protein have also been suggested including degradation of other proteins or inhibition of other proteases. However, these conclusions arose probably due to inefficient purification of PAPP-A, complexation with proMBP that was still unknown and the reactivity of polyclonal anti-PAPP-A sera with proMBP, use of conditions divergent from the physiological situation or inaccurate prediction of function from structure. For example, it has been shown that PAPP-A derived from pregnancy serum can inhibit human leukocyte elastase and human cathepsin G (Sinosich and Zakher, 1991; Oxvig *et al.*, 1994b). However, this is seen only at an ionic strength significantly lower than the physiological concentration and the inhibition is most likely due to interactions with the GAG moiety of proMBP (Oxvig *et al.*, 1994b).

Another example is linked to the complement cascade of the immune system. Bischof and colleagues (1984) showed that PAPP-A inhibited complement-induced haemolysis and that this phenomenon was a result of direct interaction with complement C3. However, in this study PAPP-A was purified from third trimester pregnancy heparin plasma or with heparin

affinity column from ethylenediaminetetraacetic acid (EDTA) anticoagulated plasma. This provided the possibility of contamination with heparin, a potent complement inhibitor. In the same study experiments were also conducted with unpurified PAPP-A in pregnancy plasma in which labelled C3 was found to bind to PAPP-A that was captured by rabbit anti-PAPP-A antibodies. However, this polyclonal PAPP-A antisera was produced with PAPP-A purified from pregnancy serum i.e. PAPP-A/proMBP complex used as the antigen and probably also had anti-proMBP reactivity. Another group has later shown that complexes form in serum between proMBP and C3-derived C3dg (Christiansen *et al.*, 2000). Thus, in the study of Bischof and colleagues interaction between C3 and proMBP could have been possible instead of interaction between PAPP-A and C3.

There have been some suggestions that PAPP-A might modulate immune reactions. However, the evidence published on this matter in international peer-reviewed journals is scarce and contradictory. For instance, PAPP-A has been suggested to have an effect on the spontaneous proliferation of peripheral blood polymorphonuclear cells. However, the effect seems to change from suppressive to stimulation depending on the dose of PAPP-A as well as the gender and age of the donors or the phase of pregnancy. (Reviewed by Zhabin *et al.*, 2003.)

Despite the fact that the sequence of the active site of PAPP-A is almost identical with that of matrix metalloproteinases, to date there is no published evidence that PAPP-A would degrade structural matrix proteins.

The enzyme activities of the proteins that resemble PAPP-A have also been studied. Ulilysin shows similar pattern of IGF-modulated proteolysis of IGFBP-4 suggesting that conserved structural features in the proteolytic domain are responsible for substrate recognition (Gyrup and Oxvig, 2007). However, in addition to IGFBP-2, -4 and -5, ulilysin is also able to cleave various other substrates such as IGFBP-3, IGFBP-6, insulin β chain, azoalbumin, gelatine, actin and elastin and itself for autoactivation of the inactive proform proulilysin (Tallant *et al.*, 2006; Tallant *et al.*, 2007). The splice variant of PAPP-A found in mice, PAPP-Ai, degrades IGFBP-5 IGF-independently in a similar manner to PAPP-A, but the IGF-dependent IGFBP-4 degradation is highly reduced, possibly due to the positive charge of the insert (Soe *et al.*, 2002; Bunn *et al.*, 2004). PAPP-A2, which does not bind to cell surfaces (Laursen *et al.*, 2002b) due to three basic residues of the CCP3 module replaced by acidic residues (Weyer *et al.*, 2004), degrades IGFBP-5 IGF-independently similar to PAPP-A but does not cleave IGFBP-4 even in the presence of IGF-II (Overgaard *et al.*, 2001).

2.2.3 Activity regulation

Inhibition by proMBP

ProMBP has been identified as an inhibitor of the proteolytic activity of PAPP-A (Overgaard *et al.*, 2000; Chen *et al.*, 2002). Inhibition of the proteolytic activity of PAPP-A by proMBP requires covalent bonding, as proMBP which lacks Cys-51 and Cys-169, the residues responsible for PAPP-A – proMBP intermolecular disulphide bridges, does not cause inhibition (Overgaard *et al.*, 2004). Apparently, this is the only known example of a case in which inhibition of enzyme activity is mediated through covalent disulfide bridges between the enzyme and the inhibitor (Glerup *et al.*, 2005).

The actual mechanism of the inhibition of PAPP-A activity by proMBP is still unknown. The complexation may abrogate the access of the substrate to the active site of PAPP-A or induce a conformational change leading to inactivation (Overgaard *et al.*, 2004). Moreover, substrate-like interactions of the basic portion of proMBP may interfere with the binding of the substrate to PAPP-A (Laursen *et al.*, 2002a). Notably, one of the residues of PAPP-A involved in the complexation with proMBP, Cys-381, lies within the proteolytic domain of PAPP-A (Overgaard *et al.*, 2003). However, results of the study by Overgaard and colleagues (2004) imply that the cysteine residues of proMBP do not interact directly with the zinc atom of the active site of PAPP-A.

Overgaard and colleagues (2000) found that in late pregnancy serum the vast majority of PAPP-A is present in the complexed inactive form. The activity of recombinant PAPP-A dimer is 100-fold higher than that of PAPP-A purified from pregnancy serum or the activity in unpurified late pregnancy serum. However, in pregnancy serum some residual activity is still detectable and it is assumed to be caused by a minor fraction (<1%) of uncomplexed PAPP-A. Interestingly, pregnancy serum derived PAPP-A/proMBP complex separated from uncomplexed PAPP-A still shows some very low activity. This is possibly due to the presence of a minor subpopulation of partly inhibited PAPP-A in a 2:1 complex with proMBP or the incomplete inhibitory effect of proMBP.

A protein activator of PAPP-A that forms a covalent complex with PAPP-A has been suggested in one study by Sivanandam and colleagues (2004b). Human skin fibroblasts and human osteoblasts were treated with a phorbol ester tumor promoter acting as protein kinase C activator, phorbol 12-myristate-13-acetate (PMA). This reduced IGFBP-4 proteolysis without significantly decreasing the PAPP-A level in the culture medium. However, production of proMBP was not induced. SDS-PAGE analyses implied that PAPP-A was expressed in normal conditions by fibroblasts as a 470 kDa active complex with a protein other than proMBP. It was suggested that PMA reduced the synthesis or increased the degradation of this protein and thus induced conversion of PAPP-A to a less active ~400 kDa form. However, to date neither the members of this group nor other groups have published anything further on this mysterious protein activator.

Common protease inhibitors and optimal conditions

Of the common inhibitors of proteases the activity of PAPP-A against IGFBPs has been shown to be inhibited only by 1,10-phenanthroline (a specific chelator of zinc ions) and EDTA (a chelator of divalent cations) (Chandrasekher *et al.*, 1995; Besnard *et al.*, 1996; Besnard *et al.*, 1997; Mazerbourg *et al.*, 2000; Overgaard *et al.*, 2000; Mazerbourg *et al.*, 2001; Rivera and Fortune, 2001; Rivera and Fortune, 2003b; Gerard *et al.*, 2004). The activity of PAPP-A is not inhibited by phenylmethylsulfonyl fluoride (an inhibitor of serine proteases), pepstatin A (an inhibitor of aspartic proteases), E64 (a highly specific inhibitor of cysteine proteases), aprotinin (a serine protease inhibitor), benzamide (a serine protease inhibitor), phosphoramidon (an inhibitor of endopeptidases), bestatin (an inhibitor of aminopeptidases), tissue inhibitor of metalloproteinase-1 and -2 (natural inhibitors of matrix metalloproteinases), B-2116 (a synthetic inhibitor of matrix metalloproteinases) and leupeptin (an inhibitor of trypsin-like serine and some cysteine proteases) (Chandrasekher *et al.*, 1995; Besnard *et al.*, 1996; Besnard *et al.*, 1997; Mazerbourg *et al.*, 2000; Overgaard *et al.*, 2000; Mazerbourg *et al.*, 2001; Rivera and Fortune, 2003b; Gerard *et al.*, 2004).

After inhibition caused by EDTA, activity was restored by the addition of calcium chloride and zinc chloride while zinc chloride alone was sufficient to restore activity after 1,10-phenanthroline-induced inhibition. Thus, before any detailed structural data was available, PAPP-A was classified as a calcium- and zinc-dependent metalloprotease (Besnard *et al.*, 1996). Interestingly, only the proteolytic activity of PAPP-A against IGFBP-4 is dependent on calcium while calcium is not required for the degradation of IGFBP-5 (Besnard *et al.*, 1996; Besnard *et al.*, 1997; Boldt *et al.*, 2004). Activity increases in *in vitro* conditions with increasing Ca^{2+} concentration until the concentration of 1 mM is reached, but even very low trace amounts (<6 µM) are enough for detectable activity (Boldt *et al.*, 2004). The dependence on calcium may be related to the binding of calcium by the LNR3 motif as this motif is required for IGFBP-4 degradation but not for IGFBP-5 degradation (Boldt *et al.*, 2004).

Even though as a protease PAPP-A is dependent on zinc, it was unexpectedly shown that high zinc chloride concentration completely inhibits activity (Besnard *et al.*, 1996; Besnard *et al.*, 1997). When optimal conditions for PAPP-A activity measurements were determined by Laursen and colleagues (2002a) with wild-type recombinant protein, it was noticed that an added Zn^{2+} concentration as low as 5 µM inhibited activity to some extent and 50 µM almost completely blocked the degradation reaction. As a possible explanation for this phenomenon it was suggested that high zinc concentration leads to the binding of another zinc ion close to the active site, which has been seen with thermolysin.

Surprisingly, NaCl at physiological concentration was also found deleterious for the degrading activity of PAPP-A in *in vitro* experiments (Laursen *et al.*, 2002b; Gyrup *et al.*, 2007). A NaCl concentration of 100 mM led to a 65% decrease in activity compared to the reaction without additional NaCl (Laursen *et al.*, 2002a). This implies that the substrate binding possibly depends on interactions between charged residues (Gyrup *et al.*, 2007).

The pH optimum of PAPP-A in natural materials has been shown to lie between 7.4-7.6 (Mazerbourg *et al.*, 2000; Mazerbourg *et al.*, 2001; Gerard *et al.*, 2004). In *in vitro* studies with recombinant human PAPP-A, Laursen and colleagues (2002a) found the pH optimum for activity at pH 8. They also showed that when the pH was decreased from neutral to acidic, the activity of PAPP-A decreased quickly leading to complete abrogation of the activity at pH 6. Protonation of His residues and subsequent loss of zinc ion at the active site was suggested as a possible explanation for this.

Inhibition by IGFBPs

As the cleavage of IGFBP-4 is dependent on the binding of IGF, other IGFBPs seem to be able to inhibit the activity of PAPP-A against IGFBP-4 indirectly by sequestering the available IGFs. Accordingly, in a study by Fowlkes and colleagues (1997), IGFBP-3, -5 and -6 inhibited IGFBP-4 degradation in MC3T3-E1 murine osteoblast conditioned cell culture medium. The inhibitory effect was reversed by the addition of IGFs. As IGFBP-5 without IGFs is the best substrate for PAPP-A and the degradation of IGFBP-4 is IGF-dependent, in low concentration of IGFs PAPP-A preferably cleaves IGFBP-5 first and IGFBP-4 degradation takes place only after the local IGF concentration is high enough (Laursen *et al.*, 2007). Being the worst substrate, IGFBP-2 is degraded last (Smith *et al.*, 2001).

Inhibition by heparin binding peptides

It has been noticed that certain basic heparin binding peptides inhibit the activity of PAPP-A (Fowlkes *et al.*, 1997; Mazerbourg *et al.*, 2000; Mazerbourg *et al.*, 2001). Synthetic peptides, which contain the highly basic (calculated pI >10) C-terminal regions of IGFBP-3, -5 and -6 including the heparin-binding sequence XBBBXXBX, inhibit the IGFBP-4 degrading activity of PAPP-A. However, the homologous yet neutral (calculated pI 6.9) IGFBP-4 peptide does not cause inhibition. Heparin addition reverses such inhibitory effects. Other studies have shown that heparin-binding domain peptides derived from IGFBP-5, IGFBP-3, vitronectin or heparin/heparan sulfate –interacting protein (HIP) inhibit the IGFBP-2 degradation by PAPP-A (Mazerbourg *et al.*, 2003; Gerard *et al.*, 2004). IGFBP-5 derived heparin binding peptide P5 inhibited dose-dependently the cleavage of IGFBP-2 by PAPP-A and showed high affinity to PAPP-A (K_D: 3.85 x 10^{-11}) (Monget *et al.*, 2003). Moreover, a peptide containing the highly basic heparin-binding consensus sequence of C-terminal IGFBP-3 inhibited PAPP-A activity against IGFBP-4 as efficiently as EDTA (Bunn *et al.*, 2004). The inhibition mechanism possibly involves competition to prevent substrate binding (Laursen *et al.*, 2002a).

Inhibition by antibodies

Antibodies may also inhibit the activity of PAPP-A. Polyclonal antibodies created against the PAPP-A/proMBP complex have been shown to inhibit the cleavage of IGFBP-4 by human recombinant PAPP-A (Overgaard *et al.*, 2000). In fact, such polyclonal antibodies were widely used when PAPP-A was identified as the IGFBP protease in several systems by immunodepletion and immunoinhibition (Qin *et al.*, 2000; Bayes-Genis *et al.*, 2001b; Byun *et al.*, 2001; Mazerbourg *et al.*, 2001; Giudice *et al.*, 2002; Bunn *et al.*, 2004; Fleming *et al.*, 2005).

Furthermore, monoclonal antibodies have also been developed to specifically abrogate the activity of PAPP-A. An inhibitory antibody that presumably binds the active site of PAPP-A was created by Chen and colleagues (2007). The phage display technique was utilized with wild-type biotinylated PAPP-A used for positive selection and the inactive PAPP-A mutant E483A for negative selection. The IgG antibody created from the Fab-phage fusion inhibited the degradation of an IGFBP-4 derived peptide by PAPP-A with an inhibitory constant K_i of 0.7 nM. Mikkelsen and colleagues (2008) generated a monoclonal phage-derived single-chain variable fragment antibody which inhibits the activity of PAPP-A against IGFBP-4 but not against IGFBP-2 or IGFBP-5. A C-terminal fragment (amino acid residues 1133-1547) of PAPP-A was used for screening. The selective inhibition was achieved through antibody binding to the LNR3 module. The presence of calcium was crucial for the binding of the antibody, which supports the assumption that this module binds calcium. The antibody inhibited the activity of PAPP-A against native IGFBP-4 with an inhibitory constant K_i of 1.2 nM while the cleavage of an IGFBP-4 derived peptide was not inhibited.

2.3 Expression and physiological effects of PAPP-A

In early studies the expression of PAPP-A in different tissues was investigated with polyclonal antibodies that were generated using PAPP-A purified from pregnancy serum as the antigen (reviewed by Rosen, 1986). However, after it was realized that PAPP-A in late pregnancy serum is a complex of PAPP-A and proMBP, it was confirmed that such polyclonal antibodies also bind proMBP and MBP (Oxvig *et al.*, 1993). Moreover, cross-

reactivities for other molecules such as haptoglobin were also reported for certain polyclonal antibody products that were used in the early studies (Bueler and Bersinger, 1989). Thus, the results of the early studies are not reliable because where PAPP-A was found there could have actually been other molecules containing proMBP or MBP or even other non-related cross-reactants. More reliable results of the presence and expression of PAPP-A in certain tissues have been obtained with studies analysing the expression of PAPP-A mRNA, the activity of PAPP-A (which seems to be PAPP-A specific in the case of IGFBP-4 degrading activity) and immunoreactivity with antibodies binding only to the PAPP-A subunit.

In a study by Overgaard and colleagues (1999) the expression of PAPP-A and proMBP mRNA was detected by a reverse transcription polymerase chain reaction (RT-PCR) method in all tested human tissues including female reproductive tissues (ovary, tuba, uterine, endometrium, and myometrium from postmenopausal women) and in nonreproductive tissues (kidney, colon, prostate, prostate carcinoma, bone marrow cells, breast, and breast carcinoma). However, the expression of both proteins was at least 200-fold higher in late pregnancy placenta than in the other tissues studied. Furthermore, in first trimester placenta the expression was significantly lower than in late pregnancy placenta. PAPP-A mRNA expression has also been reported in the human brain (Haaning *et al.*, 1996). In mice PAPP-A mRNA expression was detected by Hourvitz and colleagues (2002) by the RT-PCR method especially in testis, kidney, breast and embryo tissues but also in brain, heart, spleen, skin, muscle, gastrointestinal tract, ovary prostate and uterus tissues. PAPP-A mRNA was not found in the murine thymus, liver, lung and adrenal gland.

The expression of PAPP-A is regulated differently in different cells and also in the cells of the same type from individuals of different ages. For instance, PAPP-A expression in smooth muscle cells is higher in adult rats than in neonatal rats (Smith *et al.*, 2001). However, similarities are also naturally seen. For instance, tumor necrosis factor-α (TNFα) and interleukin (IL)-1β seem to stimulate PAPP-A expression in many cell types studied (Conover *et al.*, 2004b; Resch *et al.*, 2004; Conover *et al.*, 2006; Resch *et al.*, 2006a; Conover *et al.*, 2008a). The effects of various agents on PAPP-A expression in different cell types are summarized in Table 2.

Some insight into the physiological role of PAPP-A *in vivo* have been provided by studies investigating mice with knocked-out PAPP-A genes (PAPP-A KO). Conover and colleagues (2004a) reported that homozygous PAPP-A KO mice are viable but only 60% of the size of wild-type mice at birth and they remain 40% smaller during postnatal development. Thus, PAPP-A seems to be an essential growth regulator. Dwarfism is proportional, the organ-to-body weight ratios are normal. However, the PAPP-A KO mice are otherwise normal and fertile. Mice heterozygous for PAPP-A KO do not differ in size from wild-type mice (Conover *et al.*, 2004a). The phenotype of the homozygous PAPP-A KO mouse is strikingly similar to the IGF-II KO mouse (Conover *et al.*, 2004a). Interestingly, a growth deficit, even though not as severe as seen in PAPP-A KO mice, also affects homozygous IGFBP-4 KO mice (Ning *et al.*, 2008). However, this growth-deficit is completely restored in double IGFBP-4/PAPPA KO mice.

Table 2. The effect of various agents on PAPP-A expression by cells in cell culture conditions.

Agent		Stimulation[a,b]	Suppression[a,b]	No effect[a,b]	References
BFGF	basic fibroblast growth factor	-	-	OB, dermal FB	Conover *et al.* 2004b, Resch *et al.* 2004
8-Br-cAMP	8-bromo cyclic adenosine monophosphate	Choriocarcinoma cell line JAR	-	-	Haaning *et al.* 1996
Bikunin	-	-	Ovarian cancer cell line HRA	-	Suzuki *et al.* 2003
BMP-2	bone morphogenetic protein-2	OB	-	Coronary artery EC	Jadlowiec *et al.* 2005, Conover *et al.* 2008a
D3	1,23-dihydroxyvitamin D_3	-	-	OB	Conover *et al.* 2004b
dB-cAMP	dibutyryl cyclic adenosine monophosphate	Glioblastoma cell line U87MG	-	-	Moreno *et al.* 2006
DM	dexamethasone	-	-	OB	Conover *et al.* 2004b
βE2	β-estradiol	-	-	OB	Conover *et al.* 2004b
EGF	epidermal growth factor	-	-	Coronary artery EC, OB, dermal FB	Conover *et al.* 2004b, Resch *et al.* 2004, Conover *et al.* 2008a
FGF	fibroblast growth factor	-	-	Coronary artery EC	Conover *et al.* 2008a
Forskolin	-	OB	-	-	Conover *et al.* 2004b
FSH	follicle stimulating hormone	Luteinizing GC, follicle membrane GC (rat)	-	-	Iwashita *et al.* 1998, Matsui *et al.* 2004
IFNγ	interferon-γ	-	Dermal FB	-	Resch *et al.* 2004
IGF-I	insulin-like growth factor-I	-	-	Coronary artery EC, OB	Conover *et al.* 2004b, Conover *et al.* 2008a
IGF-II	insulin-like growth factor-II	-	-	OB	Conover *et al.* 2004b
IL-1β	interleukin-1β	Coronary artery SMC, coronary artery EC, OB, dermal FB	-	Peripheral blood MC, acute monocyteic leukaemia cell line THP-1	Conover *et al.* 2004b, Resch *et al.* 2004, Conover *et al.* 2006, Resch *et al.* 2006a, Conover *et al.* 2007, Conover *et al.* 2008a
IL-4	interleukin-4	OB	-	-	Conover *et al.* 2004b
IL-6	interleukin-6	Coronary artery SMC	-	Coronary artery EC, OB	Conover *et al.* 2004b, Conover *et al.* 2008a
IL-13	interleukin-13	-	-	OB	Conover *et al.* 2004b
Insulin	-	-	-	Coronary artery EC	Conover *et al.* 2008a
LDL	low-density lipoprotein	-	-	Coronary artery EC, coronary artery SMC	Conover *et al.* 2008a
MCSF	macrophage colony stimulating factor	-	-	Acute monocyteic leukaemia cell line THP-1, peripheral blood MC	Conover *et al.* 2007
OSM	oncostatin M	-	-	OB	Conover *et al.* 2004b
oxLDL	oxidised low-density lipoprotein	-	-	Coronary artery EC, coronary artery SMC	Conover *et al.* 2008a
PDGF	platelet-derived growth factor	-	-	Dermal FB	Resch *et al.* 2004
PG E2	prostaglandin E_2	OB	-	-	Conover *et al.* 2004b

(continued from previous page)

Agent		Stimulation[a,b]	Suppression[a,b]	No effect[a,b]	References
PMA	phorbol myristate acetate	-	-	Peripheral blood MC, acute monocyteic leukaemia cell line THP-1	Conover *et al.* 2007
PMSG	pregnant mare serum gonadotropin	Follicle membrane GC (rat, mouse), luteinizing GC (mouse)	-	Cumulus GC (mouse, rat)	Hourvitz *et al.* 2002, Matsui *et al.* 2004
PTH	parathyroid hormone	-	-	OB	Conover *et al.* 2004b
TGFβ	transforming growth factor-β	OB	-	Coronary artery EC	Ortiz *et al.* 2003, Conover *et al.* 2004b, Conover *et al.* 2008a
TNFα	tumor necrosis factor-α	Coronary artery SMC, coronary artery EC, OB, dermal FB	-	-	Conover *et al.* 2004b, Resch *et al.* 2004, Conover *et al.* 2006, Resch *et al.* 2006a, Conover *et al.* 2008a

[a]All cells were human derived unless otherwise stated

[b]Abbreviations: EC, endothelial cells; FB, fibroblasts; GC, granulosa cells; MC, monocytes; OB, osteoblasts; SMC, smooth muscle cells

Fibroblasts derived from PAPP-A deficient mouse embryos completely lack IGFBP-4 proteolytic activity, confirming PAPP-A as the IGFBP-4 protease in this system (Conover *et al.*, 2004a). Moreover, there is significantly more IGFBP-4 in adult serum of PAPP-A KO homozygote mice than in wild-type littermates (Ning *et al.*, 2008).

In addition to dwarfism, the genetic deletion of PAPP-A in mice leads to a 30-40% increase in lifespan (Conover and Bale, 2007). Fasting glucose and insulin, food intake or energy expenditure of aged, 18-month-old mice do not differ significantly between PAPP-A KO and wild-type mice when the smaller body weight of PAPP-A KO mice is taken into account (Conover *et al.*, 2008b). Thus, the 'rate of living' or an 'altered glucose-insulin system' do not seem to be valid explanations for the longevity of PAPP-A KO mice.

The function of PAPP-A in many systems is not yet fully understood. However, the role of PAPP-A seems to be related to the enhancement of the activities of IGF by degradation of IGFBPs (Byun *et al.*, 2001; Resch *et al.*, 2004; Qin *et al.*, 2006b). What is known about PAPP-A and its expression and role in certain physiological and pathological processes will be discussed in the following sections.

2.3.1 Pregnancy

As previously stated, PAPP-A was first found in 1974 in the third-trimester plasma of pregnant women (Lin *et al.*, 1974). In the late 1970s and early 1980s PAPP-A was localized with immunohistochemical techniques in the syncytiotrophoblasts of the placenta and also in the decidua of the uterus (Fig. 4) (reviewed by Rosen, 1986). However, these early studies may have been inaccurate due to the use of polyclonal PAPP-A antibodies that were also reactive with proMBP and possibly some other proteins (Bueler and Bersinger, 1989; Oxvig *et al.*, 1993). Importantly, proMBP is also expressed in the placenta (Bonno *et al.*, 1994) and it is found in serum throughout pregnancy in 4- to 10-fold molar excess compared to PAPP-A because proMBP is found also in other protein complexes (Oxvig *et al.*, 1995).

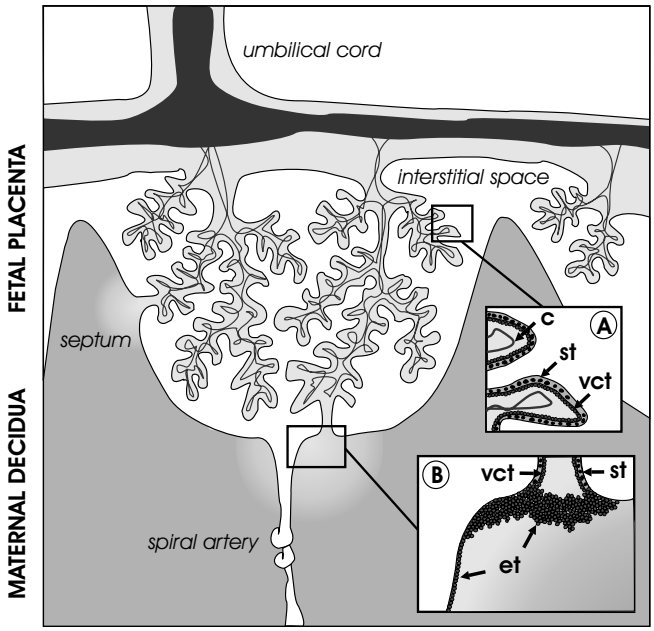

Figure 4. The structure of the placenta. **A)** The chorionic villi float in the interstitial space filled with maternal blood from which nutrients and oxygen are transferred through the syncytiotrophoblast (st) and villous cytotrophoblast (vct) layers to the capillaries (c). The capillaries transfer the nutrients and oxygen to the fetus through the umbilical cord. **B)** The anchoring villi are attached to the maternal decidua by extravillous trophoblasts (et) which also invade and transform the maternal uterine spiral arteries to enable and increase the flow of maternal blood to the interstitial space. PAPP-A protein has been found in the placenta in the septa, acnchoring villi and the syncytiotrophoblasts of chorionic villi while PAPP-A mRNA has been found in the syncytiotrophoblasts and in the extravillous trophoblasts (Bonno *et al.*, 1994).

With mRNA expression studies it was confirmed, without antibody specificity doubts, that in human tissues PAPP-A expression is the highest in the placenta (Overgaard *et al.*, 1999). Moreover, PAPP-A mRNA expression in total placental extracts increases during the course of pregnancy (Guibourdenche *et al.*, 2003). With immunohistochemical staining using PAPP-A specific antibodies that do not bind proMBP, PAPP-A protein was found to localize within the placenta in the placental septa formed of maternal decidua, in the anchoring villi, and in the syncytiotrophoblasts of the chorionic villi (Fig. 2) (Bonno *et al.*, 1994). With *in situ* hybridization PAPP-A mRNA expression was detected in the syncytiotrophoblasts and the placental X cells, i.e. the extravillous trophoblasts (Bonno *et al.*, 1994). In contrast, with immunohistochemical staining proMBP localized only to the placental septa and the anchoring villi and proMBP mRNA was found only in the extravillous trophoblasts (Bonno *et al.*, 1994).

In cell culture conditions it was shown that human villous cytotrophoblasts produce active PAPP-A (Giudice *et al.*, 2002). Moreover, PAPP-A protein and activity were found to be associated with the membranes of human trophoblasts (a mix of villous cytotrophoblasts and syncytiotrophoblasts) grown in cell culture (Sun *et al.*, 2002) and in the cytoplasm of human villous cytotrophoblasts collected from placentae at different phases of pregnancy (Guibourdenche *et al.*, 2003). *In vitro* differentiation of the villous cytotrophoblasts into syncytiotrophoblasts is associated with a significant increase of PAPP-A mRNA and pro-

tein expression (Guibourdenche *et al.*, 2003). Moreover, PAPP-A protein expression is significantly higher in cultured human invasive extravillous trophoblasts than in villous cytotrophoblasts (Fournier *et al.*, 2008). Thus, the activity of PAPP-A seems to be important in the feto-maternal interface.

The group of Giudice (2002) showed that endometrial cells of the uterus produce significantly lower levels of PAPP-A than villous cytotrophoblasts in cell culture. However, the production was significantly enhanced by the decidualization (i.e. maturation of the endothelium to become a part of the placenta) achieved *in vitro* by treatment with estradiol and progesterone. This was already predicted by early studies in the 1980s with endometrium tissue samples and uterine fluid samples from women with hormonal treatment (reviewed by Rosen, 1986). Decidualization did not induce proMBP production in endometrial cells (Giudice *et al.*, 2002). However, the treatment of decidualized endometrial cells with IGF-II increased proMBP production but decreased PAPP-A production providing a possible regulatory feed-back loop for the IGF releasing activity of PAPP-A on the maternal side of the placenta.

The role of PAPP-A in pregnancy is not yet well understood. As the only currently known activity of PAPP-A entails the release of IGFs from binding proteins, some conclusions can be drawn from this. Low maternal circulating IGF is associated with slower fetal growth and it has been suggested that this is due to defective placental development and function (Forbes and Westwood, 2008). In developing placenta a strictly regulated interaction between the differentiated fetal trophoblasts and the maternal decidua is crucial for normal placental development. The regulation assures "not too shallow but not too deep" invasion of fetal trophoblasts into the uterine wall. IGFs promote the differentiation of villous cytotrophoblasts into extravillous trophoblasts and the migration of these cells to the maternal decidualized endometrium. In the decidualized endometrium extravillous trophoblasts transform the uterine spiral arteries by widening them and thus enable and increase maternal blood flow to the interstitial space of the placenta (Forbes and Westwood, 2008). IGFs also stimulate the differentiation of villous cytotrophoblasts into the multinucleated syncytiotrophoblast that lines the chorionic villi and acts as a nutrient and gas exchange membrane, a protective barrier and a source of pregnancy-specific hormones secreted to the maternal blood (Forbes and Westwood, 2008).

PAPP-A expression in syncytiotrophoblasts and extravillous trophoblasts, among possible other yet unknown effects, presumably further promotes the differentiation of these cells and extravillous trophoblast invasion. In Down's syndrome pregnancies a defect in syncytiotrophoblast differentiation has been noticed which leads to decrease in the synthesis and secretion of pregnancy-specific hormones by the placenta (Massin *et al.*, 2001). Moreover, defective trophoblast invasion is directly involved in pre-eclampsia, a major and frequent complication of human pregnancy (Fournier *et al.*, 2008). In both conditions lower PAPP-A levels have been measured in the circulation during pregnancy (Wald *et al.*, 1992; Spencer *et al.*, 2008b).

In 1979, Smith and colleagues showed that PAPP-A is predominantly secreted from the placenta to the placental compartment, not to the fetal compartment, as no PAPP-A was found in the cord artery, the cord vein or amniotic fluid. Significantly lower levels of PAPP-A were reported in peritoneal fluid than in peripheral blood implying that PAPP-A does not easily transfer from blood to interstitial fluid. Interestingly, PAPP-A concentra-

tions were lower in retroplacental blood than in peripheral blood or in the uterine vein. This suggests that PAPP-A is not simply secreted by the chorionic villi directly into the intervillous space of the placenta but possibly makes its way into the maternal circulation by a more circuitous route (Smith *et al.*, 1979).

As previously discussed, in late pregnancy plasma most PAPP-A (>99%) appears to be in complex with proMBP (Overgaard *et al.*, 2000). PAPP-A complexation probably takes place in the placenta after PAPP-A and proMBP have been secreted by cells because PAPP-A seems to be produced mostly by different cells than proMBP in the placenta (Bonno *et al.*, 1994) and active PAPP-A dimer binds to cell surfaces (Laursen *et al.*, 2002b), which promotes complex formation and detachment of the complex (Sivanandam *et al.*, 2004). Moreover, no complex formation has been detected in serum samples collected from women in the first trimester of pregnancy as PAPP-A activity in the samples remains stable (Gyrup *et al.*, 2007). In addition, the half-life of active dimeric PAPP-A in the circulation is very short compared to the half-life of the complexed form (Glerup *et al.*, 2007).

The complexation of the majority of PAPP-A prior to appearing in blood seems rational when considering the physiological role of PAPP-A. In this way PAPP-A is active in the placenta where it locally releases active IGFs from the binding proteins. When released to circulation, the complexation prevents excessive PAPP-A activity and IGF activation in the pregnant woman. However, recent evidence by Gyrup and colleagues (2007) showed that in early pregnancy the ratio of active PAPP-A over total PAPP-A detected in serum is higher: at gestational week 10 ~10% of PAPP-A is in active form. The activity of PAPP-A in pregnancy serum increased with gestational age but the relative amount of active PAPP-A decreased. The meaning and implications of these findings remain to be elucidated. One possibility is that the pregnancy related changes in the pregnant women may also require increase of free IGFs in other parts of the body.

Although PAPP-A seems important in human pregnancy, PAPP-A gene deficient mice are fertile (Conover *et al.*, 2004a). However, significant inter-species differences seem to exist especially related to the role of PAPP-A in pregnancy between mouse and human. In striking contrast to human, in mice PAPP-A mRNA expression is not significantly higher in the placenta than in other tissues and the circulating concentrations of PAPP-A protein are not elevated during pregnancy in mice (Soe *et al.*, 2002).

2.3.2 Atherosclerotic plaques

Atherosclerosis is a condition that affects the arteries of the human body. It involves formation of lesions to the innermost layer of the arteries, the *tunica intima*, which lies between the endothelium and the smooth muscle layer referred to as the *tunica media* (Fig. 5). The process of lesion formation and progression is complex and inflammation has been shown to play an important role in it. The mechanisms for lesion development initiation and progression are gradually becoming elucidated. Factors causing endothelial dysfunction and activation of inflammatory reactions in the cell wall seem essential including hypertension, smoking, high levels of low density lipoprotein (LDL) and hyperglycemia-associated products of glycoxidation in the circulation. Other factors involve proinflammatory cytokines derived from the excess adipose tissue and disturbed blood flow especially affecting the sites of curvature and branching of the arteries. (For reviews, see Lusis, 2000; Libby and Theroux, 2005; Hansson and Libby, 2006.)

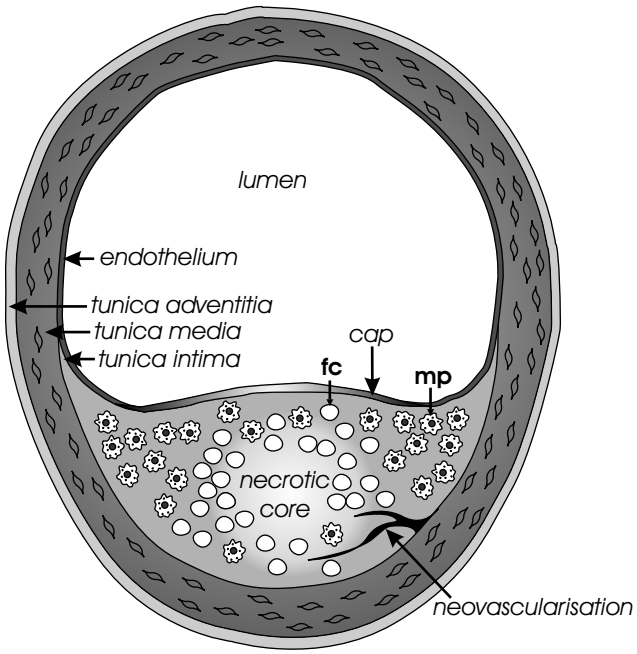

Figure 5. An atherosclerotic plaque that has formed into the wall of an artery at the *tunica intima* layer between the endothelium and the smooth muscle layer, the *tunica media*. Typical characteristics include abundance of activated macrophages (mp) and macrophage derived lipid-filled foam cells (fc), a necrotic core with lipids and debris of dead cells, new blood vessels growing into the plaque from the *tunica media* and a thin cap that covers the plaque. (Adapted from original figure by S.Wittfooth in Eriksson *et al.*, 2006)

The early forms of atherosclerotic lesions, known as fatty streaks, are characterised by accumulation of lipids and infiltration of inflammatory cells. These are already seen in young individuals. However, the fatty streaks do not cause symptoms and may either evolve further or regress. Mature lesions known as atherosclerotic plaques are characterized by lipid-filled macrophage-derived foam cells, extracellular lipids and debris of dead cells that form a necrotic core, capillary blood vessels that have grown into the plaque from the *vasa vasorum* (the arteries that supply the cells of blood vessels), high inflammatory cell infiltration (mainly activated macrophages and T cells most prominent in the shoulder area of the plaque) and a VSMC and collagen-rich endothelium-surfaced cap (covers the plaque and separates it from the lumen of the artery). (For reviews, see Lusis, 2000; Libby and Theroux, 2005; Hansson and Libby, 2006.)

Clinical symptoms due to atherosclerotic plaques appear when the cap of a plaque ruptures inducing thrombus formation within the artery or when a plaque grows to a size that restricts the blood flow through the artery rendering it insufficient. Thus, acute symptoms are usually caused by a certain plaque. However, as the reasons inducing lesion formation and progression can affect the whole vasculature, the lesions are not restricted to one site in an individual. Therefore, instead of a focal process, atherosclerosis should be seen as a systemic disease. Accordingly, patients with symptoms of a certain atherosclerosis-related condition are usually at higher risk for atherosclerosis-related symptoms also in the other parts of the body. (For a review, see Badimon *et al.*, 2009.)

PAPP-A in vascular tissue samples

Bayes-Genis and colleagues (2001a) found PAPP-A in the atherosclerotic coronary plaques of patients who died of cardiac causes. The presence of PAPP-A was determined by staining of tissue samples with a PAPP-A specific monoclonal antibody. The staining was significantly higher in the culprit (i. e. the plaque that was assumed to have caused the death) eroded or ruptured plaques than in stable plaques in the same individuals. In plaques with large lipid cores and cap rupture PAPP-A was found mostly in the inflammatory shoulder region and in the areas surrounding the lipid core. Staining was also localized to the monocyte/macrophages. In fibrous plaques PAPP-A staining was detected with the spindle-shaped smooth-muscle cells, in the extracellular matrix and in the noneroded endothelial cells.

Sangiorgi and colleagues (2006) found PAPP-A in tissue samples of the plaques of the carotid artery. The plaque tissue was collected from patients who underwent carotid endarterectomy to remove those lesions. Immunohistochemical staining with a PAPP-A specific monoclonal antibody showed significantly higher staining in tissue samples of plaques with vulnerable or ruptured structure than in samples of stable plaques. A strong and diffuse PAPP-A staining was evident mainly in the cap and the shoulder region of the plaque and colocalized strongly with the monocyte/macrophages. The intensity of PAPP-A staining correlated inversely with the cap thickness and positively with the intensity of inflammation.

In the immunohistochemical staining studies by Bayes-Genis and colleagues (2001b) the PAPP-A staining was shown to increase in porcine arteries after coronary overstretch balloon injury. The presence of PAPP-A was most pronounced in the *neointima* and in the *media* 14-28 days after the injury; i.e. at the time of active cell migration, proliferation and protein synthesis. This implies that PAPP-A was locally produced rather than transported to the site by tissue infiltration from plasma as a response to the vascular injury. Similarly, in a study conducted by Smith and colleagues (2001), adult rat femoral artery tissue extracts showed significant increase in PAPP-A activity within 12-24 h after a balloon injury and the increased activity persisted for 6 days. Moreover, Resch and colleagues (2006b) showed that unilateral carotid artery ligation in mice lead to a marked elevation in PAPP-A mRNA expression that was associated with progressive increase in neointimal hyperplasia detected 7 to 14 days later. On day 28 after the ligation injury, in many cases wild-type mice had a complete occlusion of the carotid artery while PAPP-A KO mice showed 75% reduction in the neointimal area. Furthermore, the proliferation of cells in the neointimal areas of the injured vessels was greatly reduced in PAPP-A KO mice.

Cells in atherosclerotic plaques

VSMC, endothelial cells (EC) and macrophages are cells involved in the development of atherosclerotic plaques. IGFs have been shown to have proatherogenic effects on these cells (summarized in Figure 6) and are possibly released from IGFBPs in the atherosclerotic lesions by PAPP-A. VSMC show high basal expression of PAPP-A (Bayes-Genis *et al.*, 2001b; Conover *et al.*, 2008a). In VSMC cell culture studies it has been noticed that the proinflammatory cytokines TNFα, IL-1β and IL-6 stimulate the expression of PAPP-A mRNA, protein and activity (Conover *et al.*, 2006; Conover *et al.*, 2008a). Increase in PAPP-A activity has also been shown in VSMC cultures which have been injured by scraping *in vitro* (Bayes-Genis *et al.*, 2001b). IGFs released by PAPP-A induce changes in

VSMC cell cycle that lead to proliferation and migration of the cells – actions important in plaque formation after vascular injury (Bayes-Genis *et al.*, 2000). However, VSMC from PAPP-A KO mice lack IGFBP-4 protease activity and fail to respond this way to treatment with IGF-I in the presence of IGFBP-4 (Resch *et al.*, 2006b).

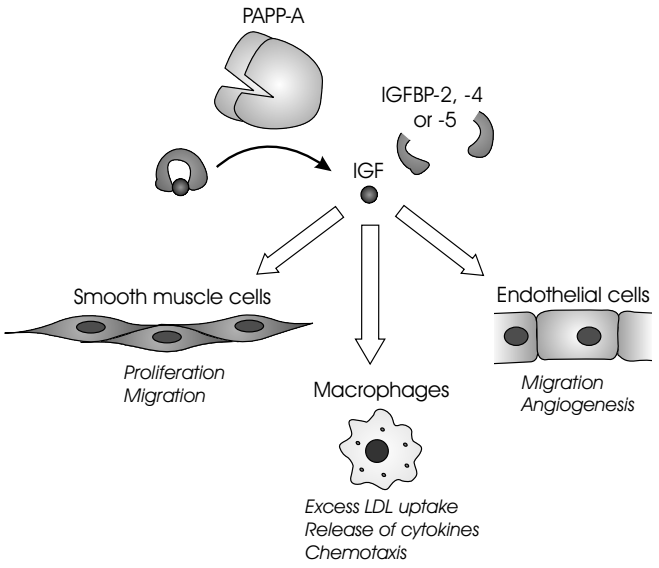

Figure 6. The proatherogenic effects of IGFs on cells found in atherosclerotic plaques. PAPP-A may mediate these effects by releasing IGF from the binding proteins.

Human coronary artery EC show little basal expression of PAPP-A (Conover *et al.*, 2008a). However, the proinflammatory cytokines TNFα and IL-1β also stimulate PAPP-A expression in EC (Conover *et al.*, 2008a). The IGF effects on EC, possibly mediated by PAPP-A degradation of the binding proteins, include promotion of migration and tube-formation. The tube-formation serves angiogenesis, i.e. growth of new blood vessels, which is important in the development of plaques (Bayes-Genis *et al.*, 2000). However, in addition to these potently proatherogenic effects, IGFs may also protect against endothelial dysfunction, an important cause of atherogenesis (Conti *et al.*, 2004).

Although otherwise expected from earlier immunohistochemical studies (Sangiorgi *et al.*, 2006), it was recently shown by Conover and colleagues (2007) that macrophages do not express PAPP-A protein or mRNA. No PAPP-A expression was detected in human peripheral blood monocytes and cells of acute monocyteic leukaemia cell line THP-1 which were differentiated into macrophages and further activated *in vitro*. The case was similar with activated macrophages isolated from a hypecholesterolemic rabbit model of atherosclerosis. However, as previously mentioned, macrophages were found to bind and internalize PAPP-A. Moreover, activated macrophages were much more efficient in this than unactivated macrophages or monocytes (Conover *et al.*, 2007). In macrophages IGFs promote chemotaxis, excess LDL uptake and release of proinflammatory cytokines such as TNFα. These effects are possibly mediated by PAPP-A through the release of active IGFs from the binding proteins (Bayes-Genis *et al.*, 2000). Thus, although macrophages do not seem to

produce PAPP-A themselves, they produce cytokines that stimulate PAPP-A production in the surrounding EC and VSMC. This may lead to a positive feed-back loop, as the PAPP-A produced by EC and VSMC and bound by macrophages may increase the amount of active free IGFs that stimulates the cytokine production in the macrophages.

PAPP-A deficient mice

ApoE KO mice serve as a model of atherosclerosis because when fed with a high-fat diet, the ApoE KO mouse has 10-fold higher total serum cholesterol levels than wild-type mice and produce aortic lesions that are similar to atherosclerotic lesions found in humans. Harrington and colleagues (2007) noticed that in ApoE KO mice PAPP-A mRNA was expressed at 20-fold higher levels in lesional areas compared to other areas of the aorta. In double ApoE PAPP-A KO/KO mice total serum cholesterol increased similarly as in ApoE KO mice but the lesion area was reduced 70-80% after 10 weeks on high-fat diet. However, there was no significant difference in the lesion number. Thus, PAPP-A seems to have a predominant effect on lesion progression rather than on the lesion initiation. The lesions of ApoE KO mice were more complex with significantly greater necrotic core areas, while the extent of inflammation determined as macrophage staining or IL-1β or TNFα expression did not differ between ApoE KO and double ApoE PAPP-A KO/KO mice (Harrington *et al.*, 2007).

Effects of cardioprotective resveratrol

Recently, Conover and colleagues (2006) showed that pretreatment with resveratrol, a polyphenol found in the skin of grapes and in red wine, inhibited the TNFα and IL-1β-induced PAPP-A mRNA and PAPP-A activity expression in VSMC. However, resveratrol did not affect basal PAPP-A mRNA expression or activity. The induced inhibition of IGFBP degrading activity was not due to induction of proMBP. It was suggested that the cardioprotective effects of resveratrol may, in part, be a result of the blocking of cytokine-stimulated PAPP-A expression.

2.3.3 Folliculogenesis

Folliculogenesis represents the process in which oocytes mature in the female ovaries to become ready for ovulation and fertilisation (Fig. 7). The process proceeds from the primordial follicles, through the stages of preantral, antral and preovulatory follicles, to the graafian follicle that releases the oocyte in ovulation. The process is controlled by the follicle stimulating hormone (FSH) produced by the pituitary gland. Although many follicles start their maturation within a certain menstrual cycle, the growth of the majority of the follicles ceases at some stage of the process. These follicles become atretic and degenerate. Due to signals not yet well understood only one follicle is chosen to become the dominant follicle and to ovulate in the monovulatory species. After ovulation, the remaining follicle tissue degenerates to form a corpus luteum. (Reviewed by Zeleznik, 2004.)

The disappearance of IGFBP-2, -4 and -5 in the fluid of preovulatory follicles and the increase in their levels in atretic follicles is a conserved phenomenon among mammalian species (Mazerbourg *et al.*, 2003). Protease-induced decreases in IGFBP-2, -4 and -5 and, in the case of IGFBP-2, reduced expression, most likely cause increased levels of bioavailable IGFs. This seems to stimulate steroidogenesis and mitogenesis in the developing dominant follicles, which ultimately prepare the follicles and oocytes for successful ovulation and fertilization (Monget *et al.*, 2003; Gerard *et al.*, 2004; Spicer, 2004). In atretic

follicles IGFBPs sequester the available IGF and degeneration of the follicle takes place (Monget *et al.*, 2003).

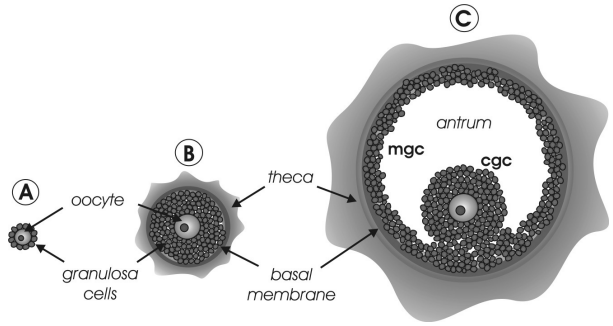

Figure 7. The stages of folliculogenesis. **A)** A primordial follicle with one granulosa cell layer and the oocyte. **B)** A preantral follicle with several layers of granulosa cells and theca cells forming the theca that surrounds the basal membrane around the granulosa cells. **C)** An antral / a preovulatory follicle with an antrum, cumulus granulosa cells (cgc) around the oocyte, membrane granulosa cells (mgc) next to the basal membrane and theca around the basal membrane.

PAPP-A has been identified as the IGFBP-2, -4 and -5 protease in the follicular fluid of preovulatory follicles of various mammalian species (Chandrasekher *et al.*, 1995; Rivera *et al.*, 2001; Gerard *et al.*, 2004). Importantly, this activity has been found to be significantly higher in the estrogen producing follicles destined to proceed further than in the subordinate follicles destined for atresia (Chandrasekher *et al.*, 1995; Rivera *et al.*, 2001; Rivera and Fortune, 2001; Matsui *et al.*, 2004). Moreover, higher activity of PAPP-A in the follicular fluid of competing antral follicles even before apparent size divergence or increased estrogen production predicts dominance (Rivera and Fortune, 2003a). Thus, PAPP-A seems to have an important role in the selection of the dominant follicle due to ovulate.

Hourwitz and colleagues (2000) found the expression of PAPP-A mRNA to follow a similar pattern as the detected activity. PAPP-A mRNA expression was detected in the healthy human ovaries of normal cycling women exclusively in the granulosa cells of healthy antral follicles from a diameter of 5 mm to the preovulatory stage. Expression was also seen in a subset of large luteal cells assumed to be granulosa lutein cells in healthy *corpora lutea*. On the other hand, PAPP-A mRNA expression is low or undetectable in preantral follicles, in atretic antral follicles and in connective tissue cells.

In developing follicles, PAPP-A expression seems to be induced by FSH. FSH injection just before the time of dominant follicle selection in heifers and in cows led to formation of codominant follicles with IGFBP-4 degrading activity similar to dominant follicles of untreated animals (Rivera and Fortune, 2001; Rivera and Fortune, 2003a). Accordingly, in rat follicle membrane granulosa cells PAPP-A mRNA expression was markedly stimulated by FSH (Matsui *et al.*, 2004) and FSH increased the IGF-dependent IGFBP-4 degradation in cell cultures of luteinizing human granulosa cells (Iwashita *et al.*, 1998).

Spatial specific control mechanisms over PAPP-A activity seem to exist in the developing follicle which arise from variable expression regulation. The stimulation of PAPP-A mRNA expression in rat follicle membrane granulosa cells by FSH is inhibited by bone morphogenetic protein (BMP)-15. BMP-15 is produced by the oocytes and this results in lower PAPP-A activity near the oocyte (Matsui *et al.*, 2004). Moreover, although the ex-

pression of PAPP-A by the granulosa cells of the small and large follicles of bovine ovaries is not affected by estradiol, in theca cells estradiol decreases PAPP-A mRNA levels (Aad *et al.*, 2006). Pregnant mare serum gonadotropin stimulates PAPP-A expression in mouse membrane granulosa cells of healthy antral / dominant follicles and in the granulosa-lutein cells of *corpus luteum*; however, this effect was not seen with cumulus granulosa cells (Hourvitz *et al.*, 2002). Similarly, pregnant mare serum gonadotropin induces PAPP-A expression in the membrane granulosa cells of healthy dominant follicles of rat ovary but not in cumulus granulosa cells juxtaposed to the oocyte or any granulosa cells of atretic follicles (Matsui *et al.*, 2004).

A small number of studies have focused on PAPP-A in relation to *in vitro* fertilization. It has been shown that the ovulation hyperstimulating agent (either gonadotropin releasing hormone agonist or antagonist) does not affect the PAPP-A levels of the follicular fluid. However, the PAPP-A concentration on the day of oocyte retrieval is inversely related to the growth and survival of the oocyte (Choi *et al.*, 2006). In another study, low concentration of PAPP-A in follicular fluid on the day of oocyte retrieval predicted better oocyte maturation and early embryo development (Wang *et al.*, 2006). Thus, in spite of the promoting role in the selection and growth of dominant follicle, the activity of PAPP-A may only play a minor role, if any, in final oocyte maturation and subsequent embryo development.

The issue of PAPP-A inhibition by proMBP in relation to folliculogenesis has been little studied. In immunohistochemical studies, Rhoton-Vlasak and collegues (2003) detected PAPP-A and proMBP expression with distinct patterns in human ovarian tissue during folliculogenesis. However, in luteal tissue the localization patterns of PAPP-A and proMBP were similar. In the study by Kalli and colleagues (2004) ovarian surface epithelial cells were found to express high levels of PAPP-A mRNA, protein and activity but no proMBP mRNA or protein. The results imply that PAPP-A is most likely not inhibited by proMBP around the cells where activity is required during folliculogenesis, while in luteal tissue inhibition may take place and be important to avoid the adverse effects of excessive PAPP-A activity.

2.3.4 Osteogenesis

PAPP-A mRNA and protein are expressed by human osteoblasts and osteoprogenitor cells, the cells important in the formation of bone tissue, i.e. osteogenesis (Lawrence *et al.*, 1999). PAPP-A seems to act as a potent anabolic factor in the regulation of bone formation because recombinant PAPP-A significantly increases the proliferation of osteoblasts and free IGF-1 concentration in the culture medium of human ostosarcoma cell line MG63 (Qin *et al.*, 2006b).

PAPP-A mRNA expression, PAPP-A protein secretion and IGFBP-4 degrading activity in the primary cultures of human osteoblastic cells are stimulated by BMP-2 and proinflammatory cytokines transforming growth factor-β, TNFα, IL-1β and IL-4 (Ortiz *et al.*, 2003; Conover *et al.*, 2004b; Jadlowiec *et al.*, 2005). BMP-2 and PAPP-A are probably involved in the mediation of end-stage osteoblastic differentiation as they can together stimulate matrix mineralization by human adult mesenchymal cells in the absence of other osteogenic supplements (Jadlowiec *et al.*, 2005). PAPP-A also seems to promote bone angiogenesis as noticed in the chick chorioallogenic membrane model (Jadlowiec *et al.*, 2005). Thus, cytokine-regulated PAPP-A may play a role in normal bone remodelling, fracture

repair and in abnormal bone remodelling associated with inflammation (Conover *et al.*, 2004b).

In PAPP-A KO mice the development of bones is delayed during embryogenesis (Conover *et al.*, 2004a). Adult PAPP-A KO mice show skeletal insufficiency of mass, density, architecture and strength (Tanner *et al.*, 2008). The bones are smaller and weaker because more bone is destroyed than formed (Tanner *et al.*, 2008). Moreover, fracture healing is significantly delayed, although eventually the fractures also heal in PAPP-A KO mice (Miller *et al.*, 2007). Thus, PAPP-A KO mice may represent a model of age-related osteoporosis with increased fracture risk.

PAPP-A has also been found by Gruber and colleagues (2008) in human intervertebral discs where it seems to be expressed in inner anulus cells especially when the discs are degenerating. The role of PAPP-A has been suggested to be linked to the repair of the degerated disc tissue by releasing active IGF. IGFs have been reported to prevent or retard apoptosis of cultured human anulus cells and to stimulate proteoglycan synthesis within the disc.

2.3.5 Myogenesis

PAPP-A may be important in myogenesis, in the developmental program that generates and regenerates skeletal muscle. Rehage and colleagues (2007) showed that in normal wild-type mice with induced muscle injury the expression of PAPP-A was shown to be significantly increased five days after injury, while the expression was normal on day 14. In a study by Kumar and colleagues (2005) exogenous recombinant PAPP-A and transient overexpression of PAPP-A in a mouse myoblast cell line C2C12 increased the proliferation in a dose- and time-dependent manner. PAPP-A also enhanced myotube formation and the activity of creatine kinase implying that PAPP-A promotes myoblast differentiation. As IGFs potently stimulate the proliferation and differentiation of myogenic cells, the stimulatory effect seems to depend on the ability of PAPP-A to increase the amount of free IGF-I through IGFBP degradation

In vivo studies with genetically modified mice by Rehage and colleagues (2007) have also provided evidence that PAPP-A is a promoter of skeletal muscle formation and growth. Targeted overexpression of PAPP-A in the muscles of mice was achieved with the use of muscle-specific promoter (human skeletal α-actin promoter). The prenatal and the postnatal growth were significantly enhanced in the genetically modified mice. At the age of 10 weeks the genetically altered mice were 20-50% heavier and 9% longer than wild-type mice at that age. The skeletal muscle weight and muscle fiber area were also increased. Moreover, free IGF-I concentration was remarkably increased in the culture medium of *ex vivo* cultured muscle cells from transgenic mice and the proliferation of C2C12 myoblasts significantly increased in the presence of this culture medium.

2.3.6 Skin wound healing

With PAPP-A specific immunohistochemical staining, PAPP-A has was found by Chen and colleagues (Chen *et al.*, 2003) in human skin at the epidermis, in sweat and sebaceous gland epithelial cells, in hair follicles and in endothelial cells of the blood vessels. Injury led to an increase in the localization of PAPP-A in the dermal granulation tissue within and adjacent to the injury, in small blood vessels, on newly synthesized collagen and at the

advancing epithelial edge of the healing epidermis. The PAPP-A staining colocalized with activated wound fibroblasts showing spindle-shaped morphology and with activated wound macrophages but not with lymphocytes. In the healing skin, PAPP-A is presumably involved in the tissue healing process by releasing IGF that acts as a stimulant of keratinocyte and fibroblast proliferation and matrix protein synthesis.

2.3.7 Cancer

As a mediator of IGF availability, PAPP-A is a potent cell growth promoter and accordingly some studies have shown that PAPP-A activity is important in malignancy. In a study by Suzuki and colleagues (2003) glycoprotein bikunin suppressed cancer cell invasion and metastasis and transiently suppressed the expression of PAPP-A in human ovarian cancer cell line HRA. Treatment of the cells with 18-base phosphorothioate antisense PAPP-A oligodeoxynucleotide had a similar effect. Moreover, Tanaka and colleagues (2004) showed that, after intraperitoneal tumor cell inoculation into mice, PAPP-A mRNA and protein became markedly upregulated in intraperitoneal disseminated metastatic tumors. In contrast, antisense PAPP-A significantly suppressed the growth of these cells. Genetic deletion of PAPP-A in mice has been shown to lead to 30-40% increase in life-span and a sharp reduction in the incidence of spontaneous tumors (Conover and Bale, 2007; Conover *et al.*, 2008b). In the study by Conover and Bale (2007), at the age of 23- to 28- months ~70% of the wild-type mice had developed tumors affecting primarily liver, lung, kidney and colon while only 15% of the PAPP-A KO mice had small tumors that were located in the liver.

In some cases, however, PAPP-A has been shown to be negatively associated with malignancy. PAPP-A mRNA and protein are expressed by human osteoblasts and osteoprogenitor cells but not by osteosarcoma cells (Lawrence *et al.*, 1999). PAPP-A expression is also lower in ovarian tumours than in normal ovarian cells (Alexiadis *et al.*, 2006). Moreover, while dibutyryl cyclic adenosine monophosphate (dB-cAMP) reverses the transformed malignant phenotype of human glioblastoma cells U87MG, it also increases the expression PAPP-A among other genes (Moreno *et al.*, 2006). Induction of PAPP-A mRNA expression by another cAMP derivative, 8-bromo-cAMP, has also been reported in a choriocarcinoma cell line JAR (Haaning *et al.*, 1996).

Apparently, some tumor promoters seem to induce production of proMBP, the specific inhibitor of PAPP-A. Chen and colleagues (2002) showed that in cultured human fibroblasts a phorbol ester tumor promoter β-phorbol 12,13-didecanoate (β-PDD) induced inhibition of the activity of PAPP-A against IGFBP-4 by stimulating proMBP expression. Transformation with simian virus large T antigen or treatment with another phorbol ester tumor promoter PMA also had a similar effect.

Inhibition of PAPP-A activity in malignancy has also been shown in a study by Kalli and colleagues (2004). Ovarian tumor cells were found to express variable levels of PAPP-A but relatively high amounts of proMBP protein and mRNA and, probably as a result, negligible PAPP-A activity. Similarly, in a reversible premalignant ovarian surface epithelial cell model system, the premalignant cells proliferated but produced low amounts of PAPP-A mRNA, protein and activity. When the premalignant state was reversed, proliferation ceased and PAPP-A mRNA and protein expression and PAPP-A activity increased significantly. Thus, there seems to be an association between the normal phenotype of ovarian cells and PAPP-A activity.

2.4 PAPP-A as a circulating biomarker

In this section are reviewed studies related to the use of PAPP-A as a circulating biomarker in various conditions. However, it should be noted that currently PAPP-A has been adopted into clinical use only for prenatal screening of Down's syndrome during the first trimester of pregnancy. For this purpose PAPP-A is widely used in the developed countries worldwide.

2.4.1 Pregnancy

The circulating levels of PAPP-A have been found to increase throughout pregnancy until delivery (Smith *et al.*, 1979; Folkersen *et al.*, 1981; Rosen, 1986). Elevated levels are detected already at the 4th gestational week (median 11 µg/L, 31 mIU/L) (Qin *et al.*, 1996) and the concentrations seem to reach at least 30 000 µg/L (~100 000 mIU/L) by the time of delivery, although variation in the determined concentrations is high between individuals and the detection methods used (Folkersen *et al.*, 1981; Rosen, 1986; Oxvig *et al.*, 1995). After delivery, the PAPP-A levels normalize with an average half-life of 51 h within 5 weeks (Smith *et al.*, 1979).

Down's syndrome

In the early 1990s it was realised that lower levels of serum PAPP-A were associated with Down's syndrome (DS) pregnancies (Wald *et al.*, 1992). In this first study by Wald and colleagues serum PAPP-A was compared between DS cases and controls matched with gestational age as multiples of the median (MoM) of the control group and this has remained the practice since. In samples collected in the first trimester of pregnancy, between gestational weeks 9 and 12, the median MoM of 19 DS cases was 0.23. Subsequently, it was soon noticed that PAPP-A was not a good marker in the second trimester but showed better discrimination in the first trimester (Cuckle *et al.*, 1992). By the end of the first decade of the 21st century PAPP-A has been widely implemented into the Down's syndrome screening regimes in developed countries. Although the discriminating power of PAPP-A alone is not high (52% of DS cases detected with a false positive rate of 5%), it is of significant benefit in multimarker approaches (Cuckle and van Lith, 1999).

Previously, second trimester screening (15-18 weeks of gestation) including maternal age and maternal serum concentrations of α-fetoprotein, unconjugated estriol and human chorionic gonadotropin (hCG) was the mainstay of prenatal DS screening (Benn, 2002). However, currently the best prediction of the incidence of DS pregnancy is obtained with first trimester screening (10-14 weeks of gestation) protocol that combines serum PAPP-A with maternal age (DS risk increases with age), the diameter of the fetal non-echogenic nuchal edema commonly referred to as nuchal translucency (DS risk increases with larger diameter) and serum free β-form of hCG (DS risk increases with elevated concentration). With this procedure a detection rate of ~90% for DS has been achieved with a false-positive rate of 5% in retrospective and prospective studies (Spencer *et al.*, 1999; Bindra *et al.*, 2002; Spencer *et al.*, 2003; Nicolaides *et al.*, 2005; Canick *et al.*, 2006). Such non-invasive screening procedures help in choosing the women in high DS pregnancy risk for invasive confirming examination with amniocentesis or chorionic villi biopsy. In addition to offering better risk prediction with lower false-positive rate compared to second trimester screening, the first trimester screening provides earlier reassurance for those with a normal result and possibly less traumatic pregnancy termination for those receiving abnormal results also from the confirming test.

Certain factors may affect PAPP-A concentrations during pregnancy and thus confound the risk calculations. For example, the number of fetuses should be taken into account as well as the chorionicity because PAPP-A levels are generally higher in twin pregnancies but in monochorionic twin pregnancies PAPP-A levels are lower than in dichorionic twin pregnancies (Spencer *et al.*, 2008c). The ethnic origin of the pregnant woman also has an influence as, for instance, the circulating concentrations in the first trimester are significantly higher in Afro-Caribbean than in Caucasian women, which possibly leads to underestimation of DS risk in pregnant women of Afro-Caribbean origin (Kagan *et al.*, 2008). On the other hand, smoking and the use of assisted reproductive technologies decrease PAPP-A levels thus increasing the rate of false-positive screening results (Kagan *et al.*, 2008; Amor *et al.*, 2009).

Other pregnancy abnormalities

In addition to DS caused by the trisomy of the 21st chromosome, lower PAPP-A values are also associated with other genetic anomalies and birth defects. Lower levels of maternal serum PAPP-A have been seen in pregnancies affected by the trisomy of chromosomes 13 and 18 as well as by maternal and paternal triploidy. It has been shown that maternal serum PAPP-A can be used together with maternal serum βhCG and nuchal translucency thickness to identify such cases (Spencer *et al.*, 2000; Spencer, 2007). Low maternal circulating PAPP-A levels are also associated with Cornelia de Lange syndrome, a rare congenital defect with typical physical characteristics (Westergaard *et al.*, 1983a; Chong *et al.*, 2009). Interestingly, PAPP-A protein was not detected in the plancentae of pregnancies complicated by Cornelia de Lange syndrome in striking contrast to normal pregnancy with abundant PAPP-A in syncytiotrophoblast tissue (Westergaard *et al.*, 1983a). Chong and colleagues (2009) recently reported that Cornelia de Lange syndrome may be detected by low maternal serum PAPP-A and following detailed ultrasound examination in first trimester screening.

Montanari and colleagues (2009) noticed that in fetuses with normal karyotype low first trimester serum PAPP-A concentrations are also associated with reduced fetal size and higher risk of intrauterine fetal growth restriction with Doppler signs of impaired placental perfusion. However, only 10-16% of women with low PAPP-A values in the first trimester have a pregnancy complicated with fetal growth restriction (Montanari *et al.*, 2009). In the study by Spencer and colleagues (2008b) low maternal circulating PAPP-A levels in the first trimester (11 to 13 weeks of gestation) were associated with developing pre-eclampsia and the risk for pre-eclampsia increased with decreasing PAPP-A concentrations. At a PAPP-A MoM of 0.415, 15% of the cases with pre-eclampsia were identified.

Among pregnancies without chromosomal abnormalities Spencer and colleagues (2008a) reported an adjusted risk ratio of 2.1 [95% confidence interval (95% CI) 1.3-3.6] for the overall power of low PAPP-A levels (<0.4 MoM) to predict fetal loss, pre-eclampsia, severe pregnancy-induced hypertension and small for gestational age infant. Thus, women with low PAPP-A early in pregnancy have twice the risk of an adverse outcome. Gestational age of 10-14 weeks was shown to be the only time period when low PAPP-A is significantly associated with these adverse outcomes. The confounding factors affecting the ability of PAPP-A to predict risk included maternal weight, smoking and caffeine intake. Although the predictive power of PAPP-A for these adverse outcomes is low, it has been suggested that women with low first-trimester PAPP-A MoM and a fetus with normal karyotype should be monitored carefully to further assess the risk of pre-eclampsia and

poor fetal growth (Spencer *et al.*, 2008b; Montanari *et al.*, 2009). Extremely high PAPP-A levels sometimes encountered in the first trimester screening do not seem to be associated with adverse pregnancy outcome and they may possibly arise, at least in some cases, from detection method related bias (Cuckle *et al.*, 2003).

2.4.2 Acute coronary syndromes

Acute coronary syndromes (ACS) are characterized by symptoms, most commonly severe chest pain, caused by a sudden obstruction of blood flow through a coronary artery that supplies oxygen to the heart muscle tissue. Most often the reason is thrombus formation within the coronary artery that was initiated by a rupture or superficial erosion of a coronary atherosclerotic plaque. ACS are further classified as myocardial infarction (MI, a total block of the blood flow that results in necrosis of myocardial tissue) and unstable angina (UA, only partial or quickly resolving obstruction of blood flow that does not lead to detectable myocardial necrosis). Moreover, MI can further be classified as MI with elevation of the ST-segment in ECG (STEMI) indicating severe dysfunction of the heart muscle and MI without elevation of the ST-segment in ECG (NSTEMI). NSTEMI and UA are both forms of ACS without ST-segment elevation and can therefore together be referred to as NSTE-ACS. (Reviewed by Morrow *et al.*, 2007.) Pronounced atherosclerosis of the coronary arteries, i.e. coronary artery disease (CAD), can also present as stable angina (SA). In SA the coronary plaques are large enough in size to obstruct blood flow and cause symptoms when the oxygen demand is increased, for example with physical stress. The symptoms are alleviated with rest and medication that reduces the oxygen demand or dilates the blood vessels. (Reviewed by Libby and Theroux, 2005.) It is noteworthy that SA is not considered as an ACS because the symptoms can be predicted. The classification of the forms of symptomatic presentation of CAD is summarized in Figure 8.

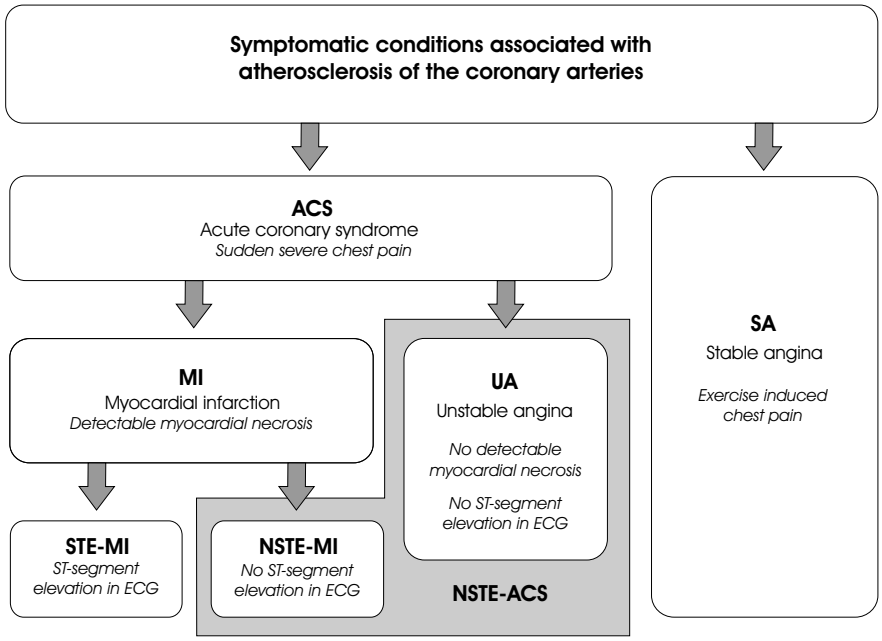

Figure 8. Classification of the forms of coronary artery disease with symptoms.

Elevation in the circulation of ACS patients

In 2001, in the same study by Bayes-Genis and colleagues (2001a) in which they revealed that PAPP-A is exclusively found in eroded and ruptured atherosclerotic plaques, it was noted for the first time that the circulating levels of PAPP-A were elevated in ACS patients. PAPP-A concentrations were significantly higher in blood samples from patients diagnosed with MI or UA than in samples from SA patients or control subjects with normal coronary arteries. The PAPP-A levels were not associated with age, gender, traditional risk factors, medication, markers of cardiac necrosis including cardiac troponin I (cTnI) and MB isoform of creatine kinase (CK-MB) while association was noticed with the inflammation marker C-reactive protein (CRP) ($r=0.61$, $p=0.02$). At a cut-off level of 10 mIU/L, PAPP-A detected ACS cases with a sensitivity of 89.2% and a specificity of 81.3%. The details of the studies related to PAPP-A and ACS are summarized in Table 3.

PAPP-A elevation in ACS has subsequently been confirmed by others. Khosravi and colleagues (2002) reported on higher PAPP-A concentrations in patients with positive cTnT and CK-MB than in CK-MB negative cardiac patients or apparently healthy controls. However, there was not a significant correlation between PAPP-A and cTnT or CK-MB concentrations. Laterza and colleagues (2004) also showed higher serum concentrations of PAPP-A in MI patients than in non-MI patients among patients admitted to hospital with ACS symptoms. The correlation between PAPP-A and cTnT was also poor in this study.

In a study by Rossen and colleagues (2007) circulating PAPP-A levels were lower in NSTEMI patients than in STEMI patients and yet lower levels were seen in healthy controls. Miedema and colleagues (2008) reported on higher circulating PAPP-A levels in ACS patients than in patients with stable angina or in asymptomatic CAD patients while Liu and colleagues (2008) noticed higher serum PAPP-A in STEMI and UA patients than in SA patients and controls. Iversen and colleagues (Iversen *et al.*, 2008b) have also reported on elevated circulating PAPP-A in STEMI patients.

In a recent study by Schoos and colleagues (2009) higher PAPP-A levels were found in STEMI patients compared to high risk NSTE-ACS patients or to low-risk NSTE-ACS patients with even lower levels, although samples were available for most of the patients only 12 h after admission. Similarly, a recent study by Iversen and colleagues (2009) showed higher PAPP-A levels in high-risk NSTE-ACS patients than in low-risk NSTE-ACS patients. Moreover, the PAPP-A concentrations of this whole patient group were lower than in STEMI patients but higher than in patients with non-cardiac disease or in healthy controls whose PAPP-A concentrations had been measured with the same method by the same group previously.

Dominguez-Rodriguez and colleagues (2005) did not find significant difference in the serum concentration of PAPP-A between MI patients and controls. However, in this study the control subjects were also admitted to hospital implying that these individuals were not healthy. Moreover, the ability of the assay used by this group to reliably detect the low concentrations reported has been questioned (Wittfooth *et al.*, 2006).

Table 3. Summary of the clinical studies of PAPP-A in ACS patients.

Study	Patients[a]	Sampling time[a]	Assay[b]	PAPP-A concentrations[a,c]	Heparin interference
Bayes-Genis *et al.* (2001a)	17 MI, 20 UA, 19 SA, 13 controls	Time of coronary angiography	Bayes-Genis	MI 20.6 [9.2,46.6] mIU/L, UA 14.9 [6.3,63.4] mIU/L, SA 8.4 [4.4,22.5] mIU/L, controls 7.4 [3.8,10.4] mIU/L	Possible
Brügger-Andersen *et al.* (2007)	20 STEMI with PCI, 18 STEMI with TL	Right before treatment, immediately after PCI, 90 min after TL	DSL	Before PCI 0.62 [0.3,8.12] mIU/L, after PCI 4.26 [2.24,9.20] mIU/L, before TL 1.03 [0.31,14.71]mIU/L, after TL 8.78 [0.79,36.79] mIU/L	Highly likely
Brügger-Andersen *et al.* (2008)	298 MI	4-6 days after admission	DSL	PAPP-A 0.5 [0.3,0.7] mIU/L	Possible
Dominguez-Rodriguez *et al.* (2005)	80 AMI, 80 controls	Admission, before any medication	DRG	MI 1.29 (SEM 0.02) mIU/L, controls 1.24 (SEM 0.07) mIU/L	Not likely
Elesber *et al.* (2007)	59 intermediate to high risk ACP => final diagnosis: 19 ACS, 40 NCCP	Admission	DSL	ACS 2.0 [1.2,4.9] mIU/L, NCCP 1.2 [0.7,1.6] mIU/L	Not likely
Furenes *et al.* (2009)	20 STEMI, 10 SA	SA before PCI, all 3 and 12 h; 1, 3, 5, 7 and 14 days after PCI	DRG	SA before PCI 5.90 ng/mL, SA 3 h 9.48 ng/mL, STEMI 3 h 7.62 ng/mL	Possible
Hájek *et al.* (2008)	66 STEMI, 35 NSTEMI, 21 UA, 110 SA, 51 controls	During angiography or routine admission examination	BRAHMS Kryptor	STEMI 17.75 [0,146.8] mIU/L, NSTEMI 14.3 [0,123.0] mIU/L UA 11.4 [0,68.3] mIU/L, SA 8.1 [0,16.4] mIU/L, controls 7.5 [0,14.2] mIU/L	Possible
Heeschen *et al.* (2005)	547 with angiographically validated ACS	First available (8.7±4.9 h after symptom onset)	Roche	9.3 [0.2,105.4] mIU/L	Highly likely
	644 ACP => final diagnosis: 323 NSTE-ACS, 105 SA, 19 CHF, 197 no CAD	Admission, before initiation of treatment	Roche	ACS 4.9 [0.1,362.5] mIU/L, SA 1.9 [0.1,113.9] mIU/L, no CHD 1.4 [0.1,54.1] mIU/L	Not likely
Iversen *et al.* (2008b)	354 STEMI with PCI	At admission 0.8 [0,113.7] h after PCI, every 6-8 h until necrosis markers decreasing	Rossen	Admission 12.1 [2.0,224.2] mIU/L, 2nd sample 2.2 [2.0,31.5] mIU/L, 3rd sample 2.0 [2.0,20.2] mIU/L	Possible
Iversen *et al.* (2009)	123 high-risk NSTE-ACS, 415 low-risk NSTE-ACS	At admission, every 6-8 h until necrosis markers decreasing	Rossen	63% high-risk NSTE-ACS >4 mIU/L, 49% low-risk NSTE-ACS >4 mIU/L	Possible
Kavsak *et al.* (2009)	320 ACP	Earliest possible for PAPP-A, repeated samples for cTnI	DSL	Peak PAPP-A in heparin treated patients 4.03 [1.95,9.84] mIU/L, in patients without heparin treatment 1.56 [1.79,1.97] mIU/L; non-heparin cTnI negative patients: changing hs cTnI 1.88 [1.06,3.66] mIU/L, not changing hs cTnI 1.29 [0.86,2.27] mIU/L	Taken into account
Khosravi *et al.* (2002)	71 cardiac patients (3 serial sampling), 47 controls	Unspecified	DSL	cTnT positive patients 3.89 {0.01-97.0} mIU/L, CK-MB positive patients 4.47 {0.31-16.5} mIU/L, CK-MB negative patients 1.07 {BDL - 4.99} mIU/L	Possible

(continued from previous page)

Study	Patients[a]	Sampling time[a]	Assay[b]	PAPP-A concentrations[a,c]	Heparin interference
Laterza *et al.* (2004)	346 with ACS symptoms	Immediately after enrollment	DSL	MI 2.14 (SEM 0.39) mIU/L, non-MI 0.77 (SEM 0.08) mIU/L	Possible
Liu *et al.* (2008)	12 STEMI, 15 UA, 15 SA, 16 controls; 24 of STEMI and UA treated with PCI	ACS 30 min before PCI, SA 4 h before and 4 h after PCI	Bayes-Genis	STEMI 16.9±10.3 mIU/L, UA 15.2±10.5 mIU/L, SA 8.5±3.1 mIU/L, controls 8.4±2.0 mIU/L, before PCI 15.1±10.0 mIU/L, after PCI 19.9±10.1 mIU/L	Possible
Lund *et al.* (2003)	136 cTnI negative ACS	Admission, 6-12 h, 24 h	Qin	Admission 2.3 [1.6,3.0] mIU/L; peak concentration mIU/L in discharged 2.35 [1.6,2.9] in hospitalized 3.3 [2.1,6.5]	Possible
Lund *et al.* (2006)	62 STEMI (14 frequent sampling)	Admission, 6-12, 24 and 48 h (+1, 2, 4 h)	Qin	Frequent sampling patients: admission 8.0 [3.7,12.2] mIU/L, 1 h 11.6 [4.7,18.8] mIU/L, 12 h 4.7 [3.0,6.3] mIU/L	Possible
McCann *et al.* (2008)	415 ACP => final diagnosis: 73 STEMI, 125 NSTEMI, 124 UA, 93 NCCP	Initial sample before thrombolytics and anticoagulants	Demeditec	MI 6.7 [2.6,12.4] ng/mL, non-MI 5.0 [1.7,11.0] ng/mL	Not likely
McCann *et al.* (2009)	550 ACP	Admission sample before thrombolytic or anticoagulant	Demeditec	Death or MI in 1 year 6.7 [3.0,13.4] ng/mL, no adverse event 6.3 [2.2-12.4] ng/mL	Not likely
Qin *et al.* (2002b)	14 MI, 1 UA	Admission, 1, 2, 4, 6, 24, 48, 72 h	Qin	Highly variable levels between ~1-30 mIU/L	Possible
Rossen *et al.* (2007)	14 STEMI with PCI, 20 NSTEMI, 103 controls	NSTEMI 17-93 h from symptom onset, STEMI 4-10 h from symptom onset	Rossen	STEMI 4.5-49.8 mIU/L, NSTEMI <3.3-18.9 mIU/L, controls <3.3-4.8 mIU/L	Possible
Sanchis *et al.* (2008)	422 cTnI neg. NSTE-ACS	12-24 h after admission	DSL	1.2 [0.6,2.5] mIU/L	Possible
Schoos *et al.* (2008)	19 STEMI (17 with PCI), 13 high risk NSTE-ACS, 8 low-risk NSTE-ACS	2 h after PCI or at admission	Rossen	Median peak values in STEMI PCI patients 23.2 mIU/L, high-risk NSTE-ACS 15.3 mIU/L, low-risk NSTE-ACS 6.35 mIU/L	Possible

[a] Abbreviations not appearing outside this table in the thesis: ACP, acute chest pain; BDL, below detection limit; CHF, chronic heart failure; hs cTnI, cTnI measured with a high sensitivity cTnI assay; NCCP, non-cardiac chest pain; SEM, standard error of the mean; TL, thrombolysis

[b] The assays are described in detail in table 4.

[c] Concentrations are either median [25th,75th percentile], mean±SD, median{min-max} or within designated range

Heeschen and colleagues (2005) studied a heterogeneous population of individuals who had arrived at the emergency room with chest pain. PAPP-A concentrations were significantly higher in those patients who were later diagnosed with ACS during the index hospitalization than in those who were diagnosed with stable angina (SA) or who did not have coronary heart disease. Similarly, in a study by Elesber and colleagues (2007) the admission serum sample PAPP-A concentrations were higher in those intermediate to high risk chest pain patients who were subsequently diagnosed with ACS than in those patients who were diagnosed with non-cardiac chest pain. Moreover, McCann and colleagues (McCann *et al.*, 2008) found higher PAPP-A levels in the admission samples of those patients with acute ischaemic-type chest pain who were later diagnosed with MI than in those without MI diagnoses.

Release profile

The release profile of PAPP-A in ACS has been investigated in some studies. Qin and colleagues (2002b) reported on highly variable release profiles of PAPP-A in samples serially collected from 14 ACS patients after admission. A peak of elevation was seen in all patients. However, the time, height and duration of the elevation varied remarkably between individuals. No correlation was detected with myoglobin or CRP but significant association was seen with cTnI ($r=0.419$, $p<0.001$). In the previously mentioned study by Khosravi and colleagues (2002) serial samples were also available from three cardiac patients and increasing PAPP-A levels were seen in all of them. However, the release profiles differed from those of cTnT and remarkably between patients. Lund and colleagues (2006) studied the PAPP-A release in 14 STEMI patients with frequent sampling. Median PAPP-A showed a peak at 1 h and rapidly declined within a couple of hours after this while median cTnI increased steadily up to 12 h. Moreover, in a larger STEMI cohort of the same study late PAPP-A elevation was detected in 32% of patients. The variable release profiles may explain why significant correlations of PAPP-A with cardiac troponins or other cardiac necrosis markers have been seen in some studies but not in others.

PAPP-A elevations have also been studied in ACS patients in relation to percutaneous coronary intervention (PCI), i.e. balloon angioplasty treatment aimed for restoration of the blood flow through the blocked coronary arteries. Brügger-Andersen and colleagues (2007) found significantly higher PAPP-A levels in the plasma of STEMI patients immediately after PCI than before. In the same study, similar results were obtained with patients treated with thrombolysis instead of PCI when samples taken 90 min after the administration of the thrombolytic medication were compared to samples taken right before the treatment. Liu and colleagues (2008) also reported on higher PAPP-A concentrations after PCI than before in STEMI and UA patients. Schoos and colleagues (2009) noticed a peak in PAPP-A levels followed by an abrupt decrease after the treatment of STEMI patients with PCI. Moreover, in a very recent study by Furenes and colleagues (2009) elevated PAPP-A levels were detected after PCI in STEMI patients and the concentrations decreased rapidly subsequently. In the same study PAPP-A levels were also seen to increase significantly from the levels determined before the operation in SA patients treated with PCI.

Risk prediction

Elevated PAPP-A levels measured during index hospitalization have been shown to predict adverse cardiac events in ACS patients. In a study by Lund and colleagues (2003) in which cTnI negative ACS patients were followed for 6 months, a PAPP-A level >2.9 mIU/L measured within 24 h of admission predicted adverse cardiac events (new MI, death or revascularization) with an adjusted risk ratio of 4.6 [95% CI 1.8-11.8, $p=0.002$]. Similarly, in a study conducted by Heeschen and colleagues (2005) with angiographically confirmed ACS patients who were followed for 6 months, PAPP-A levels above 12.6 mIU/L in the first available sample predicted death and MI with an adjusted odds ratio of 2.44 [95% CI 1.43-4.15, $p=0.001$]. Moreover, when only those patients with cTnT below <0.01 µg/L were considered, the adjusted odds ratio increased to 3.97 [95% CI 1.24-12.68, $p=0.016$]. In the same study in a heterogeneous group of chest pain patients PAPP-A levels above 7.0 mIU/L predicted the risk for death and MI with an adjusted odds ratio of 3.08 [95% CI 1.85-5.02, $p<0.001$]. In the study by Laterza and colleagues (2004) the risk of patients with ACS symptoms of developing ACS within 30 days was with PAPP-A levels above 0.22 mIU/L counted to a risk ratio of 4.7 [95% CI 2.2-9.8]. However, for use of PAPP-A as a

discriminating marker the area under the curve from receiver-operating curve analysis in this study was only 0.56 [95% CI 0.50-0.61] implying insignificant discrimination power.

Kavsak and colleagues (2009) analysed the ability of PAPP-A to predict the long-term risk for adverse cardiac events in patients with suggestive cardiac ischemia. Patients in the highest PAPP-A tertile were at higher risk for death within 2 years (p=0.03) and 10 years (p=0.05) even after adjusting for age, gender and baseline cTnI. Among the patients with cTnI below 0.04 µg/L but changing cTnI analysed with a high sensitivity cTnI assay, patients in the third PAPP-A tertile had higher probability of death (p=0.02). Moreover, in the study by Lund and colleagues (2006), PAPP-A was shown to predict risk for adverse events also in STEMI patients as the cumulative risk in 12 months was 45% in the 3rd tertile of admission PAPP-A (>10 mIU/L) while the cumulative risk was only 15% in the 1st tertile (<3.0 mIU/L).

Although the prognostic power of PAPP-A to predict adverse cardiac events has been shown in the studies presented above, others have failed to find this prognostic link. Brügger-Andersen and colleagues (2008) did not find an association between PAPP-A levels and adverse events in MI patients who were followed for 45 months. However, in this study the sampling for PAPP-A measurements was performed 4-5 days after admission while in the previously presented studies the sampling had been performed earlier. Similarly, Sanchis and colleagues (2008) failed to show association between PAPP-A and death or ACS in a multivariable model when they studied patients presenting with ACS symptoms without ST-segment deviation or positive cTnI and followed them for 45 months. In a study by McCann and colleagues (2008) PAPP-A did not predict MI diagnosis in patients with acute ischemic-type chest pain. Moreover, in another study by the same group, STEMI, NSTEMI, UA and non-ischemic chest pain patients were followed for one year and PAPP-A was not found to be a predictor of adverse events even in an unadjusted model (McCann *et al.*, 2009). However, there are doubts about the sensitivity of the assay (Ultra sensitive enzyme immunoassay, Demeditec Diagnostics) used in the studies of this group as the majority of the measured levels resided below the lowest standard (11.25 ng/mL) provided by the assay manufacturer (assay information from the manufacturer web page: http://www.demeditec.com/en/products/product_details/backPID/23/preview/papp_a_ultra_sensitive_elisa/beginat/20/; accessed August 30, 2009).

Molecular diagnostics

PAPP-A has also been studied from the molecular diagnostic point of view in ACS by Park and colleagues (2007). Interestingly, they noticed that PAPP-A IVS6 + 95 C allele is associated with an increased risk of MI (dominancy odds ratio 2.13 [95% CI 1.12-4.07; p=0.022]; codominancy odds ratio 1.89 [95% CI 1.14-3.16; p=0.015). This allele was found to be an independent risk factor even after adjustment for traditional risk factors. The single nucleotide polymorphism associated with this allele is located in an intron of the PAPP-A gene and the knowledge of its functional significance is lacking. However, the polymorphism may possibly be associated with other functional gene variants in the exons of the PAPP-A gene.

2.4.3 Other vascular diseases

Atherosclerosis may affect arteries in various parts of the body leading to plaque formation, causing obstruction of the flow of blood and resulting in symptoms in the tissues affected. Typically, plaques form to the coronary arteries where they may lead to ACS or SA. However, the process of atherosclerosis may also lead to plaque formation in the carotid arteries, thus possibly causing problems in the blood supply of the brain. A sudden rupture of a plaque in the carotid artery may lead to embolisation of plaque or thrombus material to the brain inducing transient ischemic attack or more severe ischemic stroke. Pronounced atherosclerosis in the lower limbs causes pain especially associated with physical exercise and often presents as intermittent claudication. Severe peripheral atherosclerosis may even lead to necrosis in the lower limbs and need for amputation.

Coronary atherosclerosis

Cosin-Sales and colleagues (2004) found higher PAPP-A levels in patients with complex coronary stenoses than in patients who did not have complex stenoses among SA patients undergoing angiography. The concentration of PAPP-A also correlated with the number of complex stenoses found in a patient. Similar associations were noticed with the ratio of PAPP-A concentration over proMBP concentration. In another study by the same group, PAPP-A levels were found to be higher in the SA patients with multi-vessel disease than in SA patients with single-vessel disease or without significant coronary artery stenoses (Cosin-Sales *et al.*, 2005). Accordingly, the circulating PAPP-A concentrations of single-vessel disease patients were significantly higher than those of patients without stenoses. In this study, PAPP-A was found to be an independent predictor of the presence of CAD. With a PAPP-A cut-off value of 4.5 mIU/L the sensitivity of PAPP-A for CAD prediction was 45% and specificity 86%. The area under the curve from receiver-operating curve analysis was 0.75 [95% CI 0.72-0.78].

PAPP-A has also been found to predict adverse cardiac events in SA patients. Elesber and colleagues (2006) showed that PAPP-A concentrations associated significantly in a multivariable model with the occurrence of death (adjusted hazard ratio 5.29 [95% CI 1.27-22.0, p=0.023) and the combined end-point of death and ACS (adjusted hazard ratio 3.56 [95% CI 1.27-10.0, p=0.015]) in a group of SA patients referred for angiography and followed for 4.9 years. In a study by Consuerga-Sanchez and colleagues (2008) that was conducted with SA patients with a median follow-up time of 8.8 years, serum PAPP-A concentration above 4.8 mIU/L was an independent predictor of all-cause mortality with a hazard ratio of 1.95 [95% CI 1.14-3.36, p=0.016]. The sensitivity of PAPP-A to predict mortality at this cut-off concentration was 84%, but the specificity was only 33%. It is noteworthy that proMBP concentration and the PAPP-A over proMBP ratio were not found to be independent predictors of mortality.

Coronary arteries severely narrowed due to extensive coronary plaque growth in SA patients are often opened by PCI, similarly to the manner in which the blocked arteries are opened in ACS patients. A common delayed complication of this procedure is restenosis, the narrowing of the artery lumen again in time. In a study by Li and colleagues (2008) it was shown that serum PAPP-A levels were higher 6 months after PCI in those patients in which restenosis was detected with angiography at that time. Thus, PAPP-A seems to be associated with restenosis and may also be involved in the processes leading to this complication.

Carotid atherosclerosis

Circulating PAPP-A levels have been associated with carotid lesions and the vulnerable structure of carotid plaques. Beaudeux and colleagues (2003) reported on higher circulating PAPP-A levels in those asymptomatic hyperlipidemic individuals who were evaluated with ultrasonography to have hyperechoic or isoechoic, echogenic carotid lesions, i.e. advanced plaques with vulnerable structure in the carotid artery, than in patients without such lesions. No correlation was found between PAPP-A levels and *intima-media* thickness, an indicator of the size of the carotid plaque determined ultrasonographically. Similarly, Pellitero and colleagues (2009) did not find a correlation between PAPP-A and *intima-media* thickness when they studied type 2 diabetes patients. However, in a study by Aso and colleagues (2004) a significant correlation between PAPP-A and the *intima-media* thickness of carotid plaques was shown in type 2 diabetes patients. The group of Sangiorgi (2006) revealed that the plasma levels of PAPP-A were higher in carotid endarterectomy patients whose removed carotid plaques were vulnerable in structure or were ruptured with thrombus than in patients whose removed plaques were stable in structure. In the same study the plasma PAPP-A levels also correlated significantly with the extent of immunohistochemical staining for PAPP-A in tissue samples of the removed plaques.

Fialová and colleagues (2006) found higher circulating PAPP-A levels in patients who had experienced a cerebrovascular event than in controls. When certain types of cerebrovascular events were looked at separately, the differences in PAPP-A levels compared to controls were significant only in patients with intrachranial haemorrhage or in ischemic stroke patients who had CAD. Patients with ischemic stroke without CAD did not have elevated PAPP-A levels, although ischemic stroke rather than haemorrhage is often associated with plaque rupture and embolisation.

Setacci and colleagues (2008) studied patients who underwent carotid stenting to prevent new cerebrovascular events. Among patients who had experienced transient ischemic attack or stroke they found higher post-operative than pre-operative serum concentrations of PAPP-A.

Peripheral atherosclerosis

In addition to carotid and coronary atherosclerosis, an association has also been found between circulating PAPP-A and peripheral atherosclerosis by the group of Mueller (2006). They noticed higher serum PAPP-A levels in patients with symptomatic peripheral arterial disease than in age, sex and diabetes status matched controls. In this study PAPP-A predicted peripheral arterial disease in the fourth PAPP-A quartile in a multivariable model with an odds ratio of 2.86 [95% CI 1.78-4.59, $p<0.001$].

Hypercholesterolemia is a significant risk factor for cardiovascular diseases. Stůlc and colleagues (2003) reported on higher serum PAPP-A concentrations in patients with isolated cholesterolemia but without symptoms of vascular disease than in healthy controls. However, there was no significant correlation between PAPP-A and serum lipid levels. On the other hand, the group of Ceska (2003) found no difference between the serum levels of PAPP-A in patients with isolated cholesterolemia, in patients with combined hypercholesterolemia, in patients with severe but stable CAD or in patients without hypercholesterolemia.

2.4.4 Renal dysfunction

Kidney dysfunction is generally associated with increased risk for adverse cardiovascular events (Hage *et al.*, 2009). In the study by Fialova and colleagues (2004), circulating PAPP-A levels correlated with serum creatinine levels ($r=0.68$, $p<0.05$) in chronic haemodialysis patients and with 24-h creatinine clearance ($r=-0.59$, $p<0.05$) in chronic renal insufficiency patients who had not yet been dialyzed. However, the serum concentrations of PAPP-A were not significantly higher in these patients than in controls. Astrup and colleagues (2007) found elevated PAPP-A levels in type 1 diabetic patients with diabetic nephropathy presenting with persistent significant albuminuria when they were compared to matched type 1 diabetic patients with normal albumin secretion or to non-diabetic controls. In diabetic nephropathy patients the glomerular filtration rate, the indicator of kidney function, had an inverse correlation with PAPP-A ($r=-0.21$, $p=0.003$).

Significantly higher PAPP-A levels have been reported in end-stage renal disease patients undergoing haemodialysis therapy than in healthy controls (Kalousova *et al.*, 2003; Fialova *et al.*, 2004; Lauzurica *et al.*, 2005b; Hodkova *et al.*, 2006; Coskun *et al.*, 2007b). Increased levels have also been found in peritoneal dialysis patients and haemodiafiltration patients. One group reported on higher PAPP-A elevations with haemodialysis than peritoneal dialysis (Fialova *et al.*, 2004), while others have shown similar elevations with different dialysis modalities (Kalousova *et al.*, 2006; Coskun *et al.*, 2007b). The levels of PAPP-A have been shown to remain unchanged (Fialova *et al.*, 2004; Kalousova *et al.*, 2006) or slightly increase (Coskun *et al.*, 2007b) when PAPP-A concentrations before the dialysis session have been compared to levels measured at 4 h after beginning the dialysis. However, more detailed sampling revealed a significant increase in plasma PAPP-A at 15 min and decrease thereafter during the haemodialysis session and a similar effect was seen with the heamodiafiltration procedure (Hodkova *et al.*, 2005; Kalousova *et al.*, 2006). Correlations have been found in haemodialysis patients between PAPP-A and advanced oxidation protein products (Kalousova *et al.*, 2003), CRP (Fialova *et al.*, 2004), cytokines IL-6 and TNFα (Lauzurica *et al.*, 2005a), F2-isoprostanes as markers of oxidative stress (Lauzurica *et al.*, 2005b), intact parathyroid hormone, alkaline phosphatase activity and dialysis therapy duration (Coskun *et al.*, 2007c). In haemodialysis patients serum PAPP-A has been shown to associate with higher mortality rates (Kalousova *et al.*, 2004).

Elevated PAPP-A levels have also been found in renal transplant patients when compared to healthy controls (Coskun *et al.*, 2007c). However, serum PAPP-A concentrations have been shown to decrease after kidney transplantation (Kalousova *et al.*, 2007). In renal transplant patients PAPP-A correlates inversely with glomerular filtration rate ($r=-0.52$, $p<0.001$) (Kalousova *et al.*, 2007). Moreover, significant correlations with PAPP-A have been reported in transplant patients with urea ($r=0.547$, $p=0.001$), creatinine ($r=0.497$, $p=0.001$), uric acid ($r=0.452$) and CRP ($r=0.387$, $p=0.001$) (Coskun *et al.*, 2007c). However, other studies revealed no correlation with CRP (Kalousova *et al.*, 2007) or creatinine (Lauzurica *et al.*, 2005a). In a graft biopsy taken 3 months after transplantation serum PAPP-A levels seemed to associate with histological findings of interstitial inflammation ($r=0.57$, $p<0.05$) and vascular fibrous intimal thickening ($r=0.47$, $p<0.05$) possibly implying a worse fate for the graft (Kalousova *et al.*, 2007).

Delayed graft function, new-onset diabetes mellitus after transplantation (NODAT) and chronic allograft nephropathy are frequent complications after renal transplantation. Serum PAPP-A levels in samples taken 1-3 h before kidney transplantation were not significantly

higher in those non-diabetic patients who developed NODAT than in those who did not develop NODAT within a mean follow-up time of 5.7 months (Bayes *et al.*, 2007). However, in a study by Lauzurica and colleagues (2008) significantly higher PAPP-A concentrations were measured 6-8 h before renal transplantation in those patients who developed delayed graft function within the first week after transplantation than those who did not. Additionally, in a multivariate analysis PAPP-A was identified as an independent risk factor of delayed graft function with an odds ratio of 2.96 [95% CI 1.34-5.40]. PAPP-A in serum samples taken 6-8 h before renal transplantation predicted chronic allograft nephropathy with a risk ratio of 4.27 [95% CI 1.03-17.60] (Lauzurica *et al.*, 2005a). Moreover, in the same study PAPP-A was also an independent predictor of post-transplant cardiovascular events with a risk ratio of 6.4 [95% CI 1.23-33.11].

2.4.5 Other conditions

Some studies have evaluated the levels of PAPP-A in blood in other conditions than those already presented above. Pellitero and colleagues (2007) found higher circulating PAPP-A levels in type 2 diabetes mellitus patients with stable glycaemic control and without macrovascular disease than in matched controls. Interestingly, in these diabetic patients serum PAPP-A concentrations correlated inversely with HbA1c, the indicator of haemoglobin glycosylation ($r=-0.2$, $p=0.03$). Coskun and colleagues (2007a) reported on higher serum PAPP-A levels in patients with newly diagnosed asthma and without anti-inflammatory medication than in healthy controls. In this study a correlation was also found between serum PAPP-A and the severity of asthma ($r=0.581$, $p<0.01$). In the study by Bulut and colleagues (2009) the serum PAPP-A levels were found to be higher in lung cancer patients than in age, sex and smoking history matched controls. However, no differences were seen between different cancer forms (small cell carcinoma, epidermoid carcinoma, or adenocarcinoma). In the lung cancer patients serum PAPP-A levels correlated inversely with the Karnofsky performance status, the indicator of the functional status of the patient ($r=-0.330$, $p<0.001$).

Rysava and colleagues (2007) found no differences in serum PAPP-A levels between patients with rheumatological diseases and histologically proven amyloid A amyloidosis, patients with rheumatoid arthritis but without amyloidosis and healthy controls. Moreover, in a study by Iversen and colleagues (2008a) no differences in mean PAPP-A serum concentrations were detected between patients with certain diagnoses among 1448 consecutive patients admitted to hospital with diagnoses other than ACS.

2.4.6 Effect of medication

Heparin

When using PAPP-A as a marker measured in the blood, it is imperative to know how different medications affect the circulating levels to avoid confounding effects and to discover the possible beneficial or harmful influence of certain medication schemes.

As discussed previously, it has been shown in *in vitro* studies that PAPP-A is able to bind to cell surfaces by attaching to glycosaminoglycans, presumably heparan sulfate, with its GAG binding site located at the CCP3 and CCP4 domains of PAPP-A (see section 2.1.2) (Laursen *et al.*, 2002b). The binding can be reversed by heparan sulphate or heparin GAGs (Laursen *et al.*, 2002b). Westergaard and colleagues (1983b) have shown the direct interaction of PAPP-A with heparin which can be reversed with specific heparin antagonist pro-

tamine sulphate. Heparin-binding alters the electrophoretic mobility of PAPP-A and leads to dose-dependent artificial increase in the concentrations of PAPP-A measured with electroimmunoassay implying dose-dependent change in the molecule (Westergaard *et al.*, 1983b).

GAGs are heterogenous polysaccharides in terms of their molecular mass, charge, density and physico-chemical properties as they are not synthesized as homologous copies of templates. In the human body, heparin is found primarily in the mast cells and it resembles heparan sulphate in structure. Its most prominent constituent is a disaccharide of sulphated L-iduronic acid and sulphated D-glucosamine. (Reviewed by Korir and Larive, 2009.)

Due to their anticoagulative effects, heparin products are widely used in the treatment of patients in whom blood coagulation should be prevented. Unfractionated heparin (UFH) is usually recovered from porcine instestines and is always a mixture of molecules of different structures and molecule sizes. The mixture consists of mucopolysaccharide molecules with a molecular weight ranging from 3 000 to 30 000 composed of alternating sulfated disaccharide units. The sulfated groups give heparin a highly negative charge. UFH inhibits blood coagulation by catalyzing the reaction between antithrombin III (ATIII) and thrombin, thus inhibiting the conversion of soluble fibrinogen to blood clot forming fibrin. The inhibition of thrombin by ATIII also requires binding of the same heparin molecule to thrombin. In addition, heparin binding to ATIII leads to inactivation of factor Xa of the coagulation cascade. (Reviewed by Davenport, 2008.)

Low molecular weight heparins (LMWH) can be produced through chemical or enzymatic degradation of UFH to shorter polysaccharides (Korir and Larive, 2009). The average molecular weight varies from 4200 to 6000 between products. LMWH are not long enough to also bind thrombin when attached to ATIII and their anticoagulation activity is thus mediated only through inactivation of factor Xa. The half-life of LMWHs in human circulation varies between different products but is generally significantly longer than that of UFH due to differences in the metabolic routes. (Reviewed by Davenport, 2008.)

Heparin administration has been shown to increase rapidly and significantly the concentration of certain molecules in the circulation. For example, in a study by Jones and colleagues (2004) UFH elicited a rapid and dose-dependent release of activin A and follistatin in the circulation of adult female sheep. Multiple administration of heparin led to repeated responses to each injection, but the subsequent stimulation increased and extended the responses, consistent with saturation of the heparin clearance mechanism. The rapidity of the release, within 5 min, suggested a release from the luminal surface of the vascular endothelium directly into the circulation. Similarly, a rapid and robust release of activin A and follistatin into the circulation at the time of vessel clamping and administration of UFH (5000 IU) was shown by Phillips and colleagues (2000) in patients who were undergoing abdominal aortic aneurysm repair or carotid endarterectomy. The peak concentrations were reached at 5-15 min after the clamping of the artery and heparin administration. Moreover, in the same study, administration of heparin (2500 IU) to coronary angiography patients produced a similar activin A and follistatin response.

In a study by Baldus and colleagues (2006) heparin administration has also been shown to affect the circulating levels of a candidate molecule for a new ACS marker, myeloperoxidase (MPO). Plasma concentrations of MPO increased significantly in patients who under-

went coronary angiography with UFH bolus administration (70 U/kg), approximately 1.6-fold at 15 min after administration. The increase was higher in patients with CAD (p=0.01) than in patients without detectable CAD. Moreover, in the same study, *ex vivo* heparin treatment of extracellular matrix proteins, cultured endothelial cells and saphenous vein graft specimens from CAD patients resulted in MPO release to the surrounding solution implying that MPO was released into the circulation from the blood vessel wall.

Terkelsen and colleagues (2009) have reported preliminary evidence that heparin may also affect the circulating concentrations of PAPP-A. In their study, plasma PAPP-A levels were compared between 84 STEMI patients, who received UFH (10 000 U) and were treated with PCI before or after sampling, and 14 STEMI and 56 NSTEMI patients who were not given UFH or any other medication before sampling. The PAPP-A levels in the plasma samples tended to be higher in the group of STEMI patients with UFH and PCI treatment than in the STEMI patients without UFH and PCI treatment (median 18 [25^{th},75^{th} percentile: 5.8,40] vs. 5.5 [3.7,24] mIU/L, p=0.07). Plasma PAPP-A levels were significantly higher in the STEMI patients with UFH and PCI treatment than in the NSTEMI patients (5.2 [3.4,7.0] mIU/L, p<0.001). However, the exact timing information of the heparin administration in relation to sampling was lacking. Moreover, samples before and after heparin administration taken from the same individuals were not available. In the group of STEMI patients with UFH and PCI, PAPP-A seemed to be cleared from the circulation within a few hours after onset of symptoms or treatment with PCI.

In the same study Terkelsen and colleagues showed that injection of human recombinant PAPP-A in mice with concomitant UFH administration resulted in higher circulating PAPP-A peak levels than without UFH and in delayed recombinant PAPP-A clearance. When UFH administration was delayed for 5 min after PAPP-A administration, re-elevation in circulating PAPP-A concentration was seen, implying that heparin may possibly facilitate PAPP-A detachment from the vessel wall.

The finding that heparin products may affect circulating PAPP-A concentration is especially important in that the results of previous studies may have been biased by heparin medication. UFH and LMWH are widely used in the treatment of ACS patients (Mahaffey *et al.*, 2005). The likelihood of bias caused by heparin for all ACS related PAPP-A studies published thus far is estimated in Table 3. However, studies with non-ACS patients may have also been affected by the heparin effect as, for example, UFH or LMWH are generally used to prevent blood clotting during dialysis sessions (Davenport, 2008).

Other medication

The effect of certain other medications on circulating PAPP-A concentrations has also been under investigation. It has been reported that the use of atorvastatin (20 mg/day) for 10 or 12 weeks did not affect serum PAPP-A levels in isolated cholesterolemia patients although significant decrease was seen in LDL, total cholesterol and CRP levels (Ceska *et al.*, 2003; Stulc *et al.*, 2003). Similarly, in a study by Pellitero and colleagues (2007) no association was found between the concentration of circulating PAPP-A and statin use in type 2 diabetes patients. However, Miedema and colleagues (2008) showed a borderline significant (p=0.05) decrease in PAPP-A levels in ACS and stable CAD patients after use of atorvastatin (80 mg/day) for 1 month. A lower dose of 10 mg/day did not have a similar effect.

The use of fenofibrates in patients with combined hyperlipidemia caused no changes in serum PAPP-A levels and neither did LDL-apheresis in patients with hypercholesterolemia and severe but stable CAD (Stulc *et al.*, 2003). However, in a population of >80 years old hypertensive individuals PAPP-A was significantly lower in those who took calcium channel blockers to reduce hypertension than in those without this medication (Onder *et al.*, 2007). In patients undergoing off-pump coronary artery bypass graft surgery, there was a significant increase in PAPP-A blood levels measured at 72 h after the procedure in patients who received the intravenous anaesthetic propofol although with anaesthetic gas sevoflurane treatment the levels mildly decreased (Lucchinetti *et al.*, 2007). The effects on PAPP-A concentrations are currently unknown for other drugs often given to CAD or ACS patients, such as acetylsalicylic acid, β-blockers, clopidogrel, glycoprotein IIb/IIIa inhibitors, inhibitors of angiotensin-converting enzyme and nitrates.

Intravenous iron is given to haemodialysis patients to enhance the response to erythropoietin therapy aimed for preventing kidney failure and dialysis induced anaemia. Hodkova and colleagues (2005) noticed that intravenous iron given during haemodialysis session increased and prolonged the previously found PAPP-A elevation during the dialysis session. In kidney transplant patients, immunosuppressive medication is invaluable in preventing rejection reactions against the renal graft. No differences were found in the circulating concentrations of PAPP-A between the transplantation patients using cyclosporine microemulsion or tacrolimus for immunsuppression (Lauzurica *et al.*, 2007). Similarly, Coskun and colleagues (2007c) found no effect of immunosuppressive drugs on PAPP-A serum levels in renal transplant patients. It has also been noticed that vitamin E, a potent antioxidant, did not change the serum levels of PAPP-A within 5 weeks of oral therapy (400 mg / day) in haemodialysis patients (Hodkova *et al.*, 2006).

Finally, Jones and Clemmons (1995) noticed that circulating levels of PAPP-A are higher in growth hormone deficient adults than in matched control subjects. However, one year of growth hormone therapy reduced the serum PAPP-A concentrations to normal level.

3 AIMS OF THE STUDY

The overall aim of this thesis was to study the novel ACS-associated biomarker, PAPP-A. As an ACS-related form of PAPP-A was identified, the further aims were to develop an easy and rapid detection method for this molecule and to evaluate analytical and clinical performance of the method. In addition, we also studied how the heparin products commonly used as medication in ACS patients affect the PAPP-A molecule and the circulating concentrations of PAPP-A forms.

More specifically the aims were:

I To characterize the PAPP-A forms present in the circulation of ACS patients.

II To develop a point-of-care detection method for the ACS-related form of PAPP-A and to evaluate the performance of the method in serum and heparin plasma.

III To evaluate the performance of the developed point-of-care method in the detection of the ACS-related form of PAPP-A in heparinized whole blood.

IV To evaluate the clinical performance of the ACS-related form of PAPP-A measured by the developed detection method as a risk marker predicting adverse cardiac events in ACS patients.

V To investigate the effect of treatment with intravenous heparin products on the circulating concentrations of PAPP-A forms.

4 SUMMARY OF MATERIALS AND METHODS

The detailed descriptions of the materials and methods used in this study can be found in the original publications (**I-V**). A brief summary with some additional information is presented here.

4.1 Clinical samples and standard materials

The sample panels used in the studies (**I-V**) are described in Table 4. All samples were collected after informed consent of the donor and the study protocols were in conformance with the Declaration of Helsinki as revised in 1996. The serum and plasma samples were stored at -20 °C (pregnancy serum) or at -70 °C (other samples). Heparinized whole blood samples were used freshly.

Table 4. Description of the clinical sample panels used in the studies.

Collection place and time[a]	Study population		Gender (males/ females)	Age (years)[b]	Sampling	Sample matrix	Publication and use	
TUCH 2000-2001	541 consecutive patients with ACS symptoms	8 STEMI patients	4/4	males 63 (13), females 72 (16)	Admission; 1, 2, 4, 6, 24 and 48 h	serum	**I**	Study of PAPP-A forms in serum
		200 with ACS symptoms	112/88	69 [55,77]	Admission; 6-12, 24 and 48 h; discharge	serum	**II**	Developed total PAPP-A assay vs.an older total PAPP-A assay
		267 NSTE-ACS patients	136/131	70 [60,78]	Admission; 6-12 and 24 h	serum	**IV**	free PAPP-A as a prognostic biomarker in ACS patients
HUCH 2004-2005	39 STEMI patients		26/13	62 [56,70]	Prehospitally; before PCI; 0, 2, 12 and 24 h after PCI	serum and heparin plasma	**II**	Serum vs. heparin plasma as sample matrices, total PAPP-A assay evaluation
TUCH 2007-2008	14 haemodialysis patients		9/5	62 (9)	Before; 5, 15 and 120 min (+20, 30, 60, 125, 135 or 240 min from some patients)	serum	**V**	LMWH effect on circulating PAPP-A concentration
TUCH 2007-2008	10 angiography patients		5/5	65 (15)	Before; 5 and 15 min; at the end	serum	**V**	UFH effect on circulating PAPP-A concentration
SSI 1993-1996	100 women participating first-trimester prenatal screening program	3	0/3	-	Gestational age 9, 10 or 11 weeks	serum	**I**	Study of PAPP-A forms in serum
		3	0/3	-	Gestational age 5, 8 or 9 weeks	serum	**II**	Complexed PAPP-A assay evaluation
Labquality	159 apparently healthy non-pregnant individuals		79/80	56 [50,70]	-	serum	**II**	Determination of reference values
UTU-DB 2006	11 healthy volunteers		4/7	20-45	-	heparin whole blood and plasma	**III**	Heparinized whole blood as the sample matrix

[a] HUCH, Helsinki University Central Hospital, Finland; Labquality, Finland (a commercial panel); SSI, Statens Serum Institut, Denmark; TUCH, Turku University Central Hospital, Finland; UTU-DB, University of Turku / Department of Biotechnology, Finland

[b] Ages presented as mean (standard deviation), median [25th ,75th percentile] or range

A pool of third trimester pregnancy sera diluted in a buffer containing 0.05 mol/L Tris-HCl pH 7.75, 0.15 mol/L NaCl, 0.5 g/L NaN_3 and 60 g/L bovine serum albumin (BSA) was used as the standard material for the PAPP-A assays (**I**-**V**). BSA was either from Intergen (Millipore, USA) or Sigma-Aldrich (USA). The standard material was calibrated against the pooled third-trimester pregnancy serum-derived WHO IRP 78/610 standard for pregnancy-associated proteins (WHO International Laboratory for Biological Standards, Statens Serum Institut, Copenhagen, Denmark). The standard solutions were stored at -20 °C until use.

4.2 Recombinant PAPP-A

The recombinant human PAPP-A used in publication **III** and in some unpublished experiments described here was produced in human embryonic kidney 293T cells which were transiently transfected with pFLAG-CMV-1 plasmid containing the coding sequence of the whole human PAPP-A. The plasmid was a generous gift from Dr. Xuezhong Qin from J. L. Pettis Memorial Veterans' Medical Center, Loma Linda, USA. The transfection was conducted according to the instructions of the transfection reagent manufacturer using a ratio of 1 µg of plasmid DNA and 6 µL of FuGENE® 6 transfection reagent (Roche Diagnostics, Switzerland). The cells were grown in DMEM culture medium supplemented with 100 mL/L fetal bovine serum. The supernatant was collected 8 days after transfection, cleared by centrifugation and stored at -20 °C until use.

4.3 Antibodies

In the studies the six monoclonal IgG antibodies (B1-B6) used were created through immunisation of mice with PAPP-A/proMBP complex and were a kind gift from Dr. Michael Christiansen from Statens Serum Institut, Copenhagen. Eleven monoclonal antibodies (A1-A11) similarly developed were received from HyTest Ltd., Finland.

Antibodies used for detection in the immunoassays were labeled with a heptadentate europium chelate ({2,2´,2´´,2´´´-{[4-[(4-isothiocyanatophenyl)ethynyl]pyridine-2,6-diyl]-bis (methylenenitrilo)}tetrakis(acetato)}europium(III)) (Takalo *et al.*, 1994) (**I**) or with a nonadentate α-glucose europium chelate ({2,2´,2´´,2´´´-{[2-(4-isothiocyanatophenyl)ethyl imino]bis(methylene)bis{4-{[4-(α-glucopyranoxy)phenyl]ethynyl}-pyridine-6,2-diyl}bis (methylenenitrilo)}tetrakis(acetato)}europium(III)) (von Lode *et al.*, 2003) (**II**, **III**, **IV**, **V**). These inherently fluorescent chelates and the biotinylation reagent, biotin isothiocyanate, were kindly provided by Jaana Rosenberg (University of Turku, Finland).

The labeling reactions are described in detail in original publications **I** and **II**. In brief, 15- to 100-fold molar excess of Eu^{3+} chelate over the antibody was used in the reaction containing 1 mg/mL antibody in 0.05 mol/L Na_2CO_3/$NaHCO_3$ buffer, pH 9.6. Ethylene glycol in the concentration of 100 mL/L was also included in some reactions to enhance the labelling efficiency (**II**). The Eu-labeling reactions were incubated overnight at room temperature in the dark. Antibody biotinylations were conducted with 1 mg/mL antibody concentration in 0.05 mol/L Na_2CO_3/$NaHCO_3$ buffer, pH 9.6, with 15- to 50-fold molar excess of biotin isothiocyanate over the antibody by incubating for three hours at room temperature in the dark. The labeled antibodies were separated from free chelate or biotin isothiocyanate by gel filtration using a buffer containing 0.15 mol/L Tris-HCl (pH 7.75), 0.15 mol/L NaCl, and 0.5 g/L NaN_3 for elution. The labeled antibodies were stabilized with 1 g/L BSA

and stored at 4 °C. Typically, 5 to 15 Eu^{3+} chelates became attached to each antibody molecule in the labeling procedure.

4.4 Immunoassays

4.4.1 Manual assays

For the immunoassays conducted by manual pipetting (**I**), low-fluorescence 96-well plate microtitration wells (NUNC, Denmark) were coated with the capture antibody either through passive interaction of 400 ng of antibody per well (**I**, details in Qin *et al.*, 2001) or through binding of 100 - 300 ng of biotinylated antibody in 50 µL of assay buffer (**I**, details in Pettersson and Soderholm, 1990) in each microtitration well previously coated with streptavidin (Innotrac Diagnostics).

After washing with wash solution (**I**, details in Pettersson and Soderholm, 1990) sample or calibrator and 100 - 300 ng of detection antibody in assay buffer or in modified assay buffer (containing 0.1 g/L denatured mouse IgG and 0.2 g/L native mouse IgG) in a total volume of 30 µL were added to the wells. The wells were incubated for 30 - 60 minutes at 36 °C with 900 rpm shaking in iEMS shaker/incubator (Labsystems, Finland), washed six times with wash solution, dried under a stream of 60 °C air for 5 min and allowed to cool at least for 5 min. The time-resolved europium fluorescence was measured from the dry-surface with a Victor™ 1420 multilabel counter (PerkinElmer; Wallac, Finland).

4.4.2 Automated assays in all-in-one dry-cups

To prepare all-in-one dry-cups (**II**), 300 ng of biotinylated capture antibody in 50 µL of assay buffer was immobilised in streptavidin coated single-wells (Innotrac Diagnostics) overnight at ambient temperature. After washing, 40 µL of a protective solution was added to the wells and dried overnight at 35 °C and 5% relative humidity. Europium-labeled detection antibody (200 - 300 ng per well) was added in 1 µL of optimized label diluent buffer (von Lode *et al.*, 2003) on top of the insulating layer and dried immediately in a stream of hot air. The dry-reagent wells were packed in analyte pens and stored at 4 °C protected from humidity until use. The calibrator wells were prepared otherwise similarly but a known concentration of PAPP-A calibrator was added to the protective solution.

The assays were performed with an automated Aio! Immunoanalyzer with a protocol applying in each dry-cup 20 µL of sample and 10 µL of Aio! buffer (Innotrac Diagnostics). The instrument incubated the cups at 36 °C for 15 minutes with shaking and, after washing for six times and drying the cups, time-resolved fluorescence was measured from the surface of the cup. The concentrations of samples were obtained from the analyzer as it compared the fluorescence signals against a calibration curve constructed by the instrument with the calibrator wells.

4.5 Gel filtration studies

In gel filtration studies samples were fractionated with a Superose 6 PC 3.2/30 gel filtration column (GE Healthcare Life Sciences, UK) operated with the SMART system (GE Healthcare Life Sciences) at 10 °C. A buffer containing 0.05 mol/L Na_2HPO_4/NaH_2PO_4, 0.15 mol/L NaCl and 0.2 g/L NaN_3, pH 7.0 (gel filtration buffer) was used for the equilibration of the column and the elution at a flow rate of 40 µL/min. The samples were filtered through 0.45 µm and 0.22 µm filters and diluted two-fold in the gel filtration buffer prior to

the injection to the column. Fractions were collected between elution volumes 0.6 mL and 2.8 mL (100 µL fractions) or 0.9 mL and 1.9 mL (25 µL fractions) and analysed immediately.

4.6 Studies with heparin products

The effect of heparin products on the circulating levels of PAPP-A was studied with 14 haemodialysis patients and with 10 CAD patients who underwent elective coronary angiography (**IV**). The coronary angiography patients received UFH (LEO®, LEO Pharma, Denmark) at the start of the procedure as an intravenous bolus of 5000 IU. One patient was treated with PCI and thus received additional UFH boluses. The haemodialysis patients received enoxaparin (Klexane®, Sanofi-Aventis, Belgium), an LMWH widely used in haemodialysis treatment to prevent extracorporeal circuit clotting (Davenport, 2008). Following the standard patient care at Turku University Central Hospital, 40 to 60 mg of LMWH was administered intravenously in the dialysis line at the start of the dialysis session. In four of the patients with increased risk of bleeding, a heparin-free dialysis protocol was used. Two of these patients received a reduced LMWH bolus (20 mg) at 2 h after the start of the procedure.

Studies were also conducted in which enoxaparin and UFH in concentrations approximating those of treated patients (8.5 mg/L and 1.1 mIU/L, respectively) were added to whole blood samples of haemodialysis patients which were anticoagulated by citrate, EDTA, Li-heparin (standard blood collection glass vacuum tubes from Venoject®, Terumo Europe) or hirudin (recombinant hirudin 2 µg/mL, America Diagnostica, USA) (**IV**).

In addition, gel filtration studies were performed with recombinant PAPP-A in gel filtration buffer, serum or heparin plasma of a healthy individual or in serum in which enoxaparin (2 mg/mL) was added (**unpublished**). Serum and heparin plasma from ACS patients were also compared with pregnancy serum by gel filtration (**unpublished**).

4.7 Statistical analyses

Statistical analyses (**II**, **III**, **IV, V**) were performed with statistical softwares SAS version 9.1 or 9.2 with SAS Enterprise guide 3.0 (SAS institute, USA) and GraphPad Prism for Windows version 4.02 (GraphPad Software Inc., USA). All p values were reported 2-tailed and p values <0.05 were considered significant. The normality of the distribution of the values of each continuous variable was determined by the Kolmogorov-Smirnov test, the shape of the distribution and the probability plots. Pearson correlation was used to assess the linear correlation of continuous variables. Spearman rank-correlation was used with the variables with non-gaussian distribution. The Mann-Whitney *U*-test test was utilized for assessing the differences between groups with non-normally distributed continuous variables and the Student *t*-test with normally distributed continuous variables. Wilcoxon signed rank test with Bonferroni correction was used when comparing the levels of a continuous variable in samples taken from patients at different time points. Categorical variables were compared between groups with the Chi-Square test. The multivariate associations were analysed using Cox's proportional-hazards model.

5 SUMMARY OF RESULTS

5.1 PAPP-A forms

5.1.1 ACS patients (I, II)

The concentration of PAPP-A was found to be elevated in the circulation of ACS patients in 2001 (Bayes-Genis 2001). However, pronounced fluctuations in the concentration were detected in serial samples collected from ACS patients (Qin *et al.*, 2002b). Therefore, we tested various antibody combinations in order to detect possible blood borne degradation or complexation products of PAPP-A in serial serum samples of ACS patients. Although no such products were found, it was noticed that, in the samples showing elevated PAPP-A levels with assays using PAPP-A reactive antibodies, antibodies reactive with the proMBP subunit of the PAPP-A/proMBP complex did not detect this elevation.

Based on the epitope map that was constructed with the available monoclonal antibodies, assays were designed to specifically detect all molecules with PAPP-A subunits (Assays T (**I**), total PAPP-A assay (**II**, **III**, **IV**, **V**)) or PAPP-A only in complex with proMBP (Assay C (**I**), PAPP-A/proMBP assay (**II**, **III**, **IV**), complexed PAPP-A assay (**V**)) (Fig. 9). Significant concentration elevation was only detected in ACS samples with the total PAPP-A assay while the low levels detected with the complexed PAPP-A assay remained stable (Fig. 10A). Moreover, the correlation between the total PAPP-A and complexed PAPP-A levels detected in a large panel of serum samples from ACS patients was not strong (r = 0.532) (Fig. 10B).

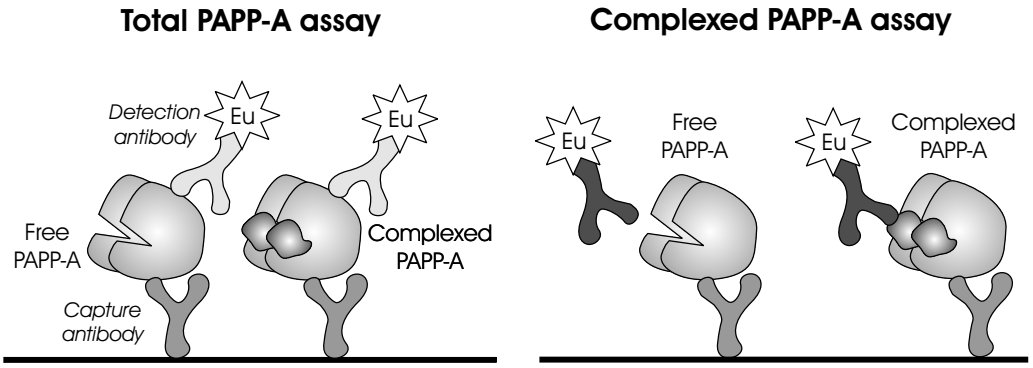

Figure 9. The principle of the assays designed for total PAPP-A and complexed PAPP-A detection. The capture antibody in both assays binds to the PAPP-A subunit. Thus, all PAPP-A subunit containing molecules in the sample become attached to the surface. In total PAPP-A assay the labeled detection antibody binds to the PAPP-A subunit at a separate epitope. Subsequently, all PAPP-A subunit containing molecules whether or not complexed with proMBP are detected. In complexed PAPP-A assay the labeled detection antibody binds to the proMBP subunit and thus only the PAPP-A/proMBP complex is detected. The concentration of free PAPP-A is determined by calculating the difference of the results given by these two assays. The labeled antibodies are labeled with europium chelates (Eu) which enable signal detection by time-resolved fluorescence.

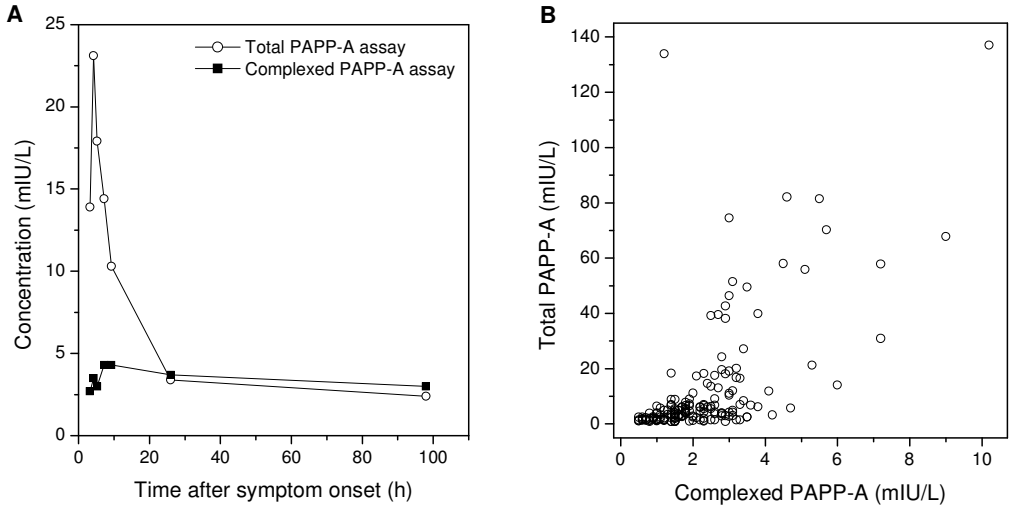

Figure 10. Total PAPP-A and complexed PAPP-A in ACS patients. **A)** The concentrations of total PAPP-A and complexed PAPP-A of serial serum samples collected from an ACS patient at certain time points after the onset of symptoms. **B)** Correlation of the total PAPP-A concentrations and the complexed PAPP-A concentrations in 651 serum samples of 200 ACS patients (r = 0.532).

With gel filtration studies it was revealed that the molecular weight of the PAPP-A form causing the elevation in the total PAPP-A assay signal in ACS patient serum also differed from the molecular weight of PAPP-A in pregnancy serum. PAPP-A in pregnancy serum eluted earlier from the gel filtration column than PAPP-A in ACS serum implying that PAPP-A in pregnancy serum was larger in molecular size. The PAPP-A form in fractionated pregnancy serum was equally detectable by the total PAPP-A and complexed PAPP-A assays, while the PAPP-A form in fractionated ACS serum was not detected by the complexed PAPP-A assay.

5.1.2 Healthy individuals (II)

We found, in an apparently healthy population, the total PAPP-A median level to be 2.4 [25^{th},75^{th} percentile: 2.0,3.1] mIU/L and the 97.5^{th} percentile upper reference limit to be 4.9 mIU/L (Fig. 11). Detected median complexed PAPP-A concentration was 2.2 [1.8,3.0] mIU/L and the median free PAPP-A concentration was 0.2 [-0.16,0.47] mIU/L in the same population (Fig. 11). The 97.5^{th} percentile upper reference limit for free PAPP-A was 1.3 mIU/L. A weak but statistically significant correlation was found between total PAPP-A concentration and age ($r=0.290$, $p<0.001$) and complexed PAPP-A and age ($r=0.230$, $p=0.004$). However, such a correlation was not found between free PAPP-A and age ($r=0.075$, $p=0.348$). Total PAPP-A and free PAPP-A concentrations were significantly higher in men than in women (total PAPP-A men 2.8 [2.4,3.5] mIU/L vs. women 2.1 [1.7,2.5] mIU/L, $p<0.001$; free PAPP-A men 0.28 [0.0,0.7] mIU/L vs. women 0.04 [-0.2,0.6] mIU/L, $p=0.003$).

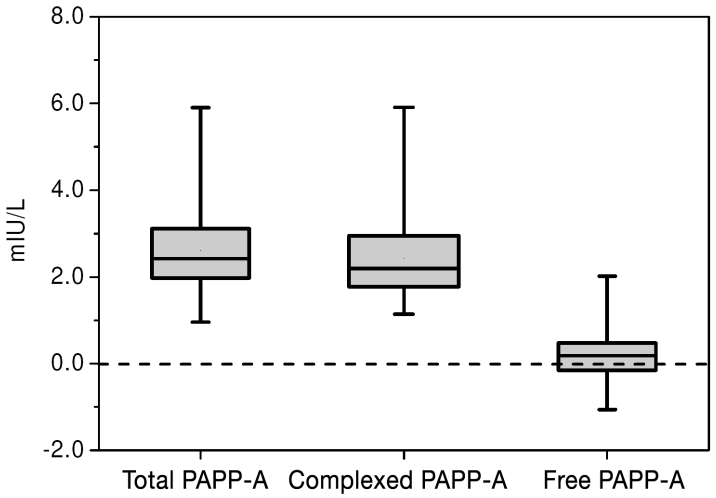

Figure 11. Distribution of total PAPP-A, complexed PAPP-A and free PAPP-A in a population of 159 apparently healthy non-pregnant individuals. (The top and bottom whiskers indicate the minimum and maximum concentration; the other horizontal lines represent the 25th, 50th and 75th percentiles.)

5.2 Point-of-care assays for free PAPP-A measurement

5.2.1 Evaluation of analytical performance (II, III)

To enable easy and rapid determination of free PAPP-A, time-resolved immunofluorometric point-of-care assays were developed for total PAPP-A and complexed PAPP-A. The concentration of free PAPP-A was calculated from the results given by these assays by subtracting the measured complexed PAPP-A concentration from the measured total PAPP-A concentration.

The analytical performance characteristics of the developed assays are shown in Table 5. Both assays gave linear signal response with the pregnancy serum derived calibrators diluted in buffer as well as with ACS and 1st trimester serum samples in the range relevant in the context of ACS samples, 0-300 mIU/L. ACS serum was used for the evaluation of the total PAPP-A assay. However, the evaluation of the complexed PAPP-A assay was mainly conducted with pregnancy serum because the concentration of complexed PAPP-A in ACS serum is generally low (<5 mIU/L). There was a good agreement between the concentrations measured from serum samples and respective heparin plasma samples collected from STEMI patients (Table 5). The assays also performed well when evaluated with heparinized whole blood samples from apparently healthy individuals into which either recombinant PAPP-A as surrogate of the free PAPP-A in ACS serum (total PAPP-A assay) or third trimester pregnancy serum containing complexed PAPP-A (complexed PAPP-A assay) was added (Table 5).

Table 5. Analytical performance characteristics of the developed assays for total PAPP-A and complexed PAPP-A.

	Total PAPP-A assay	**Complexed PAPP-A assay**
Analytical detection limit **(II)**[a]	0.18 mIU/L	0.23 mIU/L
Functional detection limit **(II)**[b]	0.27 mIU/L	0.70 mIU/L
Recovery in serum **(II)**[c]	102% - 107%	88% - 96%
Total assay imprecision **(II)**[d]		
Lower pool	7.2%	9.7%
Higher pool	6.0%	6.7%
Within-run imprecision **(II)**[d]		
Lower pool	5.4%	6.9%
Higher pool	3.6%	4.1 %
Mean relative difference (95% CI)		
Between serum and heparin plasma **(II)**	4.4% (-19.8% to 28.6%)	5.7% (-20.8% to 32.3%)
Between heparin plasma and heparinized whole blood with hematocrit correction **(III)**	15.7% (-10% to 42.3%)	12.5% (-9.2% to 34.3%)

[a] Mean of zero standard + 3SD
[b] Lowest sample concentration with coefficient of variation (CV) <20%
[c] ACS serum (total PAPP-A assay) or pregnancy serum (complexed PAPP-A assay) added to three serum samples of other ACS patients
[d] Determined according to GLSI guideline EP5-A (Total PAPP-A assay: ACS serum lower pool 4.9 mIU/L, higher pool 15.9 mIU/L; Complexed PAPP-A assay: pregnancy serum lower pool 3.1 mIU/L, higher pool 10.0 mIU/L)

5.2.2 Evaluation of clinical performance (IV)

For the clinical evaluation of the developed method for free PAPP-A detection we analysed serum samples from 267 NSTE-ACS patients. Median free PAPP-A in the admission samples of these patients was 1.43 [25^{th},75^{th} percentiles; 1.13,1.95] mIU/L and 65.9% of the patients exceeded the 97.5^{th} percentile of the apparently healthy individuals (**II**). Free PAPP-A in the admission sample correlated only weakly with admission cTnI (r=0.15, p=0.012), 24 h maximal cTnI (r=0.12, p=0.043) and CRP (r=0.17, p=0.005).

Within the follow-up period of 12 months, 21.3% of the patients experienced an adverse event, i.e. had a new nonfatal MI, died or had an MI and died. When the patients were grouped according to PAPP-A tertiles (<1.27, 1.27-1.74, >1.74 mIU/L) there was a significant difference between the tertiles in the number of patients who had an adverse event and the frequency of events increased with admission free PAPP-A (13.5%, 20.2%, 30.3% accordingly; p=0.02). In multivariate analysis with adjustment for age and ischemic ECG findings free PAPP-A was found to be an independent predictor of adverse events (free PAPP-A >1.74 mIU/L, risk ratio 2.0 [95% CI 1.0-3.9, p=0.05]) as were also positive maximal cTnI and CRP above 2.0 mg/L.The combination of free PAPP-A <1.27 mIU/L and negative maximal cTnI identified a low-risk patient group while the highest risk was seen in patients with free PAPP-A >1.74 mIU/L and positive maximal cTnI.

Total PAPP-A had an inferior performance when compared to free PAPP-A in predicting adverse cardiac events. The total PAPP-A concentration in the admission sample exceeded the total PAPP-A 97.5th percentile of healthy individuals only in 6.4% of the patients. The frequency of adverse events did not differ significantly between total PAPP-A tertile groups (<1.98, 1.98-2.99, >2.99 mIU/L; 19.1%, 19.1%, 23%; p=0.54). Moreover, total PAPP-A was not found to be an independent predictor of adverse events in multivariate analysis.

5.3 Effects of heparin products on PAPP-A

5.3.1 In vivo effects (V)

When PAPP-A levels were studied in haemodialysis patients, it was noticed that the circulating levels of free PAPP-A increased significantly and very rapidly after the start of the dialysis procedure (Fig.12A). This release was found to be caused by the intravenous bolus of LMWH as the increase was not seen at the beginning of the dialysis session in patients with heparin-free procedure. Moreover, in those patients who received delayed LMWH bolus the free PAPP-A increase coincided with LMWH delayed administration. After the peak value the concentration of free PAPP-A decreased rapidly. There was high variation between patients in the magnitude of the heparin product induced free PAPP-A release.

The circulating concentrations of free PAPP-A were also followed in stable CAD patients during elective angiography. The patients received UFH intravenously at the beginning of the procedure and similar rapid elevations in free PAPP-A were seen as in the dialysis patients, although the response did not seem to be equally high (Fig.12B). Interestingly, with one patient who was treated with PCI and who received additional boluses of UFH, repeated free PAPP-A increases were seen coinciding with the UFH boluses. Complexed PAPP-A did not increase significantly in any of the dialysis or angiography patients.

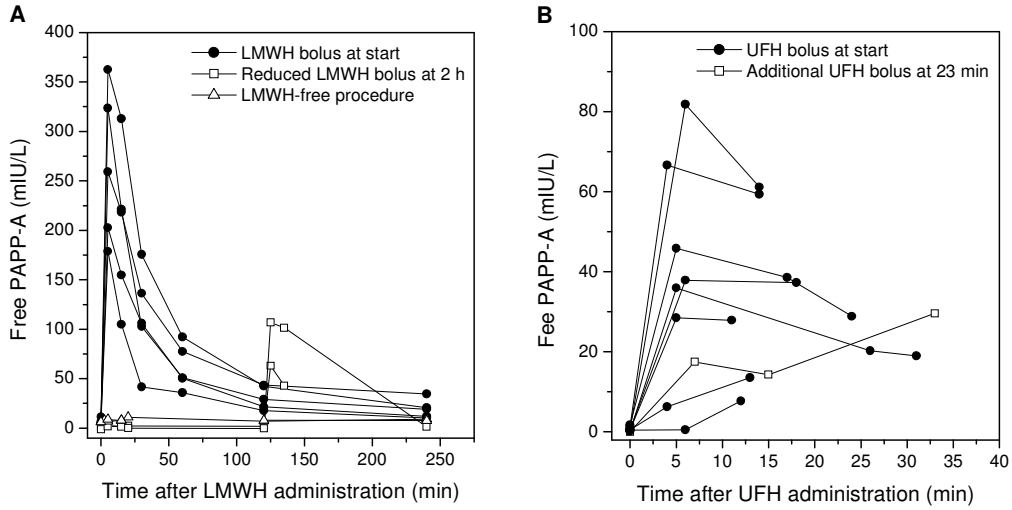

Figure 12. Free PAPP-A concentrations in serum samples of **A)** haemodialysis patients and **B)** patients undergoing elective angiography.

5.3.2 In vitro effects (V, unpublished)

The effect of heparin products on PAPP-A concentrations was also studied *in vitro*. With whole blood samples from haemodialysis patients anticoagulated by EDTA, citrate or hirudin it was revealed that LMWH or UFH in treatment concentrations did not cause an increase in free PAPP-A concentration in plasma. Moreover, no difference was seen in free PAPP-A levels of samples collected in heparin anticoagulated tubes when they were compared to samples collected in tubes with other anticoagulants or serum tubes. Thus, it is unlikely that the blood cells are the origin of free PAPP-A that becomes released into the circulation due to heparin injection. Furthermore, the results confirm that the PAPP-A increase detected is not a result of an artefact caused by the effect of heparin on the detection method.

In gel filtration studies recombinant PAPP-A in buffer or serum eluted from the column significantly later than recombinant PAPP-A in heparin plasma or recombinant PAPP-A in serum with LMWH (Fig. 13A). A similar effect was seen when the PAPP-A elution profile of an ACS patient serum sample was compared to that of an ACS patient heparin plasma sample (Fig. 13B). Thus, the apparent molecular weight of recombinant/free PAPP-A put in heparin product containing solution appears to be larger than that of recombinant/free PAPP-A without a contact with a heparin product. Moreover, the molecular weight of recombinant PAPP-A in contact with UFH (the form of heparin in heparin plasma) seemed to be larger than the molecular weight of recombinant PAPP-A in contact with LMWH (Fig. 13A).

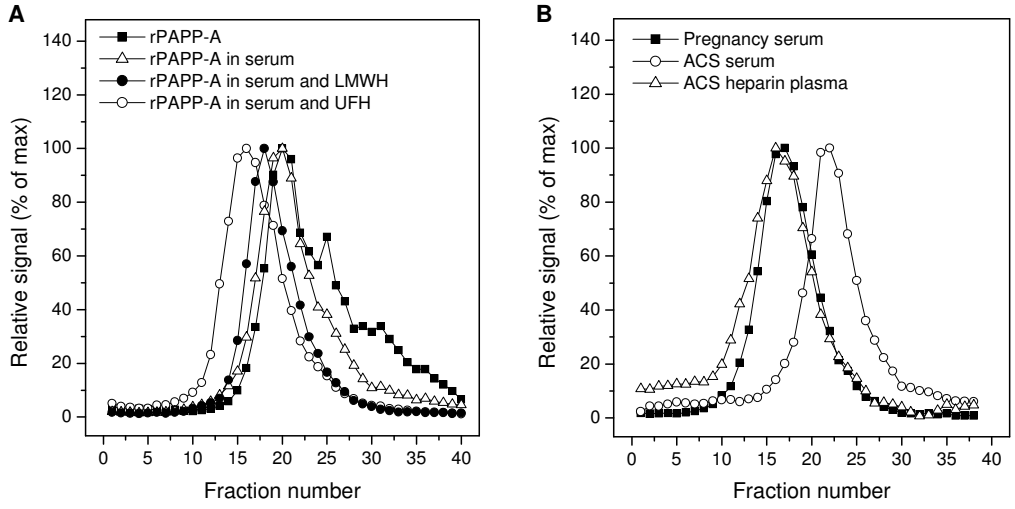

Figure 13. Total PAPP-A assay relative signals (% of maximum) of the gel filtration fractions of **A)** recombinant human PAPP-A (rPAPP-A) containing samples and **B)** samples from a pregnant woman and an ACS patient. (25 µL fractions collected between elution volumes 0.9 mL and 1.9 mL)

6 DISCUSSION

6.1 PAPP-A forms

Our results implied that the form of PAPP-A which becomes released into the circulation in ACS is not complexed with proMBP as PAPP-A in ACS serum was not detected with proMBP reactive antibodies. The difference in the molecular size between PAPP-A in pregnancy serum and PAPP-A in ACS serum suggested that the PAPP-A form in ACS circulation could represent the free noncomplexed dimeric form of PAPP-A rather than PAPP-A complexed with other molecules. Complexation with smaller molecules possibly involving only one PAPP-A subunit cannot be excluded. However, to date, such complexes have not been reported.

As PAPP-A has been found in ruptured and eroded coronary plaques but not in stable plaques (Bayes-Genis *et al.*, 2001a) and as PAPP-A concentration in blood is associated with the risk for adverse cardiac events (see section 2.4.2), it seems possible that PAPP-A has an active role in the progression of atherosclerotic plaques to vulnerable state. The role of PAPP-A in various systems appears to be linked to its enzyme activity (see section 2.3) and hence to the release of IGFs from the binding proteins. It is noteworthy that only the free form of PAPP-A seems to be active (Overgaard *et al.*, 2000; Chen *et al.*, 2002). Thus, it would be rational that PAPP-A exists in and is released from the plaque in the free form. However, the form of PAPP-A in atherosclerotic plaque has not been yet confirmed by immunohistochemical staining because free PAPP-A specific antibodies have been lacking. The mechanism of ACS associated free PAPP-A release to circulation (in the absence of heparin products) is still unknown.

We analysed the different forms of PAPP-A in a reference population. The results showed that in apparently healthy individuals free PAPP-A levels are close to zero and that the baseline total PAPP-A concentration detectable in the samples is almost exclusively comprised of the complexed form of PAPP-A. These results imply that possibly, especially at low total PAPP-A levels, free PAPP-A could better distinguish between ACS patients with an ACS-associated elevation of free PAPP-A form from those non-ACS individuals with naturally occurring higher total PAPP-A baseline caused by complexed PAPP-A (Fig. 14). Importantly, supporting evidence of this was obtained in this thesis when the point-of-care method developed for free PAPP-A detection was clinically evaluated with ACS patient samples. Free PAPP-A was shown to be superior to total PAPP-A as a prognostic marker. Thus, free PAPP-A seems to be more specific marker than total PAPP-A in reflecting the adverse processes associated with worse outcome in ACS patients.

The characteristics of the PAPP-A assays that have been used in other studies related to ACS are shown in Table 6. The assays are expected to detect total PAPP-A as specific binders for free PAPP-A have not been available, although specifications on PAPP-A forms detected are not available for all assays. It is noteworthy that for one of the assays (Kryptor BRAHMS) it has confusingly been stated that the assay detects PAPP-A/proMBP complex (Beaudeux *et al.*, 2003). However, assays initially designed for PAPP-A measurement in pregnancy, which is the case with this assay, would be of little use when studying samples from ACS patients, if they detect only the complexed form of PAPP-A. With such assays the ACS-related free PAPP-A elevations would be missed (Qin *et al.*, 2006a). In contrast, elevated levels have been reported in ACS by Hájek and colleagues (2008) using this assay.

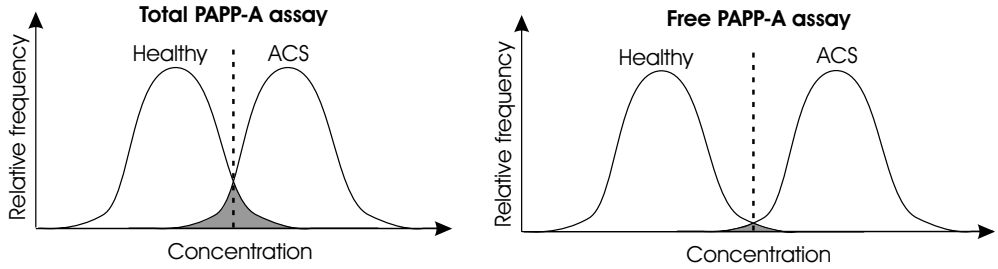

Figure 14. The hypothetical distributions of total PAPP-A and free PAPP-A in ACS patients and healthy individuals. Because complexed PAPP-A baseline levels vary between healthy individuals, it is suggested that, especially at low free PAPP-A elevations, total PAPP-A is not as good in discriminating between ACS patients and healthy individuals as free PAPP-A, which is not affected by complexed PAPP-A.

Table 6. Characteristics of the assays that have been used for PAPP-A detection in studies related to ACS.

Assay	Manufacturer/ Institute	Sample matrix	Detection limit	Calibration	Anti-bodies[a]	Detection method[b]	Assay specificity	References
Bayes-Genis	Statens Serum Institut, Denmark	Serum	0.03 mIU/L	WHO 78/610	pab, mab	Biotin-tyramide-amplified enzyme immu-noassay	Total PAPP-A	Bayes-Genis *et al.* 2001a
Qin	University of Turku, Finland	Serum, heparin plasma	0.5 mIU/L	WHO 78/610	mab, mab	Time-resolved immunofluoro-metric assay	Total PAPP-A	Qin *et al.* 2002a
Roche	Roche Diagnostics, Swizerland	Plasma	?	?	?	Electrochemi-luminescence enzyme immunoassay	?	Heeschen *et al.* 2005, Iversen *et al.* 2008
Rossen	University of Southern Den-mark, Denmark	Serum, EDTA plasma	3.3 mIU/L	WHO 78/610	mab, mab	ELISA (HRP)	Total PAPP-A	Rossen *et al.* 2007
BRAHMS Kryptor (commercial)	BRAHMS, Germany	Serum	4 mIU/L	WHO 78/610	mab, mab	Time-resolved amplified cryptate emis-sion (TRACE) assay	Unclear [c]	Spencer *et al.* 2003, Beaudeaux *et al.* 2003
Demeditec (commercial)	Demeditec Diagnostics, Germany	Serum	0.023 µg/L	?	?	ELISA (?)	?	Manufacturer web page [d]
DRG (commercial)	DRG International, USA	Serum	0.023 µg/L	?	pab, pab	ELISA (HRP)	?	Manufacturer assay infomation sheet[e]
DSL (commercial)	Beckman Coulter, Diagnostic Systems Labo-ratories (DSL), USA	Serum	0.24 mIU/L	WHO 78/610	mab, mab	ELISA (HRP)	Total PAPP-A	Khosravi *et al.* 2002, Kavsak *et al.* 2009

[a] All assays with sufficient details reported are two-site immunoassays using polyclonal antibodies (pab) or mono-clonal antibodies (mab) at each site

[b] Abbreviations: ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase

[c] Specificity for PAPP-A/proMBP complex has been reported which is in contradiction with the elevations detected by this assay in ACS patients (Hajek *et al.* 2008)

[d] http://www.demeditec.com/en/products/product_details/backPID/23/proview/papp_a_ultra_sensitive_elisa/beginat/20/; accessed 30. August 2009

[e] DRG PAPP-A US ELISA (EIA-4512)

The reported (total) PAPP-A baseline levels in healthy individuals have varied remarkably (Fig. 11). Our results of total PAPP-A concentration in an apparently healthy reference population lie near the lower end of results of the previous studies (Fig. 15). The differences between studies may be due to the use of different assays involving different antibody combinations. However, calibration related problems may also exist, as reported mean or median levels of healthy individuals have varied significantly even between studies using the same assay (Fig. 15). For some assays, the detection limit does not seem low enough to reliably detect the PAPP-A concentrations in healthy individuals (detection limits >3 mIU/L) (Table 6).

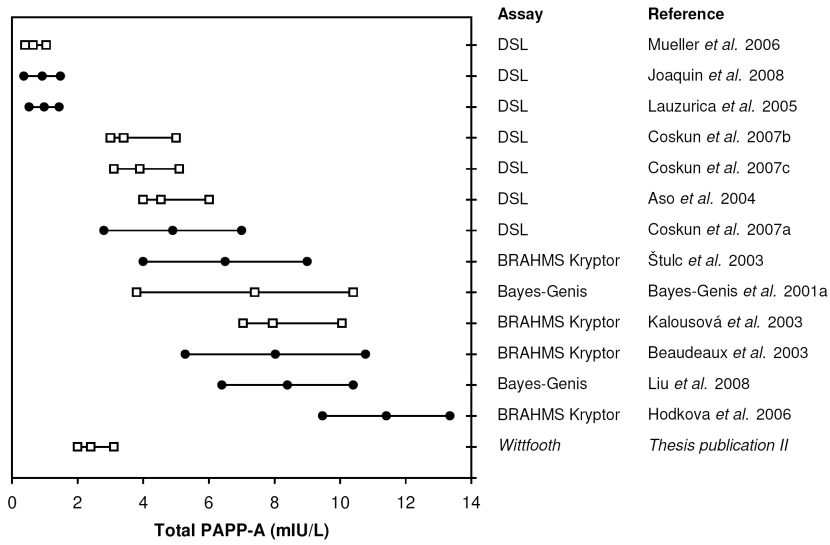

Figure 15. PAPP-A concentrations measured in apparently healthy control cohorts in different studies by different assays. (Closed circles indicate the mean concentration ±SD; open squares represent the 25th, 50th and 75th percentiles.)

6.2 Point-of-care assays for free PAPP-A measurement

In this thesis study a point-of-care method was developed for the detection of free PAPP-A. The performance characteristics were shown to be good for the purpose of ACS sample analysis. The method enables rapid and sensitive detection of free PAPP-A in serum, heparin plasma and heparinized whole blood samples. The possibility to use whole blood as the sample matrix is especially important for point-of-care assays as it removes the need of centrifugation and plasma extraction as sample preparation steps and thus enables faster sample analysis.

The method developed for free PAPP-A measurement involves the use of two assays. However, the ideal approach would be a single direct assay that specifically detects only the free form of PAPP-A. The use of two assays results in larger reagent costs, more time spent per analysis of a sample and also in a higher limit of detection due to the imprecision of the two assays accumulating in the final result. The reason for the use of the two assay approach is that free PAPP-A specific antibodies were not available at the time of the development of these assays and have only very recently started to emerge. In future studies, direct assays for free PAPP-A should be developed to avoid the limitations of the two assay

approach. Especially in the case of sensitivity it would be very interesting to see how low the circulating levels of free PAPP-A are in healthy individuals. This would presumably better reveal the power of free PAPP-A to discriminate between normal population and ACS patients at risk.

Assays for the enzyme activity of PAPP-A could also serve as an option for free PAPP-A measurement as complexation with proMBP inactivates PAPP-A. However, some PAPP-A may be ineffectively inactivated by proMBP as low residual activity has been detected with PAPP-A/proMBP complex purified from pregnancy serum (Overgaard *et al.*, 2000). This may negatively affect free PAPP-A measurement with the activity approach.

The clinical evaluation of the free PAPP-A detection method developed revealed that free PAPP-A is an independent prognostic marker in ACS patients when measured in the admission sample. The prognostic information of free PAPP-A was additive to that of cTnI and CRP implying that free PAPP-A may reflect other processes than these markers and could offer benefit when used together with these markers. However, as a new ACS related marker, free PAPP-A competes with the other emerging markers and therefore should also provide additional or superior prognostic information when compared with them. Especially, brain-type natriuretic peptide (BNP) and N-terminal proBNP, markers associated with heart failure, have also shown significant promise in improving risk prediction of ACS patients (Morrow *et al.*, 2007; Bonaca and Morrow, 2008) and thus it would be important to compare free PAPP-A with these markers. Finally, to become accepted in use, a new ACS related marker should be able to guide the treatment of patients. However, for this aim large further studies are required. As knowledge on the pathological processes leading to certain biomarker elevation in the circulation increases, new treatment methods may be discovered that specifically lower the biomarker related risk.

6.3 Effects of heparin products on PAPP-A

By analyzing samples from haemodialysis patients and patients undergoing elective angiography, it was shown that intravenously administered heparin products (LMWH and UFH) induce a rapid increase in circulating free PAPP-A. The mechanism of PAPP-A release into the circulation by heparin products is unknown. However, release from the endothelium of the vasculature has been suggested (Terkelsen *et al.*, 2009). As previously mentioned, the dimeric recombinant PAPP-A has been shown to bind to cell surfaces by the GAG binding site and heparin has been shown to release recombinant PAPP-A from the cell surface (Laursen *et al.*, 2002b). The rapid appearance of free PAPP-A in blood as a response to heparin products supports the idea of endothelium being the site of release as it is readily accessible for the intravenously administered heparin and optimal for the direct rapid release of PAPP-A into the circulation.

The magnitude of the heparin product induced free PAPP-A release seemed to differ significantly between patients in our study. The reason for this is unknown. The small number of patients included in the study does not allow reliable analysis of associations between free PAPP-A release and interesting variables such as the presence or extent of CAD or the future risk of death or cardiac events. Thus, studies with a larger number of patients would be interesting in this respect.

The reason for lower free PAPP-A responses in angiography patients than in dialysis patients is unclear. The possible explanations include lower ability of UFH than LMWH to

release free PAPP-A or lower reserves of free PAPP-A to be released by heparin in CAD patients than in dialysis patients. Studies that would involve dialysis patients receiving UFH instead of LMWH as well as angiography patients receiving LMWH instead of UFH could shed more light on this matter.

We also studied the effect of heparin products on PAPP-A *in vitro* with gel filtration and confirmed the previous findings of others obtained with other techniques that PAPP-A interacts with heparin (Laursen *et al.*, 2002b; Weyer *et al.*, 2004; Conover *et al.*, 2007). In our studies heparin products increased the apparent molecular weight of endogenous free PAPP-A and recombinant PAPP-A. A rational explanation for this phenomenon would be the binding of free PAPP-A molecules with heparin molecules. The size of UFH commonly varies between 3 and 30 kDa. Binding of two 30 kDa UFH molecules to PAPP-A dimer would increase the mass similar extent as the binding of two proMBP subunits. Accordingly, in the gel filtration studies the apparent size of PAPP-A in ACS heparin plasma was the same as that of complexed PAPP-A in pregnancy serum (Fig. 13B). However, in addition to changing the mass, heparin products may also alter the elution of PAPP-A from the gel filtration column in other ways. Being highly negatively charged, heparin changes the electric charge of the molecule it binds to. However, there was a relatively high salt concentration in the elution buffer intended to prevent the effect of electrochemical forces on the elution. The heparin binding of PAPP-A may also have some conformation altering effects on the very large PAPP-A molecule. As only the elution of globular molecules is linearly dependent on the molecular weight, a significant conformation change may have a larger effect on the elution than expected by the actual change in molecular weight.

It has been shown in the studies summarized here that LMWH or UFH increase free PAPP-A in the blood of haemodialysis and angiography patients and others have reported results implying that UFH may do so in STEMI patients (Terkelsen *et al.*, 2009). As was discussed in section 2.4.6, the effect of heparin products on circulating PAPP-A levels may have significantly influenced at least some of the previous studies related to PAPP-A in ACS and other conditions. It is very important that in the future this phenomenon is acknowledged when designing new studies. However, it should be noted that no patients who had received heparin product treatment before admission sample were included in our study described in section 5.2.2 (**IV**) and thus these results were not biased by the heparin effect. Importantly, although ACS patients are often medicated with heparin products, this effect does not render PAPP-A ineffective as a marker in this condition because the clinical relevance of PAPP-A as a biomarker would be as a very early diagnostic or prognostic marker in the situation when the diagnosis is still unknown and hence no medication has yet been administered.

The assays used for the analysis of PAPP-A may also be affected by the LMWH or UFH medication of the patients. It has been noticed that some assays are significantly negatively affected by heparin probably due to masking of the antibody epitopes on PAPP-A and therefore heparin plasma is not recommended as the sample material for such assays (Qin *et al.*, 2002a; Spencer, 2003). Thus, serum is generally the preferred sample material for the PAPP-A assays that have been used in the ACS related studies (Table 4). However, it is imperative to confirm that the PAPP-A assays used are not affected by heparin-PAPP-A interaction also when serum samples from patients medicated with heparin products are analysed. This was the case with the PAPP-A assays developed in this thesis (Table 5).

7 CONCLUSIONS

Although cardiac troponins as markers of myocardial necrosis have become a widely used, important diagnostic tool in ACS, in recent years there has been a quest to find new markers that would improve the management of patients with ACS symptoms. A new marker should fulfil certain criteria to become useful in ACS patient care: 1) It should discriminate between healthy individuals and patients at cardiac risk with high sensitivity early in the course of ACS. 2) It should be detectable by an analysis method that enables a rapid reliable measurement preferably in a point-of-care setting. 3) It should give additional information when used in a multimarker approach combining biomarkers reflecting the complementary pathological pathways associated with ACS. 4) It should be able to guide the treatment by helping to determine the aggressiveness of care (Maisel *et al.*, 2006; Bonaca and Morrow, 2008; O'Donoghue and Morrow, 2008). PAPP-A is one of the new candidate markers under investigation.

In this study it was revealed that PAPP-A is released into the circulation of ACS patients in a form that differs from the form of PAPP-A in pregnancy serum. A point-of-care detection method was developed for this ACS-related PAPP-A form and the performance of the method was evaluated with different sample materials and clinical samples from ACS patients. In addition, we also studied the effect of heparin products, which are widely used as medication in ACS patients, on the PAPP-A molecule and on the concentrations of PAPP-A forms in the circulation.

The main conclusions based on the original publications are:

I PAPP-A is released into the circulation of ACS patients in a noncomplexed free form as this form is not detected by antibodies reactive against proMBP. Further, the molecular weight of this form is significantly smaller than that of the PAPP-A/proMBP complex found in pregnancy serum.

II Free PAPP-A can be analysed rapidly and efficiently with the two-assay point-of-care approach developed based on time-resolved immunofluorometric assays. Non-pregnant individuals seem to generally have a low basal level of complexed PAPP-A in the circulation that is responsible for detectable total PAPP-A concentrations, while the concentration of free PAPP-A in non-pregnant non-ACS individuals appears to be close to zero. Therefore, free PAPP-A may better discriminate between patients with pathological elevations and healthy individuals than total PAPP-A which is also affected by the fluctuating baseline levels of complexed PAPP-A.

III In addition to serum and heparin plasma, heparinized whole blood is eligible as a sample matrix for the point-of-care method developed for free PAPP-A detection. The ability to use whole blood as the sample material obviates the need for plasma separation prior to analysis and thus saves time.

IV Free PAPP-A analysed in the admission sample (before medication with heparin products) with the method developed is an independent predictor of MI and death in NSTE-ACS patients. Furthermore, as a prognostic marker free PAPP-A seems to be superior to total PAPP-A and also gives additional prognostic information when combined with cTnI.

V Intravenous administration of LMWH or UFH induces a rapid release of free PAPP-A to the circulation in haemodialysis patients and in patients undergoing elective angiography. To avoid bias in studies due to this effect, PAPP-A levels should only be measured in patients without or before the initiation of heparin product treatment.

Additionally, with gel filtration studies with free PAPP-A containing ACS blood and with recombinant PAPP-A we confirmed that heparin interacts with PAPP-A, which leads to an increase in the apparent molecular weight of PAPP-A. This is important to be kept in mind when using PAPP-A assays with samples from patients treated with heparin products as the assays may be affected by the interaction.

As the prognostic power of free PAPP-A in ACS patients was confirmed, one objective for future studies is to investigate whether elevated free PAPP-A in the admission sample, possibly along with other new markers or troponins preferably measured with highly sensitive assays, could help in guiding the treatment of patients with ACS symptoms. Another intriguing subject for further studies that arose from the results of this thesis relates to the possible information linked to the extent of free PAPP-A release induced by heparin products. It would be interesting to investigate whether the amount of released free PAPP-A, which was shown to vary significantly between the study subjects in this thesis, reflects the burden of atherosclerotic plaques or, more specifically, the prevalence of vulnerable atherosclerotic plaques in the patients, or is associated with the risk for adverse cardiac events.

In conclusion, free PAPP-A is an interesting novel circulating biomarker that seems to perform better than total PAPP-A in predicting the risk for adverse events in ACS patients when measured at the time of admission. The developed point-of-care assays represent a rapid and easy method for the measurement of this PAPP-A form in serum, heparin plasma or heparinized whole blood. However, the interaction of heparin products with PAPP-A and the release of PAPP-A into the circulation by treatment with heparin products may have influenced the previous studies on PAPP-A in ACS and should be considered when new studies are being designed.

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