An update on the role of carboxypeptidase U (TAFIa) in fibrinolysis

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1. ABSTRACT

Since its discovery more than 20 years ago, a lot has been revealed about the biochemistry and physiological behaviour of carboxypeptidase U (CPU). Recent advances in CPU research include the unravelling of the crystal structure of proCPU and revealing the molecular mechanisms for the marked instability of the active enzyme, CPU. The recent development of two highly sensitive assays has cleared the path toward the direct measurement of CPU in circulation or the determination of CPU generation, rather than the measurement of total proCPU concentration in plasma. Finally, since CPU is known to have a prominent bridging function between coagulation and fibrinolysis, the development of CPU inhibitors as profibrinolytic agents is an attractive new concept and has gained a lot of interest from several research groups and from the pharmaceutical industry. These recent advances in CPU research are reviewed in this literature update.

2. INTRODUCTION

The coagulation and fibrinolytic systems safeguard the patency of the vasculature and surrounding tissues. Both cascades have long been considered as separate entities but the discovery of procarboxypeptidase U (proCPU) or thrombin activatable fibrinolysis inhibitor (TAFI) greatly improved our understanding of cross regulation of both systems.

Carboxypeptidase U (CPU) was first described in 1989 by Hendriks et al. who reported the presence of a novel unstable basic carboxypeptidase in fresh human serum (1). This enzyme was named procarboxypeptidase U (proCPU), where the ‘U’ stands for ‘unstable’. This discovery was confirmed by three independent research groups. Campbell et al. named the newly identified carboxypeptidase proCPR, since it showed a preference for arginine (R) containing substrates (2). In 1991, Eaton et al. provided a first clue of the important role of CPU in
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fibrinolysis. This group was able to purify a new plasminogen-binding protein (3). After isolation of the cDNA and sequence analysis, the protein was named plasma procarboxypeptidase B (plasma proCPB), based on its high sequence similarity to the well-known pancreatic CPB. The binding of the proenzyme to plasminogen, combined with the role of C-terminal lysine residues in the binding and activation of plasminogen on fibrin suggested a role for plasma CPB in fibrinolysis (3). The final link between coagulation and fibrinolysis was disclosed by Bajzar et al. in 1995 (4). They found that the antifibrinolytic effect of thrombin during fibrinolysis was due to the activation of a proenzyme, which they named thrombin activatable fibrinolysis inhibitor (TAFI) (4). Amino-terminal sequencing demonstrated that proCPU, proCPR, plasma proCPB and TAFI are identical (3-6).

Venous and arterial thromboembolism is the largest cause of disease and death in the Western World. Therapy available today includes thrombolytics, anticoagulants and antiplatelet drugs. Because of its prominent bridging function between coagulation and fibrinolysis, the development of CPU inhibitors as profibrinolytic agents is an attractive new concept (7). Furthermore, since the coagulation cascade is unaffected, CPU inhibition may result in fewer bleeding complications than conventional therapy. In recent years several small synthetic and naturally occurring CPU inhibitors have been evaluated in animal thrombosis models and existing in vivo data are intriguing and call for further evaluation in humans (7).

3. BIOCHEMICAL CHARACTERIZATION OF PROCPU

3.1. General considerations

Metallocarboxypeptidases (EC 3.4.17) are a group of enzymes capable of cleaving a single amino acid from the C-terminus of peptide or protein substrates. Their catalytic activity depends on a zinc atom in the active site (8). CPU (EC 3.4.17.20) is a basic metallocarboxypeptidase, meaning that only basic carboxy-terminal amino acids i.e. arginine (Arg) and lysine (Lys) are cleaved off from peptides and proteins (1, 8-11).

ProCPU is a zymogen, circulating in plasma with an apparent molecular mass of 60 kDa on SDS-page (3, 12, 13). ProCPU is synthesized in the liver as a prepropeptide consisting of 423 amino acids (aa), composed of a 22 aa signal peptide, a 92 aa activation peptide and a 309 aa catalytic domain. During secretion, the N-terminal signal peptide is efficiently cleaved off, resulting in the release of proCPU in circulation (3). ProCPU is a glycoprotein with 4 asparagine (Asn) linked glycosylation sites in the activation peptide (Asn22, Asn51, Asn63 and Asn86) (3, 4, 13, 14). Also in the catalytic domain a N-linked glycosylation site (Asn219) has been described, but since it is entirely buried in the protein structure, glycosylation is restricted to the activation peptide (13). After a single cleavage at Arg92, the highly glycosylated activation peptide is cleaved off, releasing the 36 kDa catalytic unit, CPU (3, 4, 13, 14).

Synthesis of proCPU is not entirely restricted to the hepatocytes. Also in megakaryocytes proCPU synthesis has been described during the process of megakaryocytopoiesis (15). This proCPU is present in the α-granules of circulating platelets in a concentration of 50 ng/1 x 10^9 platelets and is released upon platelet activation (15). Recently, it has been demonstrated that platelet derived proCPU is capable of attenuating platelet-rich thrombus lysis in vitro independently of plasma proCPU, and thus could be significant in regions of vascular damage or pathological thrombosis, where activated platelets are known to accumulate (16).

3.2. Genetic features

The proCPU gene, denoted the CPB2 gene, is located on chromosome 13q14.11 (5, 17). The 11 encoding exons stretch over approximately 48 kb of genomic DNA (5, 17). Transcription is initiated from multiple sites in the 5’-flanking region, which does not seem to carry a conserved TATA box (17). Transcription seems to be under control of liver-specific transcription factors, such as C/EBP (18), and also hepatocyte nuclear factor-1α (HNF-1α) is involved in the liver specific expression of CPB2 (19). However, proCPU synthesis is not restricted to the liver, since it is also reported in megakaryocyte cell lines (15). Several single nucleotide polymorphisms (SNPs) have been identified in the proCPU gene (20-23). Polymorphisms in the 5’-flanking region could influence the binding of specific transcription factors and thus promoter activity, whereas mRNA abundance could be altered by SNPs in the 3’-untranslated region (24). Of the polymorphisms in the coding regions, two result in an amino acid substitution, i.e. Thr147Ala and Thr325Ile (21, 23, 25), where only the latter has an influence on the enzyme characteristics (25). These polymorphisms were thought to be the major cause of proCPU variability seen in a normal population (plasma proCPU concentration ranging between 75 – 275 nM) . However, using antigen assays insensitive to genotype dependent artefacts, Frère et al. reported that only approximately 25 % of the variation can be explained by the proCPU polymorphisms (26). This implicates that non-genetic factors are more important for the explanation of variation in plasma proCPU concentration, such as glucocorticoids, interleukins, sex hormones, etc. (27-32).

3.3. Intrinsic enzymatic activity

A single-site cleavage of proCPU leads to the release of the activation peptide, generating the active enzyme CPU. Although being an inactive zymogen, it has been shown that proCPU can exert some intrinsic enzymatic activity toward small synthetic substrates (33). This feature is shared by proCPA, but is completely absent in proCPB. Comparison of the structure of proCPU with that of proCPA2 and proCPB revealed that, in contrast to the latter, the catalytic sites of proCPU and proCPA2 are in the active conformation (34). This will result in the reported intrinsic enzymatic activity of the zymogen (33).

Further comparison of the three crystal structures revealed a striking difference in the rotation of the activation peptide in proCPU compared to proCPA2 and
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proCPB (35). In proCPU, the activation peptide is rotated further away from the catalytic cavity as compared to proCPA2 and proCPB, suggesting that the active site in proCPU is more accessible to substrates compared to the other CPs (35). This observation was in line with a recent publication which reported that proCPU does not only show intrinsic activity toward small synthetic substrates, but that the catalytic centre is also accessible for high molecular substrates, thus implying that the zymogen proCPU should also be considered as being an antifibrinolytic enzyme (36). However, in two publications from independent research groups, this finding was contradicted. These papers demonstrated that the zymogen proCPU is not able to down-regulate fibrinolysis and suggested that the previously published results are compromised by in vitro activation of proCPU (37, 38). Additional structural analysis has demonstrated that although slightly larger, the access tunnel to the catalytic site in proCPU is not accessible for high molecular substrates, due to the larger side chains of the activation peptide (35). Further structural and functional research will be necessary to definitely conclude whether the zymogen proCPU has antifibrinolytic properties.

4. CHARACTERISTICS OF CPU

4.1. Generation of CPU

A single-site proteolytic cleavage at Arg92 of proCPU is necessary to generate the active enzyme CPU. There are several enzymes able to activate proCPU in vitro, including thrombin, plasmin, trypsin and neutrophil-derived elastase. However, there remains some debate on which of these proCPU activators are physiologically relevant. Leurs et al. demonstrated that during in vitro clot lysis CPU generation follows a biphasic pattern, where a first peak of CPU activity appeared after initiation of coagulation (through the action of thrombin) and a second rise in CPU activity was observed during fibrinolysis with plasmin as activator (39). Since thrombin generation will usually precede plasmin formation, the importance of plasmin-mediated proCPU activation may be limited (39). With the construction of mutant variants of proCPU resistant to activation by either thrombin or plasmin, Miah et al. were able to confirm these observations (40). In an in vitro clot lysis assay in the absence of thrombomodulin, they demonstrated that thrombin was the only relevant activator of proCPU, under the conditions described, resulting in an attenuation of fibrinolysis (40). The authors suggest that plasmin-mediated proCPU activation becomes more important in situations where there is a reduced fibrinolytic activity and hence a prolonged lysis time (40). Recently, monoclonal antibodies (41, 42), as well as nanobodies (43), against proCPU were developed, which could discriminate between the various modes of proCPU activation. In this respect, these could be regarded as excellent research tools to identify the in vivo relevant activators of proCPU in several pathophysiological conditions (41-43).

The catalytic parameters of proCPU activation by thrombin (IIa) are a $K_m$ of 2.14 nM and a $k_{cat}$ of 0.0021 s$^{-1}$, implying that IIa is a relatively weak activator of proCPU (44). Following the initial phase of coagulation – tissue factor (TF)-pathway or extrinsic pathway – small amounts of IIa are generated, leading to fibrin formation. However, these low amounts of IIa are insufficient for proCPU activation (44-49). In the second phase of coagulation – through the intrinsic pathway – IIa is able to boost its own generation through the activation of factor XI. This positive feedback loop results in the release of high amounts of IIa, leading to proCPU activation (44-49). In the presence of an intact intrinsic pathway of coagulation, the CPU concentration that is generated, will be sufficient to prolong clot lysis substantially (45, 50). Bajzar et al. demonstrated that half-maximal inhibition of clot lysis time was achieved at a CPU concentration of 1 nM (44). Since proCPU is present in plasma in a concentration of 75 - 275 nM, it is clear that even a minimal activation of proCPU will lead to a substantial attenuation of fibrinolysis (44). To demonstrate the importance of the intrinsic pathway in proCPU activation by IIa, Minnema et al. demonstrated that CPU dependent retardation of clot lysis could be attenuated in vivo by neutralization of factor XI through the addition of specific antibodies (51).

In the presence of thrombomodulin (TM), activation of proCPU by IIa is enhanced 1250 times, almost entirely through an effect on the $k_{cat}$ ($k_{cat}$ = 0.4 - 1.2 s$^{-1}$ in the presence of TM) (44). Since the cofactor TM is able to accelerate proCPU activation by IIa so drastically, it has been postulated that the IIa-TM complex is the physiological activator of proCPU (44). A recent study by Wu et al. identified several positively charged residues of proCPU to be important in its activation by IIa-Tm, through their interaction with negatively charged residues on the C-loop of the TM-EGF-like domain 3 (52). Besides the generation of the antifibrinolytic enzyme CPU, the IIa-TM complex is also responsible for the conversion of protein C to the anticoagulant enzyme activated-protein C (APC) (53). By inactivating factor Va and VIIIa, APC inhibits further IIa formation. These two actions of the IIa-TM complex seem to have opposing effects and are demonstrated to be under strict regulation (Figure 1). (54-56).

A second important activator of proCPU is plasmin. This key enzyme of fibrinolysis is a more potent activator of proCPU compared to IIa alone, with a 10-fold increased catalytic efficiency ($k_{cat}/K_m = 0.008$ µM$^{-1}$s$^{-1}$ for plasmin vs. $k_{cat}/K_m = 0.00098$ µM$^{-1}$s$^{-1}$ for IIa) . In the presence of glycosaminoglycans, such as are found in the subendothelial matrix upon arterial injury, plasmin-mediated proCPU activation becomes 20-fold more efficient. Nevertheless, the catalytic efficiency for plasmin activation remains 10-fold lower compared to IIa-TM, whereby its physiological relevance remains unclear.

To date, there remains some debate on whether the activation peptide is actually released from or stays attached to the catalytic moiety upon activation of proCPU. In attempts to purify CPU, Mao et al. used a Concanavalin A Sepharose column, which interacts with sugar residues in the protein. Since glycosylation of proCPU is restricted to the activation peptide, purification of CPU is only possible
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Figure 1. Activation of proCPU by the thrombin-thrombomodulin complex (IIa-TM). The IIa-TM complex is able to efficiently activate proCPU, resulting in the generation of the antifibrinolytic enzyme CPU. In parallel, this complex is able to activate protein C into a potent anticoagulant enzyme, activated protein C (APC).

...if the activation peptide is still attached to the catalytic domain (57). In an attempt to provide additional evidence that the activation peptide remains noncovalently attached to the catalytic residue upon activation, Buehns et al. performed Western blotting experiments on activated CPU, using an activation peptide specific monoclonal antibody (58). This work showed that the activation peptide stays in close proximity of the catalytic moiety upon activation. Moreover, this interaction was suggested to affect CPU activity (58). The latter was challenged in recent work by Marx et al. where it was demonstrated that the activation peptide was not required for CPU activity, nor did it have an effect on CPU stability (59). The exact role of the highly glycosylated activation peptide remains unclear, and is in need for further investigation. Some of its ascribed functions are ensuring structural integrity of the proenzyme, shielding the catalytic site for physiological substrates, ensuring cellular secretion and increasing the solubility of proCPU (59).

4.2. Instability of CPU

As indicated by its name, CPU is characterized by a profound thermal instability (1). At body temperature, the half-life of CPU is approximately 10 minutes, whereas at room temperature the instability is much less distinct with a half-life of 2 hours. At 0 °C, CPU is highly stable (1, 60-62). Not only a decrease in temperature could result in a significant increase in CPU stability, also the presence of competitive inhibitors (62) and an excess of substrate has been shown to improve CPU stability (63).

It is generally accepted that the marked thermal instability of CPU is the result of conformational changes within the enzyme, rather than a proteolytic cleavage (60, 61). This hypothesis was endorsed by the observation that there are no known physiologic inhibitors of CPU, in contrast to most coagulation and fibrinolytic enzymes. Thrombin is able to proteolytically cleave CPU at Arg302, however this cleavage seems to appear after a conformational change within the enzyme leading to its inactivation (60, 61). This was confirmed with an Arg302Gln mutant showing marked instability at 37 °C, without proteolytic cleavage by thrombin on SDS-page (60, 61). In contrast, proteolytic degradation of proCPU and CPU by plasmin can occur prior to the conformational change (64). However, this represents only a minor pathway for the regulation of CPU activity.

Comparison of CPU to the stable pancreatic CPB - a highly homologous enzyme with 48% sequence similarity – has revealed that the 300-330 amino acid region shows the least sequence similarity (65). In this region, a naturally occurring variation in human proCPU was discovered at position 325 (21). Threonine (Thr) at this position results in a CPU half-life of 7 min, whereas substitution to isoleucine (Ile) at position 325 leads to a 2-fold increase in half-life, i.e. 15 min (25). Extensive mutagenesis studies have been conducted to unravel the mechanism of CPU instability, where all mutations with a stabilizing effect were located in the above mentioned 300-330 amino acid region (60, 61, 65-70).

It wasn’t until 2008 that a more in-depth explanation for CPU instability was revealed by Marx et al., with the resolution of the crystal structure (35). The crystal structure of proCPU shows that CPU stability is...
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directly related to a highly dynamic region between residues 296-350. This region includes the cryptic thrombin cleavage site Arg302 and interacts with the activation peptide through hydrophobic interactions between Tyr341 and residues Val35 and Leu39 (35). Through these interactions, the dynamic region is stabilized by reducing its mobility. Upon activation of proCPU, these stabilizing interactions are lost, leading to increased mobility of the dynamic region. This will result in irreversible unfolding of the protein, resulting in disruption of the catalytic site and thus in loss of activity (35). This conformational change also leads to exposure of the cryptic thrombin cleavage site Arg302, making further proteolytic cleavage of inactivated CPU possible. Moreover, the dynamics of this region are markedly reduced by substrates as well as by reversible inhibitors, such as guanidinoethylmercaptosuccinic acid GEMSA, known stabilizers of the active enzyme CPU (35, 62). The structural data of Marx et al. were confirmed by two publications of another group (71, 72).

4.3. CPU at the interface between coagulation and fibrinolysis

After damage of the vasculature, coagulation is initiated through the tissue-factor pathway, leading to the formation of thrombin. This key enzyme of the coagulation will activate soluble fibrinogen generating fibrin monomers, which will ultimately form a stable thrombus after cross-linking through factor XIIIa. In order to prevent excessive clot formation and safeguard the fluidity of the blood, the fibrinolytic cascade will be activated. The key enzyme of the fibrinolysis is plasmin, which will break down the thrombus, leading to the generation of soluble fibrin degradation products (73-75). Plasmin is formed by the action of tissue-type plasminogen activator (t-PA) on its inactive precursor plasminogen and cleaves fibrin specifically after arginine or lysine residues. This initial phase of fibrinolysis results in the generation of partially degraded fibrin containing C-terminal arginine and lysine residues (73-75). These residues participate in a multifaceted positive feedback loop, since they (i) increase the binding-affinity for plasminogen on the fibrin surface and therefore increase plasmin formation (76-79), (ii) convert Glu-plasminogen to Lys-plasminogen and (iii) promoting the inhibition of plasmin by α2-antiplasmin (80, 81). This feedback loop results in the acceleration phase of fibrinolysis.

High amounts of thrombin – as generated upon the intrinsic pathway of the coagulation – are able to activate proCPU. By continuously removing the C-terminal lysine residues generated by the action of plasmin on fibrin, CPU prevents the fibrinolysis from proceeding into the acceleration phase (77, 78). Through this system, CPU has been shown to push the fibrinolytic system toward the antifibrinolytic state by (i) abrogating the enhanced cofactor activity of partially degraded fibrin on Glu-plasminogen activation, (ii) inhibiting the conversion of Glu-plasminogen to Lys-plasminogen and (iii) promoting the inhibition of plasmin by α2-antiplasmin (77, 78, 80, 81). It was shown independently by two research groups that CPU attenuates fibrinolysis through a threshold dependent mechanism (82, 83). As long as the plasma CPU activity remains above a certain key threshold value – which depends on the t-PA concentration - fibrinolysis stays in its initial phase, only to accelerate once the CPU activity decays to a level below this threshold value (82, 83). The time interval over which the CPU level will stay above the threshold is determined by the plasma proCPU concentration, the extent of proCPU activation by the coagulation cascade and most importantly by the stability of CPU (66, 82, 83). The process of fibrinolysis and the action of CPU are presented schematically in Figure 2.

5. MEASUREMENT OF PROCPU IN PLASMA

For the measurement of proCPU in plasma several assays exist, including immunoassays (ELISAs) as well as activity-based assays. All of these methods have their own inherent advantages and disadvantages and none benefits from the existence of an internationally recognized reference standard for proCPU. The complicated matter of proCPU measurement is extensively reviewed in (84) and here discussed briefly.

Immunoassays for the measurement of proCPU are widespread and have the major advantage of being easy to perform (84). However, one has to take into account that proCPU and CPU are present in plasma in different forms. Antibodies constructed to target proCPU could also have a nonnegligible reactivity toward the active moiety CPU, the activation peptide, inactivated CPU, proCPU bound to plasminogen, etc. (85-87). A second important disadvantage of immunoassays is the genotype dependent reactivity. Several antibodies have been described to show an altered reactivity toward a naturally occurring polymorphism at position 325 of the proCPU protein (85). It is therefore of utmost importance that these assays are well characterized with respect to genotype dependent reactivity. Although ELISAs are very attractive and easy to use, care should be taken with the interpretation of the generated results.

Most available activity-based assays are based on the principle that CPU is able to cleave off C-terminal lysine (Lys) or arginine (Arg) residues from small synthetic substrates. The released Lys or Arg on the one hand or the des-Arg or des-Lys product on the other hand can be quantified (84). A major advantage of activity-based assays is that only the enzymatically active CPU is measured, without the interference of the profragment or inactivated CPU. However, this approach requires quantitative activation of the zymogen proCPU (44, 88). Moreover, activity-based assays are compromised by the interference of carboxypeptidase N (CPN). This plasmatic enzyme is constitutively active and can interfere with CPU measurement due to its similar basic carboxypeptidase activity (89). Finally, in activity-based assays one has to take into account the pronounced thermal instability of CPU, which is genotype dependent (25). Stabilization of the active enzyme can be obtained by placing the samples on ice, by incubating with an excess of substrate or by using a short incubation interval during which linear substrate conversion can be guaranteed (63, 90). It is of
Figure 2. Molecular mechanism of the action of CPU on fibrinolysis. Plasminogen (PLG) and tissue-type plasminogen activator (t-PA) bind to fibrin forming a ternary complex. Plasmin thus formed, initiates lysis by cleaving the fibrin fibres especially after lysine residues (initial phase of fibrinolysis). The generated C-terminal lysine residues (represented as circles) are high affinity binding sites for PLG, leading to the accumulation of PLG on the fibrin surface. Moreover, they induce a conformational change within the PLG molecule, making it a more favourable substrate for activation by t-PA. This is known as the acceleration phase of fibrinolysis. CPU abrogates this feedback enhancement of plasminogen activation by cleaving these C-terminal lysine residues. Hereby, CPU prevents the fibrinolysis from proceeding into the acceleration phase.

Due to the lack of well characterized assays for the measurement of proCPU, results of several studies evaluating the possible role for the plasma proCPU concentration as risk factor for the occurrence of thrombotic disorders have to be interpreted with caution (84). This concern is shared by the Scientific Standardisation Committee (SSC) on fibrinolysis of the International Society of Thrombosis and Haemostasis (ISTH), which expressed the high need for a thorough characterization of proCPU assays (84). Recently, we have evaluated various commercially available proCPU assays, especially with regard to genotype dependent reactivity and were able to show that several assays that are on the market today still display these genotype dependent artefacts. As a result, great care should be taken when choosing an assay for proCPU measurement in a clinical setting, as well as with the interpretation and comparison of reported results (data in submission).

6. MEASUREMENT OF CPU IN CIRCULATION

In normal physiological conditions, CPU circulates in plasma as an inactive precursor, proCPU. Upon activation of the coagulation cascade, CPU is generated which will exert its antifibrinolytic activity and
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thus helps to stabilize the clot. The measurement of the proenzyme in plasma is well established (see above (84)). With the discovery of the threshold phenomenon more evidence was revealed that the direct measurement of CPU in circulation or the extent of CPU generation could be more relevant than the measurement of the proenzyme itself. However, the direct measurement of the active enzyme CPU is not straightforward. A first critical step in CPU measurement is the sample collection. Since CPU concentrations in venous circulation are expected to be very low (low pM range, see infra), ex vivo activation of proCPU must be avoided (91). Blood must be collected on citrate anticoagulant with the addition of D-phenylalanyl-L-prolyl-arginyl chloromethyl ketone (PPACK) and aprotinin, inhibitors of thrombin and plasmin, respectively. Recently, we demonstrated the importance of correct sample collection (91). A second obstacle is the intrinsic instability of CPU (25). To prevent rapid decay of CPU, samples must be placed on ice immediately after collection and subsequent centrifugation must be performed at 4 °C. Despite its thermal instability, CPU activity does not seem to be affected by freeze-thaw cycles, provided that the samples are thawed on ice (92). Apart from the preanalytical problems, there are also several analytical challenges that must be addressed. First, there is a need to measure a CPU concentration in circulation that is expected to be very low. The half-maximal effect of CPU on clot lysis occurs at 1 nM, whereas the maximal effect occurs at 20 nM. Since the circulating concentration of proCPU is 75 – 275 nM, only a small portion needs to be activated to have a significant effect on clot lysis (44). At the site of the thrombus, local CPU concentrations are most likely to be much higher than the CPU concentration in venous circulation. ProCPU is present in both plasma and in platelets and is secreted when platelets are activated by thrombin (15), causing a boost in the local proCPU concentration at the site of blood clotting. Moreover, proCPU can be cross-linked to fibrin by factor XIIa and this interaction can possibly lead to a stabilization of this carboxypeptidase increasing its antifibrinolytic potential dramatically (93). Finally, the assay procedure must be specific enough to measure low amounts of CPU when set against a rather high background activity of CPN circulating in plasma at a concentration of 30 µg/mL or 100 nM (94).

In 2004, Neill and co-workers developed a functional assay for the measurement of CPU in circulation (92). This assay is based on the fact that CPU decreases the cofactor activity of high-molecular-weight fibrin degradation products in the stimulation of plasminogen activation in a concentration-dependent manner (95-97). In a later publication of the same group, Kim and co-workers modified this functional assay to overcome its shortcomings (98). The starting point of this modified assay is similar; it is based on the ability of CPU to remove C-terminal lysine residues that are exposed on plasmin modified fibrin, thereby releasing fluorescently labelled plasminogen that was bound to these lysines by its kringle domains. The present assay directly measures the release of plasminogen rather than measuring the extent of plasminogen activation (98). The functional assay as described by Kim et al. was shown to be sensitive for CPU at a concentration as low as 12 pM. Moreover, it was not confounded by the naturally occurring proCPU Thr325Ile polymorphism or by endogenous plasminogen in the plasma. (98). With this assay, Kim et al. were able to detect basal CPU levels in the plasma of healthy individuals at a concentration of 20.3 ± 9.1 pM (n=5) (98).

Another approach for the direct measurement of CPU in circulation is the use of an activity-based assay. In a study on ischemic stroke patients, an HPLC-assisted activity-based assay was used to demonstrate CPU generation during thrombolytic treatment (91). This assay uses hippuryl-L-arginine (HipArg) as a substrate. However, since this substrate is not selective for CPU, this assay will suffer from rather high background activities from the CPN present in the plasma sample. To overcome this analytical challenge of CPN interference, a specialized procedure was used where a set of two CP-inhibitors were combined, resulting in a limit of detection (LOD) of 1 U/L or 200 pM (91, 99). A more straightforward approach would be the development of a substrate with improved selectivity toward CPU combined with minimal residual activity toward CPN. Recent screening of Bz-Xaa-Arg peptides with an aromatic amino acid at the P1 position and further modifications in this position, lead to the discovery of a selective CPU substrate, benzoyl-ortho-cyano-phenylalanyl-arginine (Bz-o-cyano-Phe-Arg) (100). Very recently, our group published a novel activity-based assay for the measurement of CPU in plasma using the selective substrate Bz-o-cyano-Phe-Arg, thereby limiting the interference of CPN as well as excluding the intrinsic activity of proCPU. The novel assay is easy to perform and its high specificity is translated into a LOD as low as 0.05 U/L or 10 pM. With this assay, basal CPU levels in healthy individuals were found to be below 10 pM (n=15).

In 2005, Ceresa and co-workers reported on the development of ELISAs measuring the extent of proCPU activation (101). A variety of monoclonal antibody (MA) based ELISAs were evaluated for their preferential reactivity toward proCPU before and after activation, identifying immunologic assays that measure the amount of CPU (active as well as inactivated form CPUi) or that are directed toward the released activation peptide (101). In a large study on 300 patients with hyperlipidemia, higher levels of both the activation peptide and CPU/CPUi were observed compared to normolipidemic controls, using the newly identified ELISAs. In contrast, no association was found between total proCPU antigen and hyperlipidemia, suggesting that the extent of activation is a more relevant parameter in CPU research (101).

7. PROCPU/CPU SYSTEM IN THROMBOSIS

In a review article by Leurs et al., published in 2005, an extensive overview of the role of proCPU in thrombotic and haemorrhagic conditions was given (102). Since then, the proCPU/CPU system gained more interest by the scientific community, as indicated by the exponential increase in publications on its pathophysiological role. In Table 1, we focus on recent
### Table 1. Overview of studies investigating the role of the proCPU plasma concentration as a risk factor for thrombotic disorders

<table>
<thead>
<tr>
<th>Author</th>
<th>Description</th>
<th>Technique</th>
<th>Ref</th>
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<tbody>
<tr>
<td><strong>Venous thromboembolism (VTE)</strong></td>
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<tr>
<td>Zee et al.</td>
<td>There is no evidence for an association between six proCPU polymorphisms and the risk of venous thromboembolism.</td>
<td>PCR</td>
<td>(103)</td>
</tr>
<tr>
<td>Lichy et al.</td>
<td>No association was found between the proCPU G438A SNP and the risk of cerebral venous thrombosis.</td>
<td>PCR</td>
<td>(104)</td>
</tr>
<tr>
<td>Verdu et al.</td>
<td>ProCPU levels above the 90th percentile of the controls increased the risk of VTE 4-fold compared with proCPU levels below the 90th percentile (OR = 4.0, 95% CI 1.4-10.9). In patients with VTE, the Thr147Thr genotype was associated with a significant increase in proCPU levels.</td>
<td>Asserachrom TAFI (Diagnostica Stago)</td>
<td>(105)</td>
</tr>
<tr>
<td>Martini et al.</td>
<td>Carriers of the 505G (1474A) allele, which is associated with lower proCPU antigen levels than the 505A (1474T) allele, showed an increased risk of DVT.</td>
<td>In-house developed TAFI ELISA</td>
<td>(106)</td>
</tr>
<tr>
<td>Sucker et al.</td>
<td>The pilot study in 40 patients indicates that the G6 genotype of the C1542G polymorphism of proCPU displays a risk factor for the manifestation of thrombotic microangiopathies.</td>
<td>PCR</td>
<td>(108)</td>
</tr>
<tr>
<td>Verdu et al.</td>
<td>Patients with the Thr325Thr proCPU genotype displayed an increased risk of DVT compared to Thr325Ala and Ile325Val genotypes.</td>
<td>TAFI-1B1 ELISA (Biologics) + Zymutest TAFI</td>
<td>(109)</td>
</tr>
<tr>
<td>Folkerina et al.</td>
<td>High levels of proCPU were not associated with an increased risk of venous and arterial thromboembolism in thrombophilic families.</td>
<td>Pelakit TAFI (Pentapharm)</td>
<td>(110)</td>
</tr>
<tr>
<td>Heylen et al.</td>
<td>Patients with inherited thrombophilia tend to display lower proCPU concentrations compared to controls. High levels of proCPU confer to an almost 4-fold increased risk of spontaneous onset thrombosis in heterozygous carriers of factor V Leiden or FII G20210A mutation. The more stable Ile325Val proCPU was more present in carriers of the FII G20210A mutation compared to controls, and seemed to impose a higher risk for clinical manifestation of the thrombophilic condition.</td>
<td>In-house developed activity-based assay</td>
<td>(111)</td>
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<tr>
<td>Meltzer et al.</td>
<td>No association was seen between proCPU levels or variation in clot lysis times and recurrent venous thrombosis. The results indicate that low levels of proCPU are associated with increased risk of recurrence.</td>
<td>Clot lysis assay + in-house developed electromunnoassay</td>
<td>(112)</td>
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<tr>
<td>Meltzer et al.</td>
<td>Plasma levels of plasma proCPU were the main determinants of CLT, followed by plasminogen, proCPU, prothrombin, and alpha2-antiplasmin. ProCPU levels were associated with thrombosis risk (odds ratios, highest quartile vs lowest, adjusted for age, sex, and body mass index = 1.6).</td>
<td>In-house developed electromunnoassay</td>
<td>(113)</td>
</tr>
<tr>
<td>Kupesic et al.</td>
<td>In children with noncatheter-related deep venous thrombosis (nCDVT), proCPU concentrations were significantly higher compared to controls. Significantly decreased fibrinolysis was found in the patient group suggesting that hypofibrinolysis may play an important role in the pathogenesis of nCDVT in children.</td>
<td>Actichrome TAFI (American Diagnostica)</td>
<td>(114)</td>
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<td><strong>Arterial thrombosis and coronary artery disease (CAD)</strong></td>
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<tr>
<td>Morange et al.</td>
<td>No significant association between proCPU levels and angina pectoris or hard coronary events was present after re-analysis of the PRIME data with an ELISA which was shown to be insensitive to proCPU genotype. This group could also conclude that proCPU gene polymorphisms are strongly associated to plasma proCPU levels.</td>
<td>TAFI-1B1 ELISA (Diagnostica Stago)</td>
<td>(115)</td>
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<tr>
<td>Cruden et al.</td>
<td>Systemic plasma proCPU does not predict reperfusion in patients receiving thrombolytic therapy for acute ST elevation myocardial infarction.</td>
<td>Zymustest TAFI (Hyphen Biomed) + Actichrome TAFI (American Diagnostica)</td>
<td>(116)</td>
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<tr>
<td>Paola Cellai et al.</td>
<td>ProCPU antigen and antigen plasma levels are not increased in acute coronary artery disease patients. Moreover, no difference in proCPU levels was observed according to clinical presentation of symptoms or indication to immediate percutaneous revascularization.</td>
<td>Actichrome TAFI (American Diagnostica) + COALIZA TAFI (Chromagenix)</td>
<td>(117)</td>
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<td>Schroeder et al.</td>
<td>Reevaluation of the study in 2002 which was compromised by genotype-dependent artefacts. Significant associations between proCPU activity and cardiovascular risk factors as well as with CAD were found.</td>
<td>Pelakit TAFI (Pentapharm)</td>
<td>(118)</td>
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<td>Malyszko et al.</td>
<td>In patients with essential arterial hypertension treated with an ACE-inhibitor, significantly higher proCPU concentrations were observed in comparison to beta-blocker treated patients. Moreover, in these patients proCPU activity was inversely correlated with intervascular septal diameter.</td>
<td>Visualize TAFI (Affinity Biologicals) + Actichrome TAFI (American Diagnostica)</td>
<td>(119)</td>
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<tr>
<td>Agirbasli et al.</td>
<td>In patients with uncontrolled hypertension despite being on ACE-inhibitor therapy, the addition of an angiotensin receptor blocker resulted in a significant decrease in blood pressure, without affecting proCPU activity.</td>
<td>n.a.</td>
<td>(120)</td>
</tr>
<tr>
<td>Tregouet et al.</td>
<td>In a prospective study on patients with angiographically proven CAD, the amount of activated proCPU – measured by CPU/CPU – was associated with an increased risk of cardiovascular death, whereas total proCPU antigen was not.</td>
<td>Asserachrom TAFI + Asserachrom TAFI (Diagnostica Stago)</td>
<td>(121)</td>
</tr>
<tr>
<td>Meltzer et al.</td>
<td>Patients with proCPU levels in the first quartile (lowest levels) display an increased risk of a first myocardial infarction compared to patients with proCPU levels in the fourth quartile (OR=3.4, 95% CI 2.3-5.1). The G438A and 1040T alleles were associated with lower proCPU levels, whereas the 505G allele resulted in higher proCPU levels.</td>
<td>Pelakit TAFI (Pentapharm)</td>
<td>(122)</td>
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<tr>
<td>De Bruijne et al.</td>
<td>In young patients with arterial thrombosis, CPU(i) levels were higher compared to controls in patients with a proCPU G438A SNP and a lower risk of recurrent VTE.</td>
<td>In-house developed</td>
<td>(123)</td>
</tr>
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</table>
Role of CPU in fibrinolysis

| ATTAC Study | To healthy controls. There was no difference in total proCPU levels, levels of proCPU activation peptide, nor was there a difference in proCPU activity between patients and controls. In homoygous carriers of the 325IIe allele lower proCPU levels were observed together with a decreased risk of arterial thrombosis compared to homoygous carriers of the 325IIe allele. | ELISA TAFI / TAFI-AP / TAFIa(i) + functional assay + PCR |
| Tassies et al. | ProCPU polymorphism C+1542G and Thr325Ile are related to the type of acute coronary syndrome. | Asserachrom TAFI (Diagnostica Stago) + Actichrome TAFI (American Diagnostica) (124) |
| Ischemic stroke | **Leebek et al.** | Increased functional proCPU levels, resulting in decreased fibrinolysis, are associated with an increased risk of first ischemic stroke (OR 4.0, 95% CI 1.6-9.8). As demonstrated by the persisting elevated proCPU levels 3 months after the stroke, the increase in functional proCPU levels are not caused by an acute phase reaction. Moreover, proCPU genotype does not seem to predict the risk of ischemic stroke. | Functional TAFI clot lysis assay (125) |
| | **Kim et al.** | No difference was seen in proCPU levels in acute ischemic stroke patients with or without successful recanalization. | Zymutest TAFI (Hyphen Biomed) (126) |
| | **Ladenvall et al.** | The proCPU activation peptide shows association with all 4 major subtypes of ischemic stroke, whereas intact proCPU is correlated to large vessel disease and cryptogenic stroke. | In-house developed ELISA (127) |
| | **Rooth et al.** | In patients with ischemic stroke proCPU antigen levels were significantly elevated at admission and at day 1 compared to the levels at day 60 and compared to healthy controls. This suggests that elevation of proCPU may be the result of an acute phase reaction, leading to impaired fibrinolysis in stroke patients. | TAFI ELISA (Affinity Biologicals) (128) |
| | **Fernandez-Cadenas et al.** | The proCPU Thr325Thr polymorphism was associated with lower rates of recanalization after t-PA infusion in ischemic stroke patients. | PCR (129) |
| | **Mari-Fabregas et al.** | ProCPU concentration in plasma of ischemic stroke patients decreased significantly during thrombolytic therapy, without being associated to the achieved recanalization. | Actichrome TAFI (American Diagnostica) (130) |
| | **Biswas et al.** | In an Asian-Indian population, significantly higher proCPU antigen levels were observed in patients with acute onset non-cardioembolic stroke compared to normal individuals. Moreover, proCPU antigen was associated with disease phenotype and proCPU polymorphism. | Asserachrom TAFI (Diagnostica Stago) (131) |
| | **Brouns et al.** | In patients with ischemic stroke receiving thrombolytic therapy, the amount of CPU generated is associated with evolution of the neurological deficit as well as with achieved recanalization. The proCPU consumption is related to the risk of intracranial hemorrhage, mortality and final infarct volume. | In-house developed activity-based assay (132) |
| | **Biswas et al.** | ProCPU Ala147Thr mutation showed a significant association with pediatric non-cardioembolic stroke in Asian-Indian patients. | PCR (133) |
| | **Brouns et al.** | ProCPU concentration decreased significantly in the first 72 h after stroke onset and thereafter returned to baseline. This biphasic time course, with its nadir at 72 h, was more pronounced in patients with severe stroke, unfavourable stroke evolution in the first 72 h and poor long-term outcome. | In-house developed activity-based assay (134) |
| | **Koetan et al.** | LURIC Study Both the incidence of stroke and the risk of a premature event are higher in proCPU Ile325IIe patients with predisposing risk factors of thrombotic events such as diabetes mellitus, myocardial infarction or hypertension, alone or in combination. In contrast, no significant association was found for the proCPU Ala147Thr polymorphism. | Actiscreen TAFI (American Diagnostica) (135) |

**Sepsis and disseminated intravascular coagulation (DIC)**

| Fouassier et al. | In a case report of severe meningococemia, the patient presented with low proCPU levels during the acute phase of septic shock, presumably due to increased consumption by thrombin generation. After initiation of replacement therapy, plasma proCPU levels returned to baseline on day 3. | In-house developed TAFI ELISA (136) |
| Chen et al. | Although patients with overt DIC displayed more aberrant coagulation parameters in comparison to non-DIC sepsis patients, proCPU antigen levels did not differ significantly. Further no correlation between proCPU antigen levels and severity of organ injury in sepsis patients could be demonstrated. | TAFI ELISA (American Diagnostica) (137) |
| Zeerleder et al. | Septic patients had significantly decreased proCPU levels compared to controls. No difference in proCPU levels could be observed between severe sepsis or septic shock, nor between survivors or non-survivors. These results indicate that proCPU might be responsible for ongoing inhibition of the fibrinolysis in late stages of sepsis. | TAFI ELISA (Milan Analytica) (138) |
| Emons et al. | ProCPU levels were significantly decreased in patients with meningococcal disease at admission compared to the convalescence state. ProCPU was decreased in patients with septic shock vs. those with no shock. The proCPU 325IIe/IIe genotype was overrepresented in patients with DIC. | In-house developed ELISA (139) |

**Diabetes and obesity**

| Aso et al. | Plasma concentrations of proCPU correlate positively with serum total and LDL cholesterol in patients with type 2 diabetes. However, there was no significant association between plasma proCPU and components of the metabolic syndrome. | TAFI ELISA kit (Nagoya, Japan) (140) |
| Kitagawa et al. | In non-obese type 2 diabetic patients plasma levels of proCPU were significantly higher than in control subjects. An inverse correlation between proCPU levels and the glucose infusion rate was present. | TAFI ELISA kit (Kordia) (141) |
| Harmanci et al. | A comparison of proCPU activity between patients with type 1 diabetes and healthy individuals revealed no significant difference. In contrast to the observations in type 2 diabetes, these data suggest that fibrinolytic activity is not | Pefakit TAFI (Pentapharm) (142) |
### Role of CPU in fibrinolysis

<table>
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<tr>
<td>Rigla et al.</td>
<td>Fasting proCPU levels were lower in type 2 diabetic patients compared to controls, and showed to be inversely correlated with glycemic control. ProCPU levels decreased 4 hours after the meal and returned to baseline fasting levels after 8 hours. The decrease in proCPU concentration was correlated with fasting proCPU levels, glucose and hemoglobin A1c.</td>
<td>Zymutest TAFI (Hyphen Biomed)</td>
<td>(143)</td>
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<td>Guven et al.</td>
<td>ProCPU levels after orlistat therapy were significantly lower than basal proCPU levels (p&lt;0.001) in the obese group. Hemostatic abnormalities including proCPU alterations represent a link between obesity and vascular thrombosis.</td>
<td>Imuclone TAFI (American Diagnostica)</td>
<td>(144)</td>
</tr>
<tr>
<td>Guven et al.</td>
<td>Treatment with simvastatin showed a significant decrement in plasma proCPU levels (p&lt;0.001).</td>
<td>Imuclone TAFI (American Diagnostica)</td>
<td>(145)</td>
</tr>
<tr>
<td>Kilicarslan et al.</td>
<td>Fenofibrate decreases proCPU levels and improves endothelial function in metabolic syndrome and, thus, suggests a potential role of protection against cardiovascular effects.</td>
<td>TAFI ELISA (Diagnostica Stago)</td>
<td>(146)</td>
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<tr>
<td>Yener et al.</td>
<td>Baseline proCPU antigen level of type 2 diabetic subjects did not differ from healthy controls. Twelve weeks of metformin or rosiglitazone therapy did not cause significant changes in proCPU antigen levels.</td>
<td>TAFI ELISA (Affinity Biologicals)</td>
<td>(147)</td>
</tr>
<tr>
<td>Kucisz et al.</td>
<td>VEGF was significantly higher in normoalbuminuric (NAU) patients with type-2 diabetes mellitus compared to controls. Only proCPU correlated with VEGF in MAU.</td>
<td>Asserachrom TAFI (Diagnostica Stago)</td>
<td>(148)</td>
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#### Renal failure

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<tr>
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<tbody>
<tr>
<td>Malyzko et al.</td>
<td>Kidney transplant recipients have higher mean blood pressure, serum lipids, fibrinogen, proCPU antigen, proCPU activity, markers of coagulation and fibrinolysis, thicker IMT and lower PAP relative to healthy volunteers.</td>
<td>TAFI EIA (Affinity Biologicals) + Actichrome TAFI (American Diagnostica)</td>
<td>(149)</td>
</tr>
<tr>
<td>Malyzko et al.</td>
<td>Elevated proCPU concentrations and enhanced thrombin generation in hypertensive kidney transplant recipients may contribute to the hypofibrinolysis and progressive atherothrombosis in this population. Blood pressure was related to kidney function, maintenance prednisone dose and proCPU concentration.</td>
<td>TAFI EIA (Affinity Biologicals) + Actichrome TAFI (American Diagnostica)</td>
<td>(150)</td>
</tr>
<tr>
<td>Guo et al.</td>
<td>ProCPU levels were significantly elevated in patients suffering from chronic kidney disease.</td>
<td>Zymutest TAFI (Hyphen Biomed)</td>
<td>(151)</td>
</tr>
<tr>
<td>Malyzko et al.</td>
<td>Markers of endothelial dysfunction and inflammation, including proCPU, were significantly elevated in kidney allograft recipients compared to control patients, implying cardiovascular complications in these patients.</td>
<td>Actichrome TAFI (American Diagnostica)</td>
<td>(152)</td>
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#### Hepatic disease

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<tr>
<th>Authors</th>
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<tr>
<td>Yener et al.</td>
<td>Patients with biopsy-proven non-alcoholic steatohepatitis (NASH) had lower proCPU antigen expression than controls. Lower proCPU antigen levels may be related to the overtactivation of the proCPU pathway resulting in proCPU antigen depletion.</td>
<td>TAFI ELISA (Affinity Biologicals)</td>
<td>(153)</td>
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<tr>
<td>Gresele et al.</td>
<td>In patients with liver cirrhosis proCPU levels were significantly reduced. Moreover, proCPU levels in non-survivors were significantly lower than in survivors, implying that proCPU is a predictor of survival in cirrhotic patients.</td>
<td>Coaliza TAFI (Chromogenix)</td>
<td>(154)</td>
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#### Thyroid dysfunction

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<tr>
<th>Authors</th>
<th>Description</th>
<th>Test(s)</th>
<th>Reference(s)</th>
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<tbody>
<tr>
<td>Akinci et al.</td>
<td>Hypothyroid patients – both overt and subclinical – have higher proCPU antigen levels compared to controls. The proCPU level was correlated with the degree of thyroid failure. After normalization of the thyroid state by levothyroxine replacement therapy, proCPU antigen levels decreased significantly.</td>
<td>TAFI EIA (Affinity Biologicals)</td>
<td>(155)</td>
</tr>
<tr>
<td>Akinci et al.</td>
<td>In hypothyroid patients plasma proCPU antigen levels were significantly lower compared to controls.</td>
<td>TAFI EIA (Affinity Biologicals)</td>
<td>(156)</td>
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<tr>
<td>Erem et al.</td>
<td>Compared to healthy controls, proCPU antigen levels were significantly increased in patients with hypothyroidism.</td>
<td>TAFI ELISA (American Diagnostica)</td>
<td>(157)</td>
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<tr>
<td>Cetinkalp et al.</td>
<td>In patients with Hashimoto thyroiditis proCPU antigen levels are significantly higher compared to controls. After treatment with L-thyroxin proCPU levels tend to decrease, although not statistically significant.</td>
<td>Actichrome TAFI (American Diagnostica)</td>
<td>(158)</td>
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<tr>
<td>Erem et al.</td>
<td>Plasma proCPU antigen levels were not significantly different in patients with primary hyperparathyroidism compared to healthy controls.</td>
<td>Imuclone TAFI (American Diagnostica)</td>
<td>(159)</td>
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#### Cancer

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<tr>
<th>Authors</th>
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<th>Reference(s)</th>
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<tbody>
<tr>
<td>Vairaktaris et al.</td>
<td>The 325Thr allele confers to a reduced risk of the development of oral cancer. Thr325Thr homozygotes have about half of the probability of developing oral cancer, whereas for the Thr325Gle heterozygotes the level of statistical significance wasn’t reached.</td>
<td>PCR</td>
<td>(160)</td>
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<tr>
<td>Koldas et al.</td>
<td>The proCPU activity was significantly higher in patients with lung cancer than in subjects in the control group. However, there were no statistically significant associations between proCPU activity levels and patient age, sex, BMI, histopathology, or stage of disease.</td>
<td>n.a.</td>
<td>(161)</td>
</tr>
<tr>
<td>Bentov et al.</td>
<td>The risk of ovarian cancer was not associated with SNPs in the proCPU gene.</td>
<td>PCR</td>
<td>(162)</td>
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#### Pregnancy and complications

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<th>Authors</th>
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<tr>
<td>Uszynski et al.</td>
<td>ProCPU antigen is present in human amniotic fluid in the same concentration as in circulation, thus contributing to the antifibrinolytic potential of amniotic fluid. ProCPU activity was 3-fold lower as compared to proCPU activity in plasma.</td>
<td>TAFI/a1 ELISA (American Diagnostica) + Actichrome TAFI (American Diagnostica)</td>
<td>(163)</td>
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<td>Uszynski et al.</td>
<td>CPUPCU1 could be detected in cord blood, with levels that are 2 times higher compared to maternal blood.</td>
<td>TAFI/a1 ELISA (American Diagnostica)</td>
<td>(164)</td>
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<tr>
<td>Akinci et al.</td>
<td>Increased plasma proCPU antigen levels were found in pregnant women compared to non-pregnant controls. However, no significant difference in proCPU antigen levels was observed between women with gestational diabetes</td>
<td>TAFI EIA (Affinity Biologics)</td>
<td>(165)</td>
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Role of CPU in fibrinolysis

Gursoy et al.  ProCPU activity was significantly higher in neonates with meconium-stained amniotic fluid (MSAF) when compared with the control group and the levels correlated negatively with cord blood pH levels. Increased proCPU levels in neonates with MSAF might be due to hypoxia. Inflammation observed in MSAF may also play an additional role in increased proCPU activity.

Gursoy et al.  When examining preterm neonates with early respiratory distress syndrome no significant difference in proCPU plasma levels could be found. Mildly asphyxiated neonates displayed higher proCPU levels compared to nonasphyxiated neonates.

Folkeringa et al.  In a retrospective study it was shown that proCPU levels were not associated with fetal loss.

Masini et al.  Genotype and allele frequencies of proCPU +505 and +1583 SNPs were significantly different in women with recurrent pregnancy loss compared to control women, whereas polymorphisms at proCPU position -438, +1040 and +1542 were not different.

Kno et al.  Women with high proCPU levels have a reduced overall risk of fetal loss of 28%. The protective effect of high proCPU levels was most pronounced for early recurrent fetal loss.

Guven et al.  Composed to the control group, the mean proCPU levels were significantly increased in HELLP syndrome. The mean plasma proCPU antigen concentration 7 days after delivery was significantly lower compared to the baseline levels.

Martinez-Zamora et al.  In pregnant patients with antiphospholipid syndrome (APS) a significantly prolonged clot lysis time could be demonstrated compared to normal pregnant women. Basal levels of proCPU as well as basal clot lysis time did not differ between APS patients having an adverse or a good obstetrical outcome.

Martinez-Zamora et al.  Patients with unexplained recurrent miscarriage have an impairment in fibrinolysis demonstrated by increased CLT, that can be at least partly explained by higher proCPU antigen levels.

Pruner et al.  Women that were carriers of +1040 T/T genotype have an increased risk of fetal loss compared with T allele (OR 0.91; 95% CI 0.545-1.533; P = 0.7) and 1.34-fold compared with carriers of +1040 C/T genotype (95% CI 0.501-3.601; P = 0.6). The C allele is associated with a reduced risk of recurrent fetal loss compared with T allele (OR 0.91; 95% CI 0.545-1.533; P = 0.7).

Uszynski et al.  The proCPU antigen concentrations were higher in cord blood plasma compared to in maternal plasma. The differences between the levels in cord blood plasma and maternal plasma were statistically significant (p<0.0001).

Polycystic Ovary Syndrome (PCOS)

Erdogan et al.  Plasma proCPU levels were similar in polycystic ovary syndrome (PCOS) patients compared to healthy controls, which can be explained by their low ages and short duration of PCOS.

Karaku et al.  Women with PCOS have impaired fibrinolysis, as reflected by increased proCPU plasma levels. This impairment can contribute to the risk of cardiovascular disease in PCOS.

Oral et al.  Plasma proCPU levels of PCOS patients were found to be significantly higher than in healthy controls (93.8%+/-30.6%vs. 79.8% +/- 22.4%, p<0.05).

Adali et al.  Young overweight or obese women with PCOS have increased plasma proCPU levels. Impaired fibrinolysis may be responsible for the increased risk of cardiovascular diseases in women with PCOS.

Postmenopausal hormone replacement therapy (HRT)

Ozren et al.  Reduced proCPU antigen levels were demonstrated during short term treatment with raloxifene in postmenopausal women with osteopenia or osteoporosis.

Vogelvang et al.  Compared with placebo, a decrease in proCPU concentration was observed after 12 weeks of HMR 3339 therapy, thereby confirming the results of an earlier study with raloxifene therapy in healthy early postmenopausal women.

Post et al.  Data from randomized controlled trials indicate that both the route of administration (oral versus transdermal) and the addition of different progesterons play a role in the effect of oestrogens on plasma proCPU.

Other pathologies

Donmez et al.  Patients with Behçet’s disease display significantly higher plasma proCPU levels, compared to controls, although no difference could be observed between patients with and without thrombosis. Moreover, no correlation between proCPU levels and CRP could be detected in this pathology.

Gumus et al.  A trend toward higher proCPU activity levels was seen in patients with retinal vein occlusion (RVO) compared to controls. According to evaluation of proCPU activity in subgroups (>200%, 150-200%, and 0-150%), 36.7% with central RVO, 40.0% with branch RVO, and 30% of controls were found to have a proCPU activity of >200% (P = 0.83).

Sonmez et al.  In patients with Crimean-Congo hemorrhagic fever, proCPU activity was significantly lower compared to controls, leading to imbalanced fibrinolysis and bleeding complications in patients. It was suggested that the proCPU decrease is due to liver dysfunction during viral active disease state.

Ringwald et al.  A trend toward higher proCPU levels was seen in patients with systemic lupus erythematosus versus controls, although this trend did not reach the level of statistical significance.

Ricart et al.  ProCPU levels were reported to be significantly higher in patients with Behçet’s disease compared to controls. Moreover, patients with a history of thrombosis

TAFIA/ai ELISA (American Diagnostica) (175)

Imuclone TAFI (American Diagnostica) (179)

Zymtest TAFI (Hyphen Biomed) (178)

Imuclone TAFI (American Diagnostica) (177)

Imuclone TAFI (American Diagnostica) (176)

Asserachrom TAFI (Diagnostica Stago) (173)

Imuclone TAFI (American Diagnostica) (170)

Imuclone TAFI (American Diagnostica) (169)

TAFIA/ai ELISA (American Diagnostica) (168)

In-house developed activity-based assay (182)

In-house developed activity-based assay (181)

In-house developed activity-based assay (180)

TAFIA/ai ELISA (American Diagnostica) (183)

Pefakit TAFI (Pentapharm) (184)

Actichrome TAFI (American Diagnostica) (185)

TAFI ELISA (Haemochrom Diagnostica) (186)

Stachrom TAFI (Diagnostica Stago) (187)

Pefakit TAFI (Pentapharm) (166)

Pefakit TAFI (Pentapharm) (167)

Pefakit TAFI (Pentapharm) (167)
Role of CPU in fibrinolysis

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<td>Colucci et al.</td>
<td>In patients with mild hyperhomocysteinemia an increased proCPU concentration, as well as an enhanced antifibrinolytic activity was described.</td>
<td>Pelikit TAFI (Pentapharm)</td>
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<td>Koutroubakis et al.</td>
<td>In patients with inflammatory bowel disease (ulcerative colitis as well as Crohn’s disease) plasma proCPU levels were significantly lower compared to healthy controls.</td>
<td>Aserachrom TAFI (Diagnostica Stago)</td>
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<tr>
<td>Ladenvall et al.</td>
<td>Plasma proCPU Ag levels did not significantly change in patients with Cushing’s syndrome compared with controls.</td>
<td>TAFI ELISA (American Diagnostica)</td>
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<tr>
<td>Matus et al.</td>
<td>In patients with mucocutaneous bleeding, proCPU levels were significantly higher compared to controls.</td>
<td>In-house developed activity-based assay + PCR</td>
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<tr>
<td>Erem et al.</td>
<td>Plasma proCPU Ag levels were not different in patients with acromegaly compared to the controls.</td>
<td>TAFI ELISA (American Diagnostica)</td>
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<tr>
<td>Ikeda et al.</td>
<td>The presence of proCPU could be demonstrated in gastric mucosal epithelial cells in patients with Helicobacter pylori associated gastroduodenal disease.</td>
<td>TAFI-EIA (Kordia Laboratory Supplies)</td>
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<tr>
<td>Peters et al.</td>
<td>ProCPU levels are significantly higher in the plasma of rheumatoid arthritis patients with a high inflammatory state.</td>
<td>In-house developed activity-based assay</td>
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findings in the field (> 2005). Results of some studies may be compromised by the use of a proCPU assay that lacks thorough validation, as was discussed before.

8. CPU: A NEW DRUG TARGET?

The prominent bridging function of CPU between coagulation and fibrinolysis raised the interest of several research groups and of the pharmaceutical industry. The development of CPU inhibitors as profibrinolytic agents is an attractive new concept, as it will leave the coagulation cascade unaffected (7). Possibilities for rational drug design become more readily available as a result of the recently published crystal structure of CPU (35, 71, 72). An overview of CPU inhibitors and their potential role as profibrinolytic drugs is given in a recently published review by Willemse et al. (7).

The organic inhibitors 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MERGETPA) and guanidinoethylthiopropanoic acid (GEMSA) are two of the most widely used CPU inhibitors for both in vitro and in vivo studies (10, 12, 14, 62). Both compounds have two major drawbacks from a drug discovery point of view, i.e. they lack selectivity and also inhibit CPN, which is believed to have an important function in plasma as an inactivator of anaphylatoxins and in the processing of peptide hormones (94, 196); moreover, they have a low oral availability, due to their polar nature (102). Major efforts have been made to obtain more potent and selective drugs with favourable pharmacokinetic properties.

Apart from the synthetic inhibitors, protein inhibitors have also been described. The most familiar one is potato tuber carboxypeptidase inhibitor (PTCI), a 39 amino acid protein that competitively inhibits CPU with a Kᵢ in the nanomolar range; which is commonly used in in vitro settings as well as in in vivo animal models (197-200). Other protein inhibitors are the 66 amino acid residue leech carboxypeptidase inhibitor (LCI) found in Hirudo medicinalis (201) and the recently isolated tick carboxypeptidase inhibitor (TCI) from the soft tick Rhipicephalus bursa (202). A major advantage of this kind of inhibitors is the high selectivity toward CPU.

It has been observed that several competitive inhibitors exert a biphasic effect in in vitro experiments, where clot lysis is stimulated at high concentrations – as expected - but prolonged at low concentrations (62, 203). A possible explanation for this could be the equilibrium between free (and inhibitor) bound CPU. Free CPU is highly unstable and will inactivate irreversibly, due to increased dynamic segment mobility. The bound form on the other hand is protected from loss of activity through interaction of the inhibitor with the dynamic segment of the enzyme moiety. This stable bound form will be released to replenish the free pool, so as to maintain the equilibrium (62, 203). As long as the free CPU concentration stays above the t-PA dependent threshold value, fibrinolysis will stay in its initial phase (82, 83). Although this stabilizing effect of CPU inhibitors has raised some concerns about their use as profibrinolytic drugs, this observed paradoxal behaviour has not been substantiated in in vivo models. Moreover, recent insights reveal that this phenomenon is most probably compound-related.

Besides direct CPU inhibition, another profibrinolytic strategy is to prevent the activation of the proenzyme proCPU. In this regard, several monoclonal antibodies (MA) as well as nanobodies have been described, inhibiting proCPU activation by plasmin or by the thrombin-thrombomodulin complex (41-43). As already mentioned above, activation of proCPU by plasmin as well as by the thrombin-thrombomodulin complex occurs through a single-site proteolytic cleavage at Arg92. However, Hillmayer et al. were able to generate MA that could discriminate between plasmin and thrombin-thrombomodulin mediated activation, implying that these MA do not bind to the cleavage site. Moreover, different binding sites in the enzyme moiety have been identified for thrombin or plasmin. MA that inhibit exclusively the activation of proCPU by thrombin-thrombomodulin bind to Gly66, whereas MA that inhibit activation by both plasmin
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and thrombin-thrombomodulin bind to Val41. These MA are of particular interest for revealing the physiological activator of proCPU in in vivo models (204) as well as for therapeutic use, since they do not cross-react with CPN (42).

Surprisingly, initial in vivo data from proCPU knockout mice did not reveal an important function of the proCPU/CPU system, raising questions about its in vivo significance (205-207). A recent update on proCPU knockout mice is given by Morser et al. (207), concluding that proCPU deficient mice without being challenged displayed no overt phenotype, suggesting that proCPU can regulate fibrinolysis under defined conditions whereby its deficiency is only observable when normal fibrinolysis is compromised (207). For instance, in proCPU deficient mice with a heterozygous plasminogen-deficient background, a role of proCPU was demonstrated in models of pulmonary embolism (PE) and peritoneal inflammation (208). These results led to the conclusion that proCPU is able to modulate the in vivo functions of plasminogen in fibrinolysis and cell migration (208). Moreover, when fibrinogen was depleted in glomerulonephritis (209) and lung fibrosis models (210), the protective effects of proCPU deficiency were abrogated, demonstrating enhanced fibrinolysis to be at the basis for the protective phenotype in proCPU deficient mice. More recent data on proCPU knockout mice with a normal plasminogen status have been published describing a protective role of proCPU deficiency in a 3.5 % ferric chloride induced vena cava thrombosis model (211) and linking proCPU deficiency to enhanced endogenous fibrinolysis (212).

The search for clinical utility of CPU inhibitors has been focussed on improvement of endogenous fibrinolysis on the one hand and adjuvants for thrombolytic therapy on the other hand. Several studies have been conducted to evaluate whether a CPU inhibitor alone improves endogenous thrombolysis, albeit with contradictory results (reviewed in (7)). The in vivo profibrinolytic efficiency of a CPU inhibitor alone depends on the type of thrombosis model and the studied animal species, the type of inhibitor and whether this inhibitor is administered before or after thrombus induction (51, 200, 213-216). Recently, a phase II, single-blinded, multicentre study was presented which investigated the effect of the novel CPU inhibitor AZD9684 in PE (217). Fifty-eight patients with confirmed PE were randomized to receive AZD9684 or placebo, on top of once-daily dalteparin for 5-7 days. In the patient group receiving AZD9684, fibrinolysis biomarkers in plasma were higher and sustained for a longer period of time, implying that inhibition of CPU by AZD9684 stimulates endogenous fibrinolysis. Moreover, lung deficiency scintigraphy scores improved over the treatment period. In addition, no difference in the occurrence of adverse effects was seen between both treatment groups (217).

Also the use of CPU inhibitors as adjuvants for thrombolytic therapy is an attractive concept. Since thrombolytic therapy is still characterized by major shortcomings – large therapeutic doses, limited fibrin specificity, significant bleeding tendency and reocclusion - major benefit can be expected from adjunctive therapy that potentiates the t-PA mediated thrombolytic effect, enabling dose reduction and thus limiting unfavourable side effects of plasminogen activators (218, 219). In recent years, consistent data have been obtained in animal studies on the utility of CPU inhibitors as adjuvant therapy of thrombolytics (reviewed in (7)). Administration of a CPU inhibitor along with low-dose t-PA leads to a significant enhancement of thrombolytic efficiency, with a 3-fold reduction in time to reperfusion and similarly improved vessel patency compared to low-dose t-PA alone (199, 213, 215, 216).

An important concern about the use of CPU inhibitors as profibrinolytic drugs is the question whether inhibition of CPU leads to an increased bleeding risk. The use of a CPU inhibitor in many in vivo models however was not associated with an increased bleeding risk, either when used alone or in combination with t-PA (206).

9. CONCLUSIONS

By providing an important link between coagulation and fibrinolysis, the proCPU system is considered to be a potential target for the treatment of thrombotic disorders. Several selective CPU inhibitors have been designed and tested in animal thrombosis models, showing improved endogenous fibrinolysis and an increased efficiency of t-PA mediated thrombolysis upon inhibition of the (pro)CPU system. Recently, a phase II, single-blinded, multicentre study in patients with PE also showed enhancement of endogenous fibrinolysis upon administration of a selective CPU inhibitor. With the availability of the crystal structure, a major step forward has been made in CPU research, boosting the development of potent and selective CPU inhibitors in the near future.

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**Abbreviations:** proCPU: procarboxypeptidase U; CPU: carboxypeptidase U; TAFI: thrombin activatable fibrinolysis inhibitor; TAFIa: activated thrombin activatable fibrinolysis inhibitor; CPB: carboxypeptidase B; CPN: carboxypeptidase N; SNP: single nucleotide polymorphism; IIa: thrombin; TM: thrombomodulin; TF: tissue factor; EGF: epidermal growth factor; GEMSA: guanidinoethylmercaptosuccinic acid; MERGETPA: 2-mercapto-methyl-3-guanidinoethylthiopropanoic acid; PTCl: potato tuber carboxypeptidase inhibitor; LCI: leech carboxypeptidase inhibitor; TCI: tick carboxypeptidase inhibitor; t-PA: tissue plasminogen activator; MA: monoclonal antibody; ELISA: enzyme linked immunosorbent assay; HPLC: high pressure liquid chromatography; PPACK: D-phenylalanyl-L-prolyl-arginyl chloromethyl ketone; LOD: limit of detection;

**Key Words:** Procarboxypeptidase U, ProCPU, Carboxypeptidase U, CPU, Thrombin Activatable Fibrinolysis Inhibitor, TAFI, Activated Thrombin Activatable Fibrinolysis Inhibitor, Tafia, Fibrinolysis, Thrombosis, Review

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