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Minna Ala-Kopsala

CIRCULATING N-TERMINAL FRAGMENTS OF A- AND B-TYPE NATRIURETIC PEPTIDES: MOLECULAR HETEROGENEITY, MEASUREMENT AND CLINICAL APPLICATION

FACULTY OF MEDICINE,
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CIRCULATING N-TERMINAL FRAGMENTS OF A- AND B-TYPE NATRIURETIC PEPTIDES: MOLECULAR HETEROGENEITY, MEASUREMENT AND CLINICAL APPLICATION

Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in Auditorium F101 of the Department of Physiology (Aapistie 7), on November 3rd, 2006, at 12 noon

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Abstract

Natriuretic peptides have emerged as important candidates for the development of diagnostic tools in cardiovascular disease. Their increased concentrations have been found to be useful for ruling out disease of cardiac origin, as prognostic indicators, and in the follow-up of patients with heart failure. In order for natriuretic peptides to be efficient biomarkers, analytical problems in assay specificity and calibration need to be resolved. The aim of the present study was to elucidate circulating molecular components of N-terminal fragments of A- and B-type natriuretic peptides (NT-proANP and NT-proBNP) in human blood, and to develop reliable and novel assays for their measurement with clinical application.

Reliable immunoassays for NT-proANP and NT-proBNP were set up based on recombinant calibrators and antisera against different epitopes. A novel immunoassay for detecting the activation of A- and/or B-type natriuretic peptide systems, referred to as NT-proXNP, was also developed. The chromatographic results of human plasma and serum samples indicated that NT-proANP and especially NT-proBNP are heterogeneous in human circulation. They are truncated at both termini, causing a serious risk of preanalytical errors. Further studies with recombinant peptides confirmed that the central parts of NT-proANP and NT-proBNP are stable in plasma and serum even at harsh storage conditions. Thus the most reliable assays are directed at the central portions of the molecule only.

All developed assays were applicable to clinical samples of cardiac patients. NT-proXNP showed a diagnostic efficiency equal to or slightly better compared to individual NT-proANP and NT-proBNP assays. Furthermore, the prognostic value of NT-proANP and NT-proBNP was investigated in a population-based sample of men. Both peptides were strong predictors of mortality and its co-morbidities, adding to the prognostic value of conventional risk factors.

Keywords: cardiovascular diagnostic, heart, heart diseases, immunoassay, natriuretic peptides

To Anni and Anssi

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Oulu, October 2006 Minna Ala-Kopsala

Abbreviations

AF	atrial fibrillation
AMI	acute myocardial infarction
ANP	atrial natriuretic peptide or A-type natriuretic peptide
AUC	area under the curve
BNP	B-type natriuretic peptide
bTG	bovine thyroglobulin
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
C-	carboxyterminal
CAD	coronary artery disease
CHD	coronary heart disease
CNP	C-type natriuretic peptide
DA	Dalton
DNA	deoxyribonucleic acid
DNP	dendroaspis natriuretic peptide
EC_{50}	median effective concentration
ECLIA	electrochemiluminescent assay
EDTA	ethylenediamineteraacetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
FDA	U.S. Food and Drug Administration
Fmoc	9-fluorenylmethoxycarbonyl
GF-HPLC	gel filtration high performance liquid chromatography
GST	glutathione S-transferase
GS-NT-proANP	recombinant NT-proANP with glycine-serine dipeptide
GS-NT-proBNP	recombinant NT-proBNP with glycine-serine dipeptide
GS-NT-proXNP	recombinant NT-proXNP with glycine-serine dipeptide
GST-NT-proANP	GST-fusion protein of NT-proANP
GST-NT-proBNP	GST-fusion protein of NT-proBNP
GST-NT-proXNP	GST-fusion protein of NT-proXNP
HPLC	high performance liquid chromatography

HSC-HC	horseshoe crab hemocyanin
IFMA	immunofluorometric assay
ILMA	immunoluminometric assay
IPTG	isopropyl β-D-thiogalactoside
IRMA	immunoradiometric assay
LB	Luria-Bertani
mRNA	messenger ribonucleic acid
NEP	neutral endopeptidase
NPR-A	type A natriuretic peptide receptor
NPR-B	type B natriuretic peptide receptor
NPR-C	type C natriuretic peptide receptor or clearance receptor
N-	aminoterminal
NT-proANP	amino terminal fragment of proatrial natriuretic peptide
NT-proBNP	amino terminal fragment of pro-B-type natriuretic peptide
NT-proCNP	amino terminal fragment of pro-C-type natriuretic peptide
NT-pro-sCP	amino terminal fragment of pro-salmon cardiac peptide
NT-proXNP	hybrid peptide of NT-proANP and NT-proBNP
NYHA	New York Heart Association
PCR	polymerase chain reaction
PIA	processing-independent assay
RIA	radioimmunoassay
RNA	ribonucleic acid
ROC	receiver operating characteristic
RP-HPLC	reverse phase high performance liquid chromatography
RT-PCR	reverse transcriptase polymerase chain reaction
sCP	salmon cardiac peptide
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of mean
TFA	trifluoroacetic acid
VHD	valvular heart disease

List of original papers

This thesis is based on the following articles, which are referred to in the text by Roman numerals:

I Ala-Kopsala M, Magga J, Peuhkurinen K, Leipälä J, Ruskoaho H, Leppäluoto J & Vuolteenaho O (2004) Molecular heterogeneity has a major impact on the measurement of circulating N-terminal fragments of A- and B-type natriuretic peptides. Clin Chem 50: 1576-1588.

II Ala-Kopsala M, Ruskoaho H, Leppäluoto J, Seres L, Skoumal R, Toth M, Horkay F & Vuolteenaho O (2005) Single assay for amino-terminal fragments of cardiac A- and B-type natriuretic peptides. Clin Chem 51: 708-718.

III Ala-Kopsala M, Ruskoaho H & Vuolteenaho O (2006) Measurement of circulating N-terminal fragments of A- and B-type natriuretic peptides: the central but not terminal epitopes are stable. Manuscript.

IV Laukkanen J, Sudhir K, Ala-Kopsala M, Vuolteenaho O, Ruskoaho H, Nyyssönen K & Salonen JT (2006) Plasma N-terminal fragments of natriuretic propeptides predict the risk of cardiovascular events and mortality in men. Eur Heart J 27: 1230-1237.

In addition to the above papers, some unpublished data are included.

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1 Introduction

The prevalence of symptomatic heart failure in the general European population ranges from 0.4% to 2%, increasing rapidly with age. It is estimated that patients with myocardial dysfunction without symptoms of heart failure constitute an equally sized group. However, the accuracy of diagnosis by clinical means alone is often inadequate (Swedberg *et al.* 2005). Since their discovery, natriuretic peptides, a group of cardiac vasoactive hormones, have emerged as important candidates for the development of diagnostic tools in cardiovascular disease.

A- and B-type natriuretic peptides (ANP and BNP) are produced and secreted from the myocardium whenever the load of the heart is increased. They have many favorable physiological properties in order to reduce blood pressure and cardiac load including vasodilation, natriuresis, growth suppression and the inhibition of the sympathetic nervous as well as the renin-angiotensin-aldosterone system (for review, see Nakao *et al.* 1992a, Ruskoaho 1992, Potter *et al.* 2006). ANP and BNP are synthesized as prohormones, which are cleaved into biologically active hormones and their N-terminal counterparts, both of which circulate, reflecting cardiac health. Increased concentrations have been found to be useful for ruling out disease of cardiac origin, as prognostic indicators and in the follow-up of patients with heart failure. Especially proANP-derived peptides are good indicators of acute overload and rapid hemodynamic changes, whereas proBNP-derived peptides appear to be better markers of ventricular overload (for review, see Sagnella 1998, Ruskoaho 2003, Rademaker & Richards 2005, Vuolteenaho *et al.* 2005).

There exist a number of immunoassays for natriuretic peptides in competitive as well as in sandwich format. However, there are several analytical problems including assay specificity and calibration. The exact circulating forms of proANP and proBNP are still debated, causing question marks over assay specificities; furthermore, many assays lack a proper calibrator. Therefore, the measured concentrations vary a lot between different assays (for review, see Clerico & Emdin 2004, Apple *et al.* 2005). In addition, the available assays measure one analyte at a time, although the ANP and BNP systems are differently regulated, and combined information from the both systems might have potential for higher clinical sensitivity (for review, see Ruskoaho 2003).

The aim of the present study was to develop reliable biochemical methods for the assessment of cardiac health with diagnostic and prognostic applications. Several

immunoassays for NT-proANP and NT-proBNP as well as a novel single assay for their simultaneous measurement were set up. The circulating forms of N-terminal fragments and their *in vitro* stability were clarified. Moreover, the clinical applicability of the developed natriuretic peptide assays was tested.

2 Review of the literature

2.1 Natriuretic peptide family

The natriuretic peptides are a group of hormones which are structurally similar but genetically distinct. All known natriuretic peptides share a 17-amino-acid ring structure formed by a disulfide bridge between two cysteine residues, essential for biological activity. In mammals A-type natriuretic peptide (ANP, also known as atrial natriuretic peptide) and B-type natriuretic peptide (BNP, also known as brain natriuretic peptide) are of cardiac origin, whereas C-type natriuretic peptide (CNP) originates from the neural system or endothelial cells. ANP and BNP cause vasodilation, natriuresis, growth suppression and inhibition of the sympathetic nervous as well as the renin-angiotensin-aldosterone system (for review, see Nakao *et al.* 1992a, Ruskoaho 1992). All these actions are beneficial for the heart, reducing cardiac load and blood pressure. The gene expression of ANP and BNP is almost exclusively restricted to cardiac myocytes; only minute amounts are produced in the central nervous system and other tissues. Furthermore, the production and secretion is increased when the heart is loaded. Elevated circulating levels of these hormones are thus considered sensitive markers of cardiac function and have important diagnostic and therapeutic implications (for review, see Sagnella 1998, Ruskoaho 2003). The actions of natriuretic peptides are mediated by cellular receptors causing a rise in intracellular cGMP. Natriuretic peptide receptors lacking guanylyl cyclase activity are thought to possess a clearance function (for review, see Maack 1992, Nakao *et al.* 1992b, Potter *et al.* 2006).

2.1.1 A-type natriuretic peptide

2.1.1.1 Synthesis and release of A-type natriuretic peptide

A-type natriuretic peptide (ANP) is present as a single-copy gene located on chromosome 1 in humans. It is organized into three exons (for review, see Nakao *et al.* 1992a). ANP is synthesized and secreted primarily by the cardiac myocytes of the adult heart (for review, see Ruskoaho 1992). One to three percent of total atrial RNA corresponds to ANP

mRNA, whereas in ventricles the level is about 1% of that in the atria (Gardner *et al.* 1986). Low detectable levels of ANP or its mRNA are also expressed in the central nervous system, lung, adrenal gland, kidney, gastrointestinal tract and vascular tissue (Vuolteenaho *et al.* 1988, Gutkowska & Nemer 1989). The expression of ANP gene in myocytes is developmentally regulated. During fetal and embryonic development the ANP gene is expressed both in the atria and the ventricles. Soon after birth, the ventricular ANP expression declines rapidly, and the atrium is the major site of ANP synthesis in the mature myocardium (Argentin *et al.* 1994). Ventricular ANP gene expression can be reactivated when the ventricles are subjected to hemodynamic overload (Lee *et al.* 1988).

In humans ANP is produced as preproANP containing 151 amino acids. Removal of a hydrophobic 25-amino-acid signal peptide yields a 126-amino-acid peptide, $proANP_{1\text{-}126}$, which is the major storage form of ANP in atrial secretory granules (for review, see Rosenzweig & Seidman 1991, Ruskoaho 1992). In normal heart, very little ANP with only occasional secretory granules can be found in the ventricles (Arjamaa & Vuolteenaho 1985, Rinne *et al.* 1986, Arbustini *et al.* 1990). The amino acid sequence of proANP is highly homologous in different mammals species (Oikawa *et al.* 1985). On secretion, $proANP_{1\text{-}126}$ is split between arginine and serine residues by the serine protease corin (Wu *et al.* 2002, Chan *et al.* 2005) into equimolar amounts of the N-terminal fragment of 98 amino acids ($NT\text{-}proANP_{1\text{-}98}$) and biologically active $ANP_{99\text{-}126}$ (Vuolteenaho *et al.* 1985, Yandle 1994). Both fragments circulate in the blood (Fig. 1), and their concentrations are elevated by an increase of intravascular volume, such as in heart failure (for review, see Ruskoaho 2003).

ANP is stored in atrial secretory granules providing a rapid release of the peptide upon stimulus (Mangat & de Bold 1993). The storage and secretion of atrial ANP is suggested to utilize the regulated pathway (for review, see Ruskoaho 1992, Thibault *et al.* 1999). ANP is also synthesized in ventricles lacking the secretory granules. Thus ventricular ANP is probably released by the constitutive pathway (Bloch *et al.* 1986, Kokkonen *et al.* 2000). The major stimulus for ANP secretion is increased myocyte stretch. Both *in vitro* and *in vivo* studies show that atrial (Lang *et al.* 1985, Ruskoaho *et al.* 1986) as well as ventricular myocyte stretch (Kinnunen *et al.* 1993, Mäntymaa *et al.* 1993) or plasma volume expansion increase the release of ANP (Anderson *et al.* 1986, Tulassay *et al.* 1988). The exact mechanism of stretch-induced ANP release is not fully understood. The primary sensors for the stretch may be the cardiac myocytes, but the release may also be modulated by local or humoral vasoactive factors secreted by other cell types, e.g. endothelial cells (Knöll *et al.* 2003). The induction of the ANP gene in response to mechanical load takes hours or days to develop, and it is dependent on the general level of protein synthesis, as with other secondary response genes. Thus, the acute regulation of atrial ANP occurs at the level of hormone release, rather than at the level of gene transcription (for review, see Ruskoaho 1992, de Bold *et al.* 1996). In ventricular myocytes, ANP expression as well as immunoreactive ANP are greatly enhanced in cardiac overload associated with ventricular hypertrophy (Kinnunen *et al.* 1992). The amount of released ventricular ANP depends on the degree of hypertrophy. As the hypertrophy progresses, the source of ANP shifts more and more from the atrium to the ventricle (Ruskoaho *et al.* 1989, Thibault *et al.* 1989).

In addition to mechanical load, ANP gene expression and release is stimulated by several endocrine, paracrine, and autocrine factors. These vasoactive factors include endothelin, angiotensin II, arginine vasopressin, nitric oxide, glucocorticoids, catecholamines, some growth factors and cytokines (for review, see Thibault *et al.* 1999), most of which are activated in cardiac stress. Among these factors, endothelin appears to be the most potent stimulus for ANP secretion (de Bold *et al.* 1996). Increase in the heart rate (Tikkanen *et al.* 1985b), exposure to acute and chronic hypoxia (Toth *et al.* 1994) as well as increase in osmolality (Arjamaa & Vuolteenaho 1985) also modulate ANP synthesis and release.

2.1.1.2 ProANP-derived peptides in circulation

Early studies show that upon secretion into circulation, proANP$_{1\text{-}126}$ is cleaved to C-terminal ANP$_{1\text{-}28}$ (Currie *et al.* 1984) and N-terminal proANP, probably NT-proANP$_{1\text{-}98}$, in equimolar amounts (Vuolteenaho *et al.* 1985, Thibault *et al.* 1988). It is suggested that the circulating form of ANP in plasma is 3 kDa, corresponding to the C-terminal fragment of proANP, ANP$_{1\text{-}28}$ (Miyata *et al.* 1985, Schwartz *et al.* 1985, Thibault *et al.* 1985).

Many reports indicate that the major circulating molecular form of NT-proANP has a molecular weight of 10 kDa, presumed to be intact NT-proANP$_{1\text{-}98}$ (Fig. 1). One 10-kDa peak of NT-proANP$_{1\text{-}25}$ immunoreactivity, and in some patients a peak of 13 kDa as a minor component, were detected in human plasma by gel permeation chromatography (Itoh *et al.* 1988). NT-proANP was purified from human plasma by affinity chromatography using antiserum against NT-proANP$_{11\text{-}37}$, and amino acid sequencing suggested that it was closely related to NT-proANP$_{1\text{-}98}$, although some degraded peptides were also detected (Sundsfjord *et al.* 1988). Later on, the N-terminal product of proANP was investigated by two separate radioimmunoassays (RIAs) directed against the N-terminal and the C-terminal end of NT-proANP. Characterization of plasma samples by gel permeation and fast protein liquid chromatography demonstrated coelution of the N- and C-terminal immunoreactivities in a single chromatographic peak (Meleagros *et al.* 1989, Buckley *et al.* 1990a). Chen *et al.* (1990) used antibodies recognizing the prohormone sequences 1-30 and 31-67 and found out that extracted plasma immunoreactivity of both assays eluted at 46% acetonitrile, while the corresponding synthetic peptides (1-30 and 31-67) eluted at different positions (Chen *et al.* 1990). These early studies are consistent with more recent reports (Azizi *et al.* 1996, Numata *et al.* 1998). In addition, NT-proANP has a leucine zipper-like motif in its amino acid sequence (Seidler *et al.* 1999), and it has been suggested that NT-proANP could oligomerize under physiologic conditions forming molecular trimers.

The peptide structure of ANP is susceptible to degradation, and its removal from the circulation is rapid: the half-life of ANP is reported to be 2-5 minutes in man (Yandle *et al.* 1986b, Tonolo *et al.* 1988). ANP is eliminated from the circulation mainly by neutral endopeptidase (NEP 24.11, neprilysin) and clearance receptor, natriuretic peptide receptor type C (NPR-C) (Fig. 1). The NEP, which is a membrane-bound metallopeptidase, disrupts the cysteine ring structure and C-terminus of ANP resulting in

the loss of biological activity (Kenny & Stephenson 1988, Johnson *et al.* 1989). The major sites for NEP degradation are in the kidney, lung, and vascular endothelium (for review, see Maack 1992). Elimination of ANP by receptor-mediated endocytosis followed by lysosomal degradation occurs mainly in the vascular endothelium, although NPR-C is widely distributed (for review, see Ruskoaho 1992, Barr *et al.* 1996). Urinary excretion does not play a major role in ANP elimination in normal physiological conditions (Ruskoaho 1992).

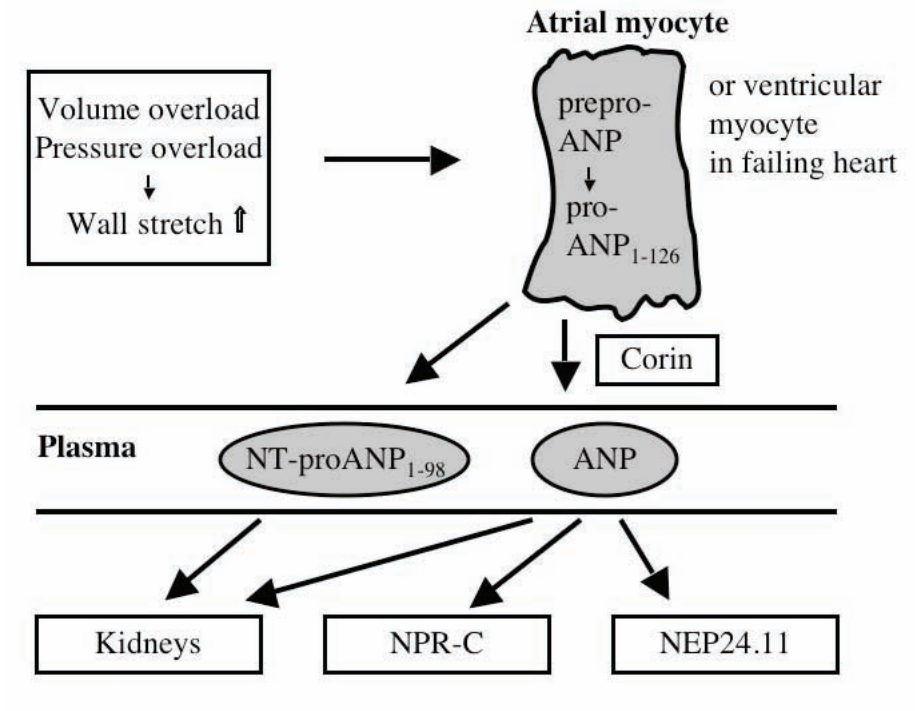

Fig. 1. Schematic representation of the production, secretion and elimination of peptides derived from proANP in the heart and circulation. NPR-C, natriuretic peptide receptor C; NEP24.11, neutral endopeptidase 24.11. For reviews, see Ruskoaho 1992, Potter *et al.* 2006.

In contrast to ANP, NT-proANP is slowly metabolized from circulation, accumulating to concentrations in plasma that are 10- to 50-fold higher than those of ANP (Thibault *et al.* 1988, Buckley *et al.* 1990a). The main elimination route for NT-proANP is considered to be passive excretion by the kidney. This hypothesis is supported by findings that the plasma level of NT-proANP is elevated in patients with chronic renal failure (Franz *et al.* 2000) and measurable concentrations can be found in urine (Hartter *et al.* 2000, Ng *et al.* 2004).

2.1.1.3 Actions of A-type natriuretic peptide

The actions of ANP are mediated by natriuretic peptide receptor A (NPR-A), which is a plasma membrane-bound guanylate cyclase. In mammals the NPR-A receptor is widely distributed in tissues including blood vessels, lung, kidney, adrenal gland, heart and adipose tissue (for review, see Maack 1992, Nakao *et al.* 1992b, Potter *et al.* 2006). The main function of ANP as well as BNP is to reduce cardiac load by modulating blood pressure, cardiac function and fluid balance (for review, see Stein & Levin 1998). At the level of kidney, ANP increases excretion of salts and water to urine by acting directly on tubular functions and renal hemodynamics. It increases the glomerular filtration rate by acting on glomerular arterioles (Weidmann *et al.* 1986) and decreases sodium absorption in the collecting duct (Zeidel *et al.* 1988). Furthermore, ANP inhibits the renin-angiotensin-aldosterone system by decreasing or inhibiting renin and aldosterone secretion, and by attenuating the stimulatory effect of angiotensin II on aldosterone release (Burnett *et al.* 1984, Atarashi *et al.* 1985). It relaxes vascular smooth muscle cells causing arterial and venous dilatation (Richards *et al.* 1985) and blocks cardiac sympathetic nervous system activity (Floras 1990). These functions of ANP cause the net effect of decreased filling of the heart (decreased preload) and lowered blood pressure (decreased afterload) (for review, see Levin *et al.* 1998).

ANP also exhibits important beneficial autocrine and paracrine functions within the heart and coronary circulation (for review, see D'Souza *et al.* 2004). ANP regulates myocyte growth inhibiting hypertrophy (Horio *et al.* 2000), suppresses cardiac fibroblast proliferation (Cao & Gardner 1995) and extracellular matrix deposition (Redondo *et al.* 1998) as well as influences coronary endothelium and vascular smooth muscle proliferation (Abell *et al.* 1989). Moreover, ANP may have cytoprotective effects in myocardial ischemia (Abell *et al.* 1989). In addition, ANP has some central effects including the ability to reduce thirst and decrease the production of antidiuretic hormone in the hypothalamus and pituitary (for review, see Ruskoaho 1992, Stein & Levin 1998). In fat cells ANP stimulates lipolysis (Sengenes *et al.* 2000).

It is uncertain whether NT-proANP$_{1\text{-}98}$ has any direct endocrine effects. However, it may be required for the proper maturation and transportation of proANP in the cardiac cells. It has been shown that acidic amino acids of proANP are critical for both calcium-mediated aggregation and regulated secretion of proANP *in vitro* (Canaff *et al.* 1996, Baertschi *et al.* 2001).

2.1.2 B-type natriuretic peptide

2.1.2.1 Synthesis and release of B-type natriuretic peptide

Similarly to ANP, B-type natriuretic peptide is present as a single copy in chromosome 1 in humans. It is organized into three exons separated by two introns (for review, see Nakao *et al.* 1992a). The highest tissue concentration of BNP is in the heart, in cardiac myocytes. In addition to myocytes, cardiac fibroblasts and the coronary vasculature have recently been shown to express the BNP gene (Casco *et al.* 2002, Tsuruda *et al.* 2002).

Some extracardiac BNP expression occurs also in the central nervous system, adrenal gland and gut (for review, see Rosenzweig & Seidman 1991, Yandle 1994). The BNP mRNA concentrations are highest in the atria, but a considerable amount is also present in the ventricles. However, the BNP mRNA level is only 2-5% of the ANP level in the atria, and 20-25% of the ANP level in the ventricles of a normal heart (Yandle 1994). When tissue weight is taken into account, the total content of BNP mRNA is greater in the ventricle than in the atrium (Ogawa *et al.* 1991). It has been proposed that ventricles are the main source of circulating BNP (Hosoda *et al.* 1991, Mukoyama *et al.* 1991).

The biosynthetic pathways of ANP and BNP resemble each other. Human BNP is synthesized as a 132-amino-acid prepropeptide containing a 24-amino-acid hydrophobic signal sequence which is processed by endoprotease cleaving to a 108-amino-acid $proBNP_{1\text{-}108}$ (for review, see Nakao *et al.* 1992a, Yandle 1994). The propeptide is further cleaved by a furin- or corin-like endoprotease (Sawada *et al.* 1997, Yan *et al.* 2000) to form active $BNP_{77\text{-}108}$ and inactive N-terminal proBNP ($NT\text{-}proBNP_{1\text{-}76}$) molecules. Both of these peptides circulate in human plasma (Hunt *et al.* 1995) (Fig. 2). In addition to BNP and NT-proBNP, proBNP may also be present in human heart and plasma, suggesting that the posttranslational processing of proBNP occurs prior to or during the secretion in the myocardium (Tateyama *et al.* 1992, Yandle *et al.* 1993b, Hunt *et al.* 1997b, Mair *et al.* 2001). The site of proteolytic cleavage is still being debated. The processing of proBNP has not been observed in the whole blood or plasma (Hunt *et al.* 1997a). In the 17-residue ring structure, 13 of 17 amino acids are identical between BNP and ANP in humans. Moreover, the C-terminus of BNP and ANP contains the same arginine-tyrosine dipeptide. However, unlike ANP, there are major species variations in the structure of mammalian BNP (for review, see McDowell *et al.* 1995).

BNP is secreted soon after its synthesis. Only small amounts of BNP may be stored in atrial secretory granules together with ANP (Suzuki *et al.* 1992, Thibault *et al.* 1992). Thus, BNP appears to utilize the constitutive secretory pathway, and increased release of BNP requires a longer stimulus to enhance its rate of synthesis and subsequent secretion. Indeed, the regulation of BNP synthesis appears to occur mainly at the level of transcription and perhaps stabilization of mRNA (for review, see de Bold *et al.* 1996). Like ANP, BNP gene is induced by pressure and volume overload, but the stimulation is much more rapid. The cardiac BNP mRNA level rises within 30 minutes during constant stretch, whereas the ANP mRNA requires several hours of stretch (Mäntymaa *et al.* 1993, Magga *et al.* 1997a). Furthermore, the stretch-induced BNP mRNA increase does not depend on protein synthesis, revealing the induction process with available factors (Magga *et al.* 1997a, Magga *et al.* 1997b). Fast induction of BNP gene resembles the induction of the immediate early genes. Like many other rapidly induced genes, BNP mRNA also contains several AU-rich sequences in the 3' untranslated region (for review, see Nakao *et al.* 1992a). These AU-rich elements are known to destabilize mRNA within the cell, and they may be involved in the translation-dependent degradation of mRNA. In fact, posttranscriptional control has been found to be an important factor in the regulation of left ventricular BNP gene expression *in vivo* (Suo *et al.* 2002).

Mechanical stretch induces BNP gene expression and it also constitutes a major determinant of BNP release from atria and ventricle (Kinnunen *et al.* 1993, Mäntymaa *et al.* 1993). Whether wall stress of cardiac cells acts directly or through local autocrine or paracrine factors to increase BNP gene expression and secretion is not fully understood.

For example, endothelin, angiotensin II, phenylephrine and arginine vasopressin have been shown to increase BNP expression (for review, see Yandle 1994, de Bold *et al.* 1996, Tokola *et al.* 2001). Likewise as with ANP, cardiac hypertrophy is associated with increased synthesis and secretion of ventricular BNP. Cardiac hypertrophy, however, leads to greater ventricular induction of BNP than ANP (Mukoyama *et al.* 1990, Yandle 1994). In addition, many other stimuli affect BNP release from the heart, including endotoxemia (Tomaru Ki *et al.* 2002), hypoxia (Toth *et al.* 1994), and ischemia (D'Souza *et al.* 2004).

2.1.2.2 ProBNP-derived peptides in circulation

The molecular forms of circulating proBNP-derived peptides have been examined by chromatography and sequence-specific immunoassays. BNP is secreted from the heart (Mukoyama *et al.* 1991, Tateyama *et al.* 1992) and circulates without binding to plasma proteins. However, BNP-32 is trimmed in whole blood to generate a truncated BNP form lacking the two N-terminal amino acids, serine and proline (Shimizu *et al.* 2002). A candidate enzyme for this truncation is dipeptidyl-peptidase IV (DPP IV/CD26; EC 3.4.14.5) (Brandt *et al.* 2005). Furthermore, blood coagulation factors, especially kallikrein, play an important role in degradation of the C-terminal structure of BNP (Shimizu *et al.* 2001). This may occur *in vivo* on the intraluminal surface of a damaged vessel or *in vitro* on the walls of glass tubes.

In addition to the biologically active BNP-32, other proBNP-derived fragments have been found in circulation (Fig. 2). The immunoreactivity of high-molecular-weight BNP (10 kDa), probably corresponding to $proBNP_{1\text{-}108}$, has been reported in the plasma of heart failure patients and healthy subjects (Tateyama *et al.* 1992, Yandle *et al.* 1993b). Size exclusion HPLC studies together with NT-$proBNP_{1\text{-}13}$ RIA indicated for the first time the presence of NT-proBNP peptide (8.6 kDa) in human plasma (Hunt *et al.* 1995). However, the high-molecular-mass BNP forms, cross-reacting with BNP-32 antisera, were not detected using the N-terminal antiserum, suggesting that the high-molecular-mass material is not intact $proBNP_{1\text{-}108}$ (Hunt *et al.* 1995, Hunt *et al.* 1997a, Hunt *et al.* 1997b). Later on Schulz and coworkers proposed, using size exclusion of plasma extracts and antiserum to the NT-$proBNP_{1\text{-}21}$ fragment, that $proBNP_{1\text{-}108}$ may circulate both as the intact prohormone and as split products, NT-$proBNP_{1\text{-}76}$ and $BNP_{77\text{-}108}$ (Schulz *et al.* 2001).

There is a leucine zipper-like motif in the proBNP sequence (Seidler *et al.* 1999). This has led to the suggestion that proBNP and NT-proBNP could oligomerize under physiologic conditions, producing either a trimer or tetramer of proBNP and a trimer of NT-proBNP (Seidler *et al.* 1999). Shimizu *et al.* (2003) also found trimers of proBNP in human plasma under physiological conditions using proBNP- and BNP-specific immunoassays for analyzing immunoreactivity in gel filtration HPLC fractions (Shimizu *et al.* 2003). Two major peaks of immunoreactivity were detected: high molecular weight (36 kDa) corresponding to trimers of proBNP and low molecular weight (4 kDa) corresponding to BNP (Shimizu *et al.* 2003). Recently, Crimmins (2005) studied synthetic NT-proBNP using analytical sedimentation, equilibrium ultracentrifugation and

circular dichroism, and demonstrated that NT-proBNP is a monomer in a physiologic buffer forming an unordered random coil with an extended-like structure (Crimmins 2005).

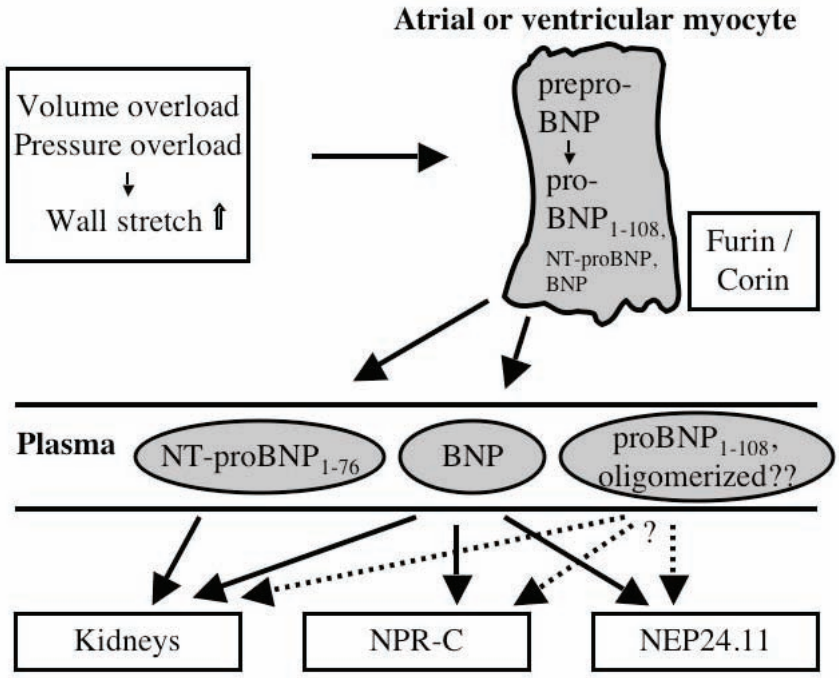

Fig. 2. Schematic representation of the production, secretion and elimination of peptides derived from proBNP in the heart and circulation. NPR-C, natriuretic peptide receptor C; NEP24.11, neutral endopeptidase 24.11. For reviews, see Ruskoaho 1992, Apple *et al.* 2005, Potter *et al.* 2006.

The half-life of BNP-32 has been reported to be about 20 minutes (Richards *et al.* 1993a), which is much longer than the half-life of ANP. Like ANP, BNP is metabolized by a membrane-bound endopeptidase (NEP 24.11) (Kenny *et al.* 1993) as well as by receptor-mediated cellular uptake (NPR-C) (Maack 1992) (Fig. 2). However, compared to ANP, BNP seems relatively resistant to NEP degradation (Kenny *et al.* 1993, Smith *et al.* 2000) and its affinity for clearance receptors is weaker (Mukoyama *et al.* 1991, Lang *et al.* 1992), leading to longer half-life. BNP-32 is also found in urine (Totsune *et al.* 1996), but the mechanism of renal excretion is not known.

There are very limited data available concerning the half-life of NT-proBNP in circulation. Studies in sheep have demonstrated that NT-proBNP has a longer clearance time than BNP (Pemberton *et al.* 2000). According to deconvolution analyses, the half-life of 70 minutes for NT-proBNP is 15 times longer than that of BNP. NT-proBNP is present in urine (Ng *et al.* 2004), and renal excretion is currently regarded as the main clearance mechanism of NT-proBNP.

2.1.2.3 Actions of B-type natriuretic peptide

The actions of BNP, like ANP, are mediated by natriuretic peptide receptor type A (NPR-A), though its potency is lower than that of ANP (Maack 1992). The natriuretic, diuretic and hemodynamic responses of BNP resemble those observed with ANP (Sudoh *et al.* 1988). ANP and BNP modulate cardiac function, blood pressure and fluid balance in order to reduce cardiac load. In addition to endocrine actions, BNP also exhibits some autocrine and paracrine functions (D'Souza *et al.* 2004). It modulates fibroblast proliferation (Cao & Gardner 1995, Tamura *et al.* 2000) and extracellular matrix formation (Tsuruda *et al.* 2002). Moreover, transgenic mice overexpressing the BNP gene exhibit systemic hypotension and bone malformations (Ogawa *et al.* 1994). BNP may also have a cytoprotective function in myocardial ischemic conditions (D'Souza *et al.* 2003). It appears unlikely that NT-proBNP has biological effects on its own (Goetze 2004a).

Table 1. Characteristics of A- and B-type natriuretic peptides. For reviews, see Ruskoaho 2003, Vuolteenaho et al. *2005, Potter* et al. *2006.*

Feature	ProANP	ProBNP
Gene expression		
mRNA concentration in normal heart	Ventricle < atrium	Ventricle > atrium
mRNA concentration in hypertrophy	Ventricle > atrium	Ventricle >> atrium
Gene induction	Slow	Rapid
mRNA turnover	Slow	Rapid
Circulating hormone		
Circulating forms	ANP, NT-proANP	ProBNP, BNP, NT-proBNP (?)
Biologically active form	ANP	BNP
Site of cleavage	Myocyte membrane	Not known
Major regulator of release	Myocyte stretch (preload)	Myocyte stretch (afterload)
Secretion	Regulated	Constitutive
Half-life	ANP 2-5 minutes, NT-proANP 40-50 minutes	BNP ~20 minutes, NT-proBNP ~70 minutes (?)

2.1.3 C-type natriuretic peptide

2.1.3.1 Synthesis and release of C-type natriuretic peptide

The gene encoding C-type natriuretic peptide (CNP) resides in human chromosome 2 and contains three exons separated by introns (for review, see Nakao *et al.* 1992a, Levin *et al.* 1998). CNP is widely distributed. The major sites for CNP expression are the central nervous system, pituitary, pineal, vascular endothelium, vascular smooth muscle cells, kidney, testis, ovary and uterus. Very little if any CNP is present in the cardiomyocytes (for review, see Barr *et al.* 1996, Walther & Stepan 2004, Scotland *et al.* 2005).

In common with ANP and BNP, CNP is synthesized as a preproCNP comprising 126 amino acids, and the first 23 amino acids are cleaved as the signal peptide. In mammals proCNP is cleaved between residues 50-51 or 81-82 to release the biologically active C-terminal peptides, CNP-53 and CNP-22 (Tawaragi *et al.* 1991). CNP-53 is presumably cleaved from proCNP by an enzyme having Arg-x-x-Arg specificity, probably furin (Wu *et al.* 2003b), and in the formation of CNP-22 the dibasic Lys-Lys sequence is important (Yandle 1994, Barr *et al.* 1996). Quite recently it has been shown that an N-terminal peptide fragment of proCNP is released into the circulation. This peptide has a molecular weight consistent with it being NT-proCNP(1-50) (Prickett *et al.* 2001). The primary structure of CNP-22 is identical in the pig, rat, human, sheep, cow and mouse (Minamino *et al.* 1990, Sudoh *et al.* 1990, Samson 1992). It is thus the most conserved peptide in the natriuretic peptide family. CNP is highly homologous with ANP and BNP within the ring structure, 11 of 17 amino acids are identical. However, CNP uniquely lacks the C-terminal extension present in the ANP and BNP molecules (for review, see Nakao *et al.* 1992a).

The exact stimuli for CNP synthesis and release are not known. *In vitro* studies have shown that many factors regulate the transcription of the CNP gene and these same factors may also regulate the release of active CNP from the cell. Many cytokines influencing vascular cell proliferation, migration and contraction increase CNP mRNA. The data on the *in vivo* regulation of CNP release are limited, but elevated levels of CNP have been found in hypoxia, sepsis and chronic renal failure (for review, see Barr *et al.* 1996).

2.1.3.2 ProCNP-derived peptides in circulation

CNP-53, CNP-22 as well as NT-proCNP exist in human plasma. CNP-53 and CNP-22 are rapidly removed from the circulation due to degradation by neutral endopeptidase and by binding to the natriuretic peptide clearance receptors. CNP is the most susceptible of the natriuretic peptides to hydrolysis by the neutral endopeptidase 24.11 *in vitro* (Kenny *et al.* 1993). CNP-22 and CNP-53 have also been detected in the urine, suggesting that they are cleared from the circulation by the kidneys (Mattingly *et al.* 1994). The half-life of CNP is short, 2.6 minutes (Hunt *et al.* 1994). CNP-22 is more abundant than CNP-53 in the circulation (Stingo *et al.* 1992b, Hama *et al.* 1994), whereas CNP-53 predominates in tissues (Yandle *et al.* 1993a).

The major NT-proCNP species circulating in humans appears to be NT-proCNP(1-50). It is the N-terminal fragment expected from processing of proCNP to CNP-53. However, smaller amounts of proCNP(1-81) may circulate in human plasma, suggesting that proCNP may be directly processed to CNP-22 (Prickett *et al.* 2001). There is also a possibility that proCNP(1-81) is further processed to CNP-53 and CNP-53 to CNP-22. The half-life of NT-proCNP is unknown, but it may be longer than that of CNP, because of the lack of receptor- and neutral endopeptidase-mediated degradation (Prickett *et al.* 2001).

2.1.3.3 Actions of C-type natriuretic peptide

CNP acts as a neuropeptide and/or a local regulator rather than a cardiac hormone in mammals, and thus it has a different role in cardiovascular physiology than ANP and BNP. The effects of CNP are mediated by natriuretic peptide receptor type B (NPR-B). CNP is a potent vasorelaxant of veins and selective arteries both *in vitro* and *in vivo* (for review, see Barr *et al.* 1996, Scotland *et al.* 2005). In humans CNP causes a significant decrease in both systolic and diastolic blood pressure with an increase in heart rate (Igaki *et al.* 1998). Additionally, CNP exerts antiproliferative and antihypertrophic actions on vascular smooth muscle cells (Furuya *et al.* 1991, Rosenkranz *et al.* 2003), neonatal cardiomyocytes, cardiac fibroblasts (Horio *et al.* 2003) and glia cells (Levin & Frank 1991). In contrast to ANP and BNP, CNP has only minimal natriuretic effects (Stingo *et al.* 1992a).

CNP is also recognized to be expressed in and to affect various extracardiovascular tissues. It promotes longitudinal bone growth in an organ culture (Yasoda *et al.* 1998). In addition, the offspring of CNP-deficient mice show severe dwarfism due to impaired endochondral ossification, indicating that CNP is a crucial molecule in bone formation (Chusho *et al.* 2001). CNP is involved in male and female reproductive processes, but its exact role in reproduction is not known (for review, see Walther & Stepan 2004).

2.1.4 Other natriuretic peptides

Dendroaspis natriuretic peptide (DNP), a 38-amino-acid peptide, is a member of the natriuretic peptide family. It was isolated from the venom of the Green Mamba (*Dendroaspis angusticeps*) and has structural similarities to other known natriuretic peptides (Schweitz *et al.* 1992). The gene for DNP has not been cloned. A 'DNP-like' peptide has been isolated from human plasma and atria, but conclusive evidence about its presence in man remains controversial (Richards *et al.* 2002).

A novel member of the natriuretic peptide family is salmon cardiac peptide (sCP), cloned and isolated from salmon (*Salmo salar*). It combines properties of ANP, BNP and CNP (Tervonen *et al.* 1998). The gene structure of sCP has been resolved and its expression is very heart-specific (Majalahti-Palviainen *et al.* 2000). The biosynthetic pathway of sCP seems to be similar to that of the other natriuretic peptides (Kokkonen *et al.* 2000). The biologically active sCP as well as the N-terminal fragment of pro-sCP (NT-pro-sCP) circulates in salmon serum, and NT-pro-sCP is a sensitive marker of the endocrine function of salmon heart (Vierimaa *et al.* 2002). sCP is stimulated by cardiac loading, endothelin-1, and beta-adrenergic stimulation (Vierimaa *et al.* 2006).

2.2 Measurement of natriuretic peptides

2.2.1 Natriuretic peptide assays

The first immunoassays for natriuretic peptides were published soon after their discovery. They were generally performed by means of competitive immunoassays, usually radioimmunoassays (RIAs) or enzyme immunoassays (EIAs), in which the analyte competes with the tracer for binding to the specific antiserum. Noncompetitive immunoassays have subsequently been set up. These sandwich assays utilize two specific monoclonal antibodies or affinity-purified polyclonal antisera prepared against sterically remote epitopes of the analyte. In recent years, fully automated and rapid point-of-care tests for natriuretic peptides have been developed for commercial use (for review, see Clerico *et al.* 1999b, Clerico *et al.* 2000, Clerico & Emdin 2004, Vuolteenaho *et al.* 2005).

2.2.1.1 Immunoassays for A- and B-type natriuretic peptides

The first competitive RIA procedures for the measurement of circulating ANP and BNP were reported at the time of the structural identification of the peptides. It soon became evident that the circulating concentration of ANP (Gutkowska *et al.* 1985, Sugawara *et al.* 1985, Vuolteenaho *et al.* 1985, Yandle *et al.* 1986a, Richards *et al.* 1987) as well as BNP (Lang *et al.* 1991, Mukoyama *et al.* 1991, Tateyama *et al.* 1992, Yandle *et al.* 1993b) is low and thus sensitive to non-specific interference requiring extraction of the peptide from the plasma before measurement. Later on, non-competitive immunoradiometric (IRMA) assays were developed allowing direct measurement of ANP (Lewis *et al.* 1989, Tattersall *et al.* 1990) and BNP (Kono *et al.* 1993) from the plasma or serum samples. Usually, the pair of antibodies directed towards the ring structure of the ANP or BNP and the N- or C-terminus of the respective peptide was utilized (Clerico *et al.* 1999a). Measurement of natriuretic peptides, especially BNP, soon proved to be clinically useful, encouraging commercial assay development. The first commercial ANP (Clerico *et al.* 1996) and BNP assays (Kono *et al.* 1993) were IRMAs developed by the Japanese Shionogi & Co. (Osaka, Japan) in the early 90s. A few years later, Biosite Diagnostics (San Diego, CA, USA) developed an automated fluorescent immunoassay, Triage® BNP Test, which is perhaps the most widely used clinical BNP assay in the world. Biosite also has the Triage® BNP Test on the Beckman Coulter platforms (Access, Synchron LXI, UniCel DXI). Bayer Diagnostics (Tarrytown, NY, USA) have licensed the use of the Shionogi antibodies and set up a fully automated immunochemiluminescent BNP assay for their ADVIA Centaur® immunoassay platform. Moreover, Abbott Laboratories (Abbott Park, Illinois, USA) has a microparticle enzyme immunoassay for BNP with fluorescent measurement on their AxSYM® system. U.S. Food and Drug Administration (FDA) has cleared the use of Triage® BNP, ADVIA Centaur® BNP as well as AxSYM® BNP as an aid e.g. in the diagnosis of heart failure (for review, see Clerico & Emdin 2004, Apple *et al.* 2005, Vuolteenaho *et al.* 2005).

2.2.1.2 Immunoassays for N-terminal A-type natriuretic peptide

Several competitive RIAs were developed for NT-proANP in the late 80s and early 90s. The antisera used were directed against N-terminus (Itoh *et al.* 1988, Meleagros *et al.* 1989, Buckley *et al.* 1990a, Chen *et al.* 1990, Azizi *et al.* 1996), middle region (Sundsfjord *et al.* 1988, Chen *et al.* 1990) or C-terminus of NT-proANP (Meleagros *et al.* 1989, Buckley *et al.* 1990a, Vuolteenaho *et al.* 1992). Later on, competitive immunoassays with non-radiometric labels were established including enzyme immunoassays (EIAs) with a horseradish peroxidase label and affinity-purified sheep antisera against NT-proANP$_{8-27}$ and NT-proANP$_{31-64}$ (Hartter *et al.* 2000), and an immunoluminometric assay (ILMA) for NT-proANP$_{1-30}$ (Squire *et al.* 2004b). The first noncompetitive immunoassay for NT-proANP was reported by Stridsberg *et al.* (1997). The assay was based on two monoclonal antibodies directed at the extreme termini of the NT-proANP peptide. The catcher antibody (NT-proANP$_{1-30}$) was biotinylated and bound to streptavidin-coated microtiter strips, whereas the detector antibody (NT-proANP$_{79-98}$) was labeled with fluorescent label (Stridsberg *et al.* 1997). Numata *et al.* (1998) established an immunoradiometric assay (IRMA) using two monoclonal antibodies against NT-proANP$_{1-25}$ and NT-proANP$_{43-66}$, and synthetic NT-proANP$_{1-67}$ as a standard (Numata *et al.* 1998). A few years later an enzyme immunosorbent assay (ELISA) utilizing antibodies against NT-proANP$_{16-23}$ and NT-proANP$_{80-88}$ was published. For the first time a recombinant NT-proANP$_{1-98}$ was used for the calibration of this assay (Missbichler *et al.* 2001). Later on, Morgenthaler *et al.* (2004) set up an immunoluminometric assay (ILMA) for the middle region of NT-proANP using affinity-purified sheep antisera against NT-proANP$_{53-72}$ and NT-proANP$_{73-90}$, and a synthetic peptide calibrator (Morgenthaler *et al.* 2004). Table 2 summarizes the published NT-proANP assays and their epitopes, calibrators and reference values.

Because the plasma concentration of NT-proANP is an order of magnitude higher than the plasma concentration of ANP and as it is also more stable in laboratory conditions (Hall *et al.* 1995a, Hall *et al.* 1995b, Davidson *et al.* 1996), it was assumed that assay development would be easier. However, immunoassays for NT-proANP are affected by several analytical problems, mainly concerning assay specificity. Most assays utilize synthetic peptide fragments, typically spanning 20-30 amino acids of the proANP peptide, as calibrators. Furthermore, the antisera are directed against these short peptide fragments without proper knowledge of how well they identify the full-length NT-proANP peptide (Clerico *et al.* 2000, Clerico & Emdin 2004).

Table 2. Reported immunoassays for NT-proANP.

Assay epitope	Assay calibrator	Assay type	Normal plasma concentration	Reference
$ProANP_{1-25}$	$ProANP_{1-25}$	RIA	174 ± 21 pmol/L (mean ± SEM)	Itoh *et al.* 1988
$ProANP_{11-37}$	$ProANP_{1-30}$	RIA	420 ± 157 pmol/L (mean ± SD)	Sundsfjord *et al.* 1988
$ProANP_{1-16}$ or $proANP_{87-98}$	$ProANP_{1-16}$ or $proANP_{87-98}$	RIAs	129 ± 13, 895 ± 213 pmol/L (mean ± SEM)	Meleagros *et al.* 1989
$ProANP_{1-30}$ or $proANP_{31-67}$	$ProANP_{1-30}$ or $proANP_{31-67}$	Sep-Pak RIAs	362 ± 173, 368 ± 160 pg/mL (34 ± 16, 35 ± 15 pmol/L, mean ± SD)	Chen *et al.* 1990
$ProANP_{1-30}$ or $proANP_{79-98}$	$ProANP_{1-30}$ or $proANP_{79-98}$	Sep-Pak RIAs	235 ± 19, 547 ± 33 pg/mL (22 ± 2, 52 ± 3 pmol/L, mean ± SEM)	Buckley *et al.* 1990
$ProANP_{79-98}$	$ProANP_{79-98}$	RIA	110 – 600 pmol/L (range)	Vuolteenaho *et al.* 1992
$ProANP_{1-30}$	$ProANP_{1-30}$	RIA	251 ± 31.5 pg/mL (24 ± 3 pmol/L, mean ± SEM)	Azizi *et al.* 1996
$ProANP_{1-30}$ and $proANP_{79-98}$	Calibrated serum pool	IFMA	277 ± 201 pmol/L (mean ± SD)	Stridsberg *et al.* 1997
$ProANP_{1-25}$ and $proANP_{43-66}$	$ProANP_{1-67}$	IRMA	188 ± 71 pmol/L (mean ± SD)	Numata *et al.* 1998
$ProANP_{8-27}$ or $proANP_{31-64}$	$ProANP_{1-30}$ or $proANP_{31-67}$	EIAs	110-470, 180-790 pmol/L (range)	Hartter *et al.* 2000
$ProANP_{16-23}$ and $proANP_{80-88}$	Recombinant NT-$proANP_{1-98}$	ELISA	0-2000 pmol/L (range)	Missbichler *et al.* 2001
$ProANP_{53-72}$ and $proANP_{73-90}$	$ProANP_{53-90}$	ILMA	9.6-313 pmol/L (range)	Morgenthaler *et al.* 2004
$ProANP_{1-30}$	$ProANP_{1-30}$	ILMA	122-1786 pmol/L (range)	Squire *et al.* 2004a

RIA, radioimmunoassay; IFMA, immunofluorometric assay; IRMA, immunoradiometric assay; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; ILMA, immunoluminometric assay.

2.2.1.3 Immunoassays for N-terminal B-type natriuretic peptide

The first measurement of NT-proBNP was reported by Hunt *et al.* (1995). It was a competitive RIA method requiring the extraction of the peptide from plasma. This assay utilized antisera raised against a peptide fragment of the extreme N-terminus of proBNP ($NT\text{-}proBNP_{1-21}$) and a corresponding peptide calibrator (Hunt *et al.* 1995). Two additional competitive RIAs against the N-terminus of proBNP were also published a few years later (Campbell *et al.* 2000, Schulz *et al.* 2001). Hughes *et al.* (1999) developed a competitive extraction based ILMA with a chemiluminscence label against the C-terminus of NT-proBNP ($NT\text{-}proBNP_{65-76}$) (Hughes *et al.* 1999). The same year, direct competitive EIAs in microtiter plate format for N-terminus ($NT\text{-}proBNP_{8-29}$) and middle region ($NT\text{-}proBNP_{39-50}$) of proBNP were reported (Missbichler *et al.* 1999). Goetze *et al.* (2002) developed a processing-independent analysis (PIA) for quantification of proBNP

fragments in plasma. Before measurement, the plasma was treated with trypsin cleaving all proBNP-derived peptides to the 1-21 fragment, which was detected by RIA using an antiserum directed against $proBNP_{1-10}$ (Goetze *et al.* 2002). The first assay for NT-proBNP in sandwich format was developed by Karl *et al.* (1999). The assay utilized affinity-purified polyclonal antisera directed against N-terminus ($NT\text{-}proBNP_{1-21}$) and mid-region of proBNP ($NT\text{-}proBNP_{30-38}$). This assay has been further tuned by Roche Diagnostics (Basel, Switzerland), which offers nowadays a fully automated electrochemiluminescence proBNP assay (ECLIA) for their Elecsys 1010, 2010 and E180 analyzers. Elecsys® proBNP has been cleared by the FDA for use as an aid in the diagnosis of heart failure. Dade-Behring has licensed Roche's patent and offers the Dimension RxL NT-proBNP method (Di Serio *et al.* 2005). Omland *et al.* (2002) described another sandwich assay for NT-proBNP in ILMA format. The antisera were against the extreme termini of NT-proBNP ($NT\text{-}proBNP_{1-12}$ and $NT\text{-}proBNP_{65-76}$) as reported previously (Hughes *et al.* 1999, Karl *et al.* 1999, Squire *et al.* 2004b). Recently, an assay for the intact $proBNP_{1-108}$ molecule was established. The monoclonal antibody specific to the hinge region of NT-proBNP and BNP was produced and used together with polyclonal antisera against BNP_{1-32} to detect proBNP in blood samples (Giuliani *et al.* 2006). Table 3 summarizes the published NT-proBNP assays and their epitopes, calibrators and reference values.

There are some major problems with the NT-proBNP assays. The circulating molecular forms of NT-proBNP are not known; furthermore, a proper full-length calibrator is difficult to prepare (Apple 2005, Apple *et al.* 2005). Similarly to the NT-proANP assays, the NT-proBNP assays are usually based on antisera raised against synthetic peptide fragments without exact knowledge of how well the antisera will recognize the extended NT-proBNP and proBNP peptides (Vuolteenaho *et al.* 2005).

Table 3. Reported immunoassays for NT-proBNP.

Assay epitope	Assay calibrator	Assay type	Normal plasma concentration	Reference
$ProBNP_{1-13}$ or $proBNP_{62-76}$	$ProBNP_{1-21}$ or $proBNP_{62-76}$	Sep-Pak RIAs	10.8 ± 1.3 pmol/L (mean ± SD), not reported	Hunt *et al.* 1995, 1997a, 1997b
$ProBNP_{1-21}$ and $proBNP_{30-38}$	Recombinant NT-proBNP	ELISA	Not reported	Karl *et al.* 1999
$ProBNP_{65-76}$	$ProBNP_{65-76}$	Sep Pak ILMA	120-245 pmol/L (range)	Hughes *et al.* 1999
$ProBNP_{8-29}$ or $proBNP_{32-57}$	$ProBNP_{8-29}$ or $proBNP_{32-57}$	EIAs	<1065, <240 pmol/L (reference)	Missbichler *et al.* 1999
$ProBNP_{1-23}$	$ProBNP_{1-23}$	Sep Pak RIA	1.0-13.8 pmol/L (range)	Campbell *et al.* 2000
$ProBNP_{1-21}$	$ProBNP_{1-21}$	RIA	12.5-74.5 pmol/L (range)	Schulz *et al.* 2001
$ProBNP_{1-10}$	Tyr-$proBNP_{1-10}$	PIA	0-15 pmol/L (range)	Goetze *et al.* 2002
$ProBNP_{1-21}$ and $proBNP_{39-50}$	Synthetic $ProBNP_{1-76}$	ECLIA (Roche)	1.7-21.1 pmol/L (range)	Karl *et al.* 1999, Clerico and Emdin 2004
$ProBNP_{1-12}$ and $proBNP_{65-76}$	Not reported	ILMA	14-933 pmol/L (range)	Omland *et al.* 2002, Squire *et al.* 2004b
$ProBNP_{75-80}$ and BNP_{1-32}	$ProBNP_{1-108}$	ELISA	42-99 ng/L (range)	Giuliani *et al.* 2006

RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; ILMA, immunoluminometric assay; EIA, enzyme immunoassay; PIA, processing-independent assay; ECLIA, electrochemiluminescent assay.

2.2.2 Circulating concentrations of natriuretic peptides

The basal plasma concentrations of ANP in humans reported by different laboratories range from 10 to 50 ng/mL (3-16 pmol/L) (Gutkowska *et al.* 1985, Richards *et al.* 1987, Lewis *et al.* 1989, Tattersall *et al.* 1990, Clerico *et al.* 1998). This variation probably relates to methodological differences such as antibody specificity, calibration and extraction procedure as well as to differences in populations studied. For example, plasma acidification before extraction increases plasma ANP about 2-fold (Buckley *et al.* 1987). Furthermore, the plasma ANP concentration is greatly affected by sample collection and storage. Blood samples should be centrifuged and frozen immediately, because some blood cells, most probably platelets, can degrade ANP even in the presence of protease inhibitors (Schiffrin *et al.* 1988). The concentration of NT-proANP is 10- to 20-fold higher than that of ANP due to its slower clearance from the circulation (Buckley *et al.* 1990b, Arjamaa *et al.* 1996) and it is also more stable in laboratory conditions compared to ANP (Hall *et al.* 1995a, Davidson *et al.* 1996). The most often reported mean levels vary from 100 to 400 pmol/L, but the range is broad (Table 2).

Plasma concentrations of BNP in healthy persons are much lower than those of ANP, although its half-life is longer. This relates to the lower expression of the BNP gene under physiological conditions, whereas the basal expression of ANP gene is considerably high.

Reported mean values for BNP by different assays range from 1 to 22 ng/L (0.35-6.4 pmol/L) (Lang *et al.* 1991, Mukoyama *et al.* 1991, Tateyama *et al.* 1992, Clerico *et al.* 1998, Clerico *et al.* 2005). BNP is unstable when collected in glass tubes because of the activation of kallikreins (Shimizu *et al.* 2001). Moreover, extremely highly variable circulating concentrations, from picomolar up to nanomolar, of NT-proBNP (up to ~100-fold) have been detected by different assays (Table 3). This indicates methodological differences and problems in antibody specificity and calibration (Goetze 2004b, Apple *et al.* 2005). However, NT-proBNP appears to be relatively stable during sample storage (Hunt *et al.* 1997b, Downie *et al.* 1999, Nowatzke & Cole 2003).

The circulating concentrations of natriuretic peptides are modified by several physiologic factors. Women have been reported to have higher natriuretic peptide levels than men (Clark *et al.* 1990, Redfield *et al.* 2002, Wang *et al.* 2002). The higher values in women during the fertile adult period may be explained by the stimulation of the renin-angiotensin system by female steroid hormones (Kuroski de Bold 1999). Increased levels are also detected in elderly subjects. This increase may be due to the fact that aging causes a decrease in the function of the myocardium and other organs, such as the kidney (Redfield *et al.* 2002, Wang *et al.* 2002). Furthermore, it has been reported that the maximum binding capacity of natriuretic peptide clearance receptors is decreased in platelets of elderly persons (Giannessi *et al.* 2001), and the molar ratio of plasma cGMP to BNP decreases progressively with aging, suggesting attenuated biological effectiveness (Kawai *et al.* 2004). Other physiologic factors, such as exercise (Huang *et al.* 2002), body posture (Hollister *et al.* 1986, Pump *et al.* 2002), sodium intake (Lang *et al.* 1991, Singer *et al.* 1991), ingestion of licorice (Forslund *et al.* 1989), and circadian variations (Leppäluoto & Ruskoaho 1990) may influence natriuretic peptide concentrations. Drugs including diuretics, angiotensin-converting enzyme inhibitors, adrenergic agonists, glucocorticoids, sex and thyroid hormones, as well as many clinical conditions can also modify circulating concentrations (for review, see Ruskoaho 2003, Rademaker & Richards 2005).

2.3 Diagnostic use of natriuretic peptides

Increased circulating levels of natriuretic peptides are associated with volume expansion and pressure overload, reduced ventricular systolic or diastolic function, left ventricular hypertrophy as well as renal impairment and neurohumoral activation (for review, see Sagnella 1998, Ruskoaho 2003). ANP is rapidly secreted from atrial granules upon stimulus, and thus it is an acutely responding hormone, whereas BNP reflects a more prolonged cardiac overload. In addition, ANP is thought of as a marker of increased cardiac preload, whereas BNP is more sensitive to increased afterload. Natriuretic peptides reflect the pump function of the heart, and the underlying disease must be further investigated by other means, e.g. echocardiography. However, the increased concentrations of natriuretic peptides are useful for ruling out disease of cardiac origin, as prognostic indicators in patients with acute coronary syndrome or heart failure, and in the follow-up of patients with heart failure (for review, see Clerico & Emdin 2004, Rademaker & Richards 2005, Vuolteenaho *et al.* 2005, Maisel *et al.* 2006). Table 4

summarizes clinical conditions in which natriuretic peptides are useful as biomarkers. The clinical utility of natriuretic peptides is reviewed in more detail in the following paragraphs.

Table 4. Clinical conditions studied for possible uses of natriuretic peptides as biomarkers. For reviews, see Ruskoaho 2003, Clerico & Emdin 2004, Vuolteenaho et al. *2005, Maisel* et al. *2006.*

Clinical use
Identification of cardiovascular patients
Diagnosis and ruling out of heart failure in primary care
Ruling out of heart failure in general population
Assessment of severity of disease and functional capacity in heart failure
Detection of left ventricular diastolic dysfunction
Differentiating between cardiac and pulmonary causes of acute dyspnea
Estimation of infarct size and left ventricular dysfunction after myocardial infarction
Identification of tachyarrhythmias
Prognostic evaluation
Prognostic outcome in heart failure
Prognostic outcome after acute coronary syndrome
Prognostic evaluation in general population (elderly)
Monitoring of therapy
Guiding the treatment in heart failure

2.3.1 Natriuretic peptides in identifying cardiovascular patients

2.3.1.1 Heart failure

Heart failure occurs whenever the heart cannot maintain cardiac output sufficient for the metabolic needs of the tissues. It is a common clinical syndrome resulting from various diseases affecting the heart. The diagnosis of heart failure is difficult because the patients often present with nonspecific symptoms, such as dyspnea, fatigue, and ankle swelling. The diagnosis is most often based on clinical history, physical examination, electrocardiogram, chest x-ray, and the evaluation of ventricular dysfunction, e.g. by means of echocardiography (Hunt *et al.* 2001, Remme & Swedberg 2001, Swedberg *et al.* 2005). The concentrations of natriuretic peptides increase even in patients with asymptomatic heart failure, and they have therefore given rise to a lot of interest as a simple biochemical tool for distinguishing patients with heart failure (Sagnella 1998, Ruskoaho 2003). As a result, the most recent European and American guidelines for the evaluation and treatment of chronic heart failure include circulating levels of natriuretic peptides as means of identification of patients with congestive heart failure (Hunt *et al.* 2001, Remme & Swedberg 2001, Swedberg *et al.* 2005).

Clinical heart failure. Pioneering studies indicated that plasma natriuretic peptide levels increase in patients with symptomatic heart failure in proportion to the severity of

the disease, reaching extremely high values in chronic conditions (Tikkanen *et al.* 1985a, Burnett *et al.* 1986, Richards *et al.* 1986, Swedberg *et al.* 1990). Natriuretic peptides and their prohormones are also significantly increased in patients with asymptomatic left ventricular dysfunction, although less so than in patients with obvious symptoms of heart failure (Lerman *et al.* 1993, Davidson *et al.* 1996, Yamamoto *et al.* 1996, Wieczorek *et al.* 2002). BNP and NT-proBNP concentrations correlate with several indices of heart failure such as the New York Heart Association (NYHA) functional class, hemodynamics, left ventricular ejection fraction and filling pressure (Morita *et al.* 1993, Richards *et al.* 1993b, Choy *et al.* 1994, Davis *et al.* 1994, Groenning *et al.* 2002). Recently, it was reported that BNP reflects left ventricular end-diastolic wall stress more accurately than the other parameters listed above in patients with systolic or diastolic heart failure (Iwanaga *et al.* 2006). Furthermore, the dynamics are favorable to proBNP-derived peptides because their basal concentrations are lower than those of proANP-derived peptides, but equal or even higher in patients with heart failure (Clerico *et al.* 1998). Thus proBNP-derived peptides seem to be a better index of ventricular impairment than proANP-derived peptides (Yamamoto *et al.* 1996, McDonagh *et al.* 1998, Doust *et al.* 2004). In addition, the increase in NT-proBNP is greater than that of BNP in patients with moderate left ventricular dysfunction (Hunt *et al.* 1997b), and NT-proBNP is claimed to be a more discerning marker of mild to moderately reduced left ventricular function than BNP (Mueller *et al.* 2004b, Seino *et al.* 2004), although the differences are modest. In severe dysfunction the markers perform equally well. In patients with chronic heart failure, plasma BNP and NT-proBNP predict functional capacity (Kruger *et al.* 2002, Jourdain *et al.* 2003, Passino *et al.* 2006), and BNP may be used as an alternative to the 6-min walk test to assess the severity of heart failure (Wieczorek *et al.* 2003). As a slight drawback, prolonged adequate treatment of heart failure may restore the BNP levels to normal despite persisting significant dysfunction (McGeoch *et al.* 2002). Moreover, natriuretic peptide levels increase with age in heart failure subjects (Masson *et al.* 2002, Hogenhuis *et al.* 2005) and are higher in women than men (Luchner *et al.* 2002a, Redfield *et al.* 2002, Wang *et al.* 2002), resulting in age-, gender- and assay-specific cut-off values. BNP is elevated in congestive heart failure patients with renal dysfunction, especially in patients with an estimated glomerular filtration rate <60 mL/min (McCullough *et al.* 2003, Tsutamoto *et al.* 2006). Decreased BNP and NT-proBNP levels have also been found in obese patients with heart failure (Mehra *et al.* 2004, Rivera *et al.* 2005), while in diabetic patients BNP is not sufficiently sensitive to identify subclinical dysfunction (Fang *et al.* 2005).

Population-based studies. The diagnostic utility of the natriuretic peptide assays as a screening method for heart failure has been tested in several different populations (Doust *et al.* 2004). Results from general population studies are conflicting, but altogether they suggest that natriuretic peptide measurements may be used for ruling out heart failure in asymptomatic individuals. In the large Framingham Heart Study cohort BNP and NT-proBNP showed good specificity and negative predictive value, but poor sensitivity and positive predictive value, especially in women, in identifying patients with left ventricular hypertrophy and systolic dysfunction (Vasan *et al.* 2002). In the Japanese population with a low prevalence of left ventricular systolic dysfunction and coronary heart disease, BNP detected cardiac abnormalities with both good sensitivity and specificity (Nakamura *et al.* 2002). More recently, however, the same study group found

that BNP testing for structural heart disease screening in Japan is more useful for cohorts with a high prevalence of heart disease (Nakamura *et al.* 2005). In a community-based study of subjects aged 45 years or older, BNP could identify only patients with moderate to severe systolic or diastolic dysfunction with appropriate sensitivity and specificity (Redfield *et al.* 2004). NT-proBNP performed at least equivalently to BNP and had significantly higher areas under the curves for detecting left ventricular dysfunction in several age and male subgroups. Optimal cut-off values for BNP and NT-proBNP varied with age and sex (Costello-Boerrigter *et al.* 2006). In other studies of subjects over middle age, however, NT-proBNP ruled out heart failure with nearly 100% negative predictive value (Hobbs *et al.* 2002, Groenning *et al.* 2004). Moreover, a pooled meta-analysis of three epidemiological studies revealed that NT-proBNP may exclude heart failure in the general population and rule out left ventricular dysfunction in breathless subjects with high sensitivity and specificity. An elevated value indicates cardiovascular or renal abnormalities and must be further investigated by other means (McDonagh *et al.* 2004). Accordingly, both plasma and urinary NT-proBNP could exclude the presence of left ventricular systolic dysfunction in a community. However, urinary NT-proBNP performed poorly in the detection of other cardiac abnormalities with preserved systolic function (Ng *et al.* 2005). Furthermore, BNP testing reduces the number of echocardiograms needed and the costs of screening in subjects at risk in the general population (Nielsen *et al.* 2003).

Primary care. In a primary care setting, natriuretic peptides may be useful as indicators of heart failure. Cowie *et al.* (1997) investigated patients with newly symptomatic heart failure and showed that BNP as well as ANP and NT-proANP could rule out congestive heart failure with high sensitivity (97% for all) and specificity (84%, 72% and 66%, respectively) (Cowie *et al.* 1997). In long-term survivors of myocardial infarction, BNP and NT-proANP could identify those patients who had severe left ventricular dysfunction but not those with moderate dysfunction (McClure *et al.* 1998). However, in elderly patients who had contacted a primary health care center for dyspnea, fatigue, and/or peripheral edema, NT-proBNP was found to be an earlier sign of abnormal cardiac function than the currently applied echocardiographic measurements. NT-proBNP increased by impaired systolic function, impaired diastolic function with pseudonormal mitral flow pattern, cardiac ischemia and enlargement, impaired renal function, and age (Alehagen *et al.* 2003). In line with this, NT-proBNP measurement significantly improved the diagnostic accuracy by a general practitioner over and above clinical review by correctly ruling out heart failure in patients with symptoms of dyspnea and/or peripheral edema (Wright *et al.* 2003).

Diastolic dysfunction. Natriuretic peptides, in particular BNP and NT-proBNP, are elevated in left ventricular diastolic dysfunction (Lang *et al.* 1994, Yamamoto *et al.* 1996, Fruhwald *et al.* 1999, Krishnaswamy *et al.* 2001, Maisel *et al.* 2001, Yamaguchi *et al.* 2004). The diagnosis of diastolic dysfunction is usually based on typical symptoms and signs of heart failure in patients with preserved left ventricular systolic function (Hunt *et al.* 2001, Remme & Swedberg 2001). It is reported that BNP can detect the presence of diastolic abnormalities on echocardiography, and a low BNP value in subjects of normal systolic function may be able to rule out clinically significant diastolic abnormalities (Lubien *et al.* 2002). Patients with diastolic dysfunction and pseudonormal filling pattern have higher BNP and NT-proBNP levels correlated with impaired relaxation (Alehagen *et*

al. 2003, Mottram *et al.* 2003, Tschope *et al.* 2005a). Moreover, BNP and NT-proBNP are correlated with increased filling pressure during exercise and may be useful for the detection of mild diastolic dysfunction in patients with exertional dyspnea (Matsumoto *et al.* 1995, Mottram *et al.* 2004, Tschope *et al.* 2005b). In addition, plasma BNP in patients with systolic heart failure reflects the severity of diastolic abnormality (Troughton *et al.* 2004, Goto *et al.* 2005). Some studies, however, suggest that BNP and NT-proBNP are mostly at normal level in patients with diastolic dysfunction and have limited diagnostic utility in stable patients with suspected diastolic heart failure (Talwar *et al.* 2000a, Mottram *et al.* 2003, Joung *et al.* 2005).

2.3.1.2 Acute dyspnea

In urgent care, it is necessary to rapidly distinguish between cardiac and pulmonary causes of acute dyspnea in order to apply appropriate medical therapy. Several studies have investigated natriuretic peptide assays in the assessment of dyspnea, suggesting that normal plasma levels of BNP or NT-proBNP most likely exclude the presence of dyspnea of cardiac origin. Initially, it was found that plasma BNP reflected the final diagnosis of heart failure in patients with acute dyspnea more accurately than left ventricular ejection fraction or plasma ANP (Davis *et al.* 1994). More recently, in two studies comprising patients presenting to urgent care with a primary complaint of shortness of breath, it was found that a rapid bedside BNP test could accurately differentiate pulmonary etiology from cardiac causes of dyspnea (Dao *et al.* 2001, Morrison *et al.* 2002). Moreover, in a study of 1,586 patients from the multinational Breathing Not Properly Study, it was found that BNP predicts heart failure above and beyond clinical variables from history and physical examination (Maisel *et al.* 2002), and adding a BNP test to careful clinical judgment improves diagnostic accuracy of congestive heart failure from 74% to 81% (McCullough *et al.* 2002). Later it was shown with the same study population that BNP was a stronger predictor of congestive heart failure in younger patients and men compared to older patients and women (Maisel *et al.* 2004). The impact of age was the strongest. However, in a smaller study of 155 subjects with shortness of breath, BNP was strongly related to the diagnosis of heart failure, but there was no significant interaction between BNP and either age or sex (Knudsen *et al.* 2004). In the BNP for Acute Shortness of Breath (BASEL) study of 452 patients the measurement of BNP reduced the need of hospitalization, intensive care and the total cost of treatment (Mueller *et al.* 2004a).

In accordance with BNP studies, also NT-proBNP can discriminate between cardiac and non-cardiac dyspnea (Lainchbury *et al.* 2003, Nielsen *et al.* 2004, Januzzi *et al.* 2006). The ProBNP Investigation of Dyspnea in the Emergency Department (PRIDE) study of 600 patients showed that NT-proBNP testing alone is superior to clinical judgment alone for diagnosing congestive heart failure irrespective of renal function (Januzzi *et al.* 2005). Moreover, NT-proBNP may serve to monitor outcome during hospitalization (Bayes-Genis *et al.* 2004). However, BNP and NT-proBNP may have reduced sensitivity to identify congestive heart failure in overweight and obese patients (Krauser *et al.* 2005, Daniels *et al.* 2006). ANP or NT-proANP data in this clinical setting

are scarce. One retrospective study indicated that the diagnostic value of NT-proANP in the diagnosis of acute heart failure in dyspneic patients is comparable to that of BNP and NT-proBNP (Gegenhuber *et al.* 2006).

2.3.1.3 Acute coronary syndromes

The various clinical syndromes caused by acute myocardial ischemia are referred to as acute coronary syndromes. They can be divided into two categories based on changes seen on electrocardiogram at presentation: acute ST-segment elevation myocardial infarction and non-ST-segment elevation acute coronary syndromes (Alpert *et al.* 2000, Braunwald *et al.* 2002). These disease states are commonly associated with neurohormonal activation. Circulating concentrations of natriuretic peptides increase in patients with acute myocardial infarction (AMI), and the extent of the increase is related to the size of the infarct (Morita *et al.* 1993, Arakawa *et al.* 1994, Uusimaa *et al.* 1999, Paelinck *et al.* 2006), even in patients with asymptomatic MI (Nakagawa *et al.* 2004). The ANP and NT-proANP concentrations are at their highest on the day of admission and decrease thereafter, with the exception that ANP level shows a small peak on day 2 to 3 (Morita *et al.* 1993, Kettunen *et al.* 1994a, Uusimaa *et al.* 1999). However, during the first hours of infarction BNP levels are considerably higher than those of ANP (Sumida *et al.* 1995). Patients with smaller infarcts tend to have a monophasic increase in BNP, peaking at 20 hours after the onset of symptoms. Patients with larger infarcts, lower ejection fraction and clinical signs of heart failure may have a second BNP peak about 5 days later, possibly reflecting the remodeling process (Morita *et al.* 1993). A similar biphasic pattern of plasma NT-proBNP is seen after anterior myocardial infarction (Talwar *et al.* 2000c). The response of NT-proBNP to AMI appears to be greater than that of either BNP or ANP, peaking at around 24 hours (Gill *et al.* 2004). Increased synthesis of the natriuretic peptides occurs in both the infarcted and non-infarcted myocardium due to an increase in regional wall tension or stretch in viable cells both within and surrounding the infarct area (Hama *et al.* 1995). In AMI BNP may regulate collagen scar formation and left ventricular remodeling. This is supported by the fact that BNP decreases cardiac collagen synthesis and activates enzymes that break down matrix proteins (Tsuruda *et al.* 2002). Furthermore, ANP, BNP and NT-proBNP correlate with collagen synthesis and breakdown in patients with AMI (Magga *et al.* 2004, Squire *et al.* 2004a, Tziakas *et al.* 2005).

In acute coronary syndromes without infarction BNP and NT-proBNP are also markedly elevated in the early phase of symptoms, decreasing thereafter. Natriuretic peptide levels are higher in patients with AMI than in those with unstable angina, and in patients with ST-segment elevation MI than in those with non-ST-segment elevation (Marumoto *et al.* 1995, Talwar *et al.* 2000b, de Lemos *et al.* 2001, Jernberg *et al.* 2002, Lindahl *et al.* 2005). A recent small study suggested that BNP values may be used to detect acute myocardial ischemia in patients with ongoing chest pain but without ST-segment elevation, and to distinguish ischemic patients from those with pain of non-ischemic origin (Nikolaou *et al.* 2005). BNP and NT-proBNP may be used as a screening test for exercise-induced myocardial ischemia (Bibbins-Domingo *et al.* 2003, Win *et al.*

2005), whereas NT-proANP may not be clinically useful in this setting (Bocek *et al.* 2005). Natriuretic peptides are potential diagnostic markers for the detection and exclusion of significant left ventricular dysfunction in patients with AMI, acute coronary syndrome or stable ischemic heart disease (Choy *et al.* 1994, Darbar *et al.* 1996, Omland *et al.* 1996, Richards *et al.* 1998, Luchner *et al.* 2002b, Richards *et al.* 2006), and elevated levels are strongly related to worse outcome (see chapter 2.3.2.1).

2.3.1.4 Other cardiovascular diseases

Natriuretic peptides can be useful biomarkers in other diseases affecting the cardiovascular system as well. The concentrations of natriuretic peptides increase in valvular heart diseases in proportion to disease severity (Gerber *et al.* 2003, Sutton *et al.* 2003), and the levels may be used for timing of corrective surgery (Qi *et al.* 2001, Watanabe *et al.* 2004). In hypertrophic cardiomyopathy the BNP concentration may reflect the degree of hypertrophy (Maron *et al.* 2004). Arterial hypertension is associated with modestly elevated natriuretic peptide levels, and BNP and NT-proBNP may be useful in assessing the progression of hypertension and in risk stratification (Freitag *et al.* 2003, Hildebrandt *et al.* 2004, Uusimaa *et al.* 2004). Furthermore, in recent studies BNP and NT-proBNP are reported to be strong prognostic markers in pulmonary hypertension and embolism (Nagaya *et al.* 1998, ten Wolde *et al.* 2003, Binder *et al.* 2005, Fijalkowska *et al.* 2006). NT-proBNP may also be a powerful tool for treatment assessment and evaluation of prognosis in primary systemic amyloidosis (Palladini *et al.* 2003). The concentration of natriuretic peptides increases in tachyarrhythmias, and atrial fibrillation appears to be an independent determinant of increased NT-proANP, but not of increased BNP (Rossi *et al.* 2000). In non-Hodgkin's lymphoma patients it may be possible to identify heart failure related to chemotherapy by natriuretic peptide assays (Nousiainen *et al.* 1998, Nousiainen *et al.* 1999, Nousiainen *et al.* 2002, Kuittinen *et al.* 2005). In pregnant women with pre-eclampsia and gestational hypertension, the concentrations of ANP and NT-proANP are higher than in those with normal pregnancies (Pouta *et al.* 1996, Pouta *et al.* 1997). However, NT-proANP was not useful in predicting pre-eclampsia in a population-based birth cohort (Pouta *et al.* 1998). Moreover, umbilical artery ANP and NT-proANP correlate with fetal cardiovascular function (Mäkikallio *et al.* 2001, Mäkikallio *et al.* 2002).

2.3.2 Natriuretic peptides as prognostic markers

When the diagnosis of cardiac disease is established, it would be important to be able to predict which patients are at risk of death or further cardiovascular events. Natriuretic peptides may be useful as prognostic markers mainly in two clinical conditions: acute coronary syndromes and heart failure (for review, see Sagnella 1998, Ruskoaho 2003, Vuolteenaho *et al.* 2005, Maisel *et al.* 2006).

2.3.2.1 Acute coronary syndromes

Myocardial infarction. Initially, ANP was reported to provide prognostic data on survival in patients with myocardial infarction or chronic heart failure (Gottlieb *et al.* 1989). Furthermore, several studies suggest that plasma ANP can be a predictor of cardiac mortality and heart failure in asymptotic patients with left ventricular dysfunction after acute myocardial infarction (AMI) (Rouleau *et al.* 1994, Omland *et al.* 1995). In patients with known heart disease, NT-proANP appears to be a much stronger predictor of mortality and morbidity after myocardial infarction than ANP (Omland *et al.* 1993, Hall *et al.* 1994, Hall *et al.* 1995b). In multivariate analysis, NT-proANP was an independent predictor of cardiovascular mortality even when age, other clinical indices of disease severity and left ventricular ejection fraction were included in the model (Hall *et al.* 1994). However, further studies suggest that BNP may be a better predictor of death and cardiovascular events after myocardial infarction than proANP-derived peptides (Darbar *et al.* 1996, Omland *et al.* 1996). Both studies showed that BNP is a significant independent predictor of cardiovascular mortality whereas ANP or NT-proANP is not. When measured within 1 week after myocardial infarction, BNP and NT-proBNP identify patients at risk for left ventricular dysfunction, heart failure and death (Omland *et al.* 1996, Richards *et al.* 1998). The NT-proBNP level measured during the chronic phase of syndromes (after 48 hours) is a better predictor of mortality than during an acute unstable phase (Talwar *et al.* 2000c, Lindahl *et al.* 2005). One study concluded, however, that failure of plasma NT-proBNP to fall in the 30 days after AMI indicates adverse prognosis (Squire *et al.* 2005). A few studies indicate that the prognostic value of BNP may be greater than that of left ventricular ejection fraction (Omland *et al.* 1996, Richards *et al.* 1998), or that NT-proBNP or BNP complements the predictive information of left ventricular ejection fraction in AMI (Richards *et al.* 2003). Plasma levels of natriuretic peptides still retain their prognostic power among survivors of AMI receiving beta-blocking medication (Jonsson *et al.* 2006), and in these patients BNP independently predicts the occurrence of sudden cardiac death (Tapanainen *et al.* 2004).

Unstable angina and non-ST-elevation myocardial infarction. More recent studies also investigate the prognostic role of proBNP-derived peptides in acute coronary syndromes in patients with unstable angina and non-ST-elevation myocardial infarction. In a large OPUS-TIMI 16 trial, BNP was measured within 72 hours after the onset of ischemic discomfort. The baseline BNP level correlated with the risk of death, heart failure and myocardial infarction at 30 days and 10 months (de Lemos *et al.* 2001). NT-proBNP was measured at admission from a consecutive series of patients with chest pain and no ST-elevation. NT-proBNP was strongly associated with long-term mortality during the follow-up time of 40 months (Jernberg *et al.* 2002). Omland *et al.* (2002) also found that NT-proBNP measured in subacute phase is a powerful indicator of long-term mortality in a heterogeneous patient population with acute coronary syndromes. It provided prognostic information above and beyond Killip class, age and left ventricular ejection fraction (Omland *et al.* 2002). In accordance with this, a recent small study consisting of patients with unstable coronary artery disease without ST-elevation and normal ventricular function confirmed that NT-proBNP on admission is an independent predictor of 2-year mortality. However, in the group with normal NT-proBNP levels, patients with

elevated NT-proANP still had a significantly higher mortality rate than patients with normal NT-proANP (Jarai *et al.* 2005). Furthermore, NT-proBNP on admission independently predicts short-term (30 days) mortality in patients either with or without persistent ST-segment elevation (Galvani *et al.* 2004b), and serial determinations of BNP or NT-proBNP levels thereafter provide important information about the further clinical course of the patients (Heeschen *et al.* 2004, Morrow *et al.* 2005). According to meta-analysis comprising several studies, the prognostic values of natriuretic peptides are similar in the short and long term, when measured at first patient contact or during hospitalization, in patients with and without ST-elevation acute coronary syndrome as well as for BNP and NT-proBNP (Galvani *et al.* 2004a). In addition to patients with unstable angina, baseline BNP and NT-proBNP are also strong predictors of cardiovascular risk in patients with stable angina independent of conventional risk factors and left ventricular systolic performance (Kragelund & Omland 2005, Schnabel *et al.* 2005, Richards *et al.* 2006, Schnabel *et al.* 2006).

Multimarker approach. The utility of proBNP-derived peptides as members of a multimarker approach in acute coronary syndromes has been evaluated in a few large studies. In addition to BNP or NT-proBNP, cardiac troponin I or T (cTnI or cTnT, biomarkers of cardiomyocyte necrosis), and/or C-reactive protein (CRP, marker of inflammation) were measured. First, it was found that the combination of BNP, cTnI and CRP adds prognostic value over that provided by any individual marker alone in non-ST elevation acute coronary syndromes (Sabatine *et al.* 2002). Later it was also concluded that BNP adds prognostic information to that of cTnI in patients with unstable angina and non-ST elevation myocardial infarction (Morrow *et al.* 2003). Mega *et al.* (2004) reported that in patients with ST-segment elevation myocardial infarction, BNP is an independent predictor of mortality risk, with performance superior to that of cTnI or CRP (Mega *et al.* 2004). In agreement with this, increasing quartiles of NT-proBNP associated with short- (30 days) and long-term (12 months) mortality more strongly than TnT, CRP and clinical markers in patients with acute coronary syndromes. The combination of NT-proBNP and creatinine clearance provided the best prediction (James *et al.* 2003).

2.3.2.2 Heart failure

The clinical assessment of prognosis in heart failure is difficult because of the lack of strong predictors of mortality or morbidity (Doust *et al.* 2005). Few studies have focused on natriuretic peptides as prognostic indicators in heart failure. Tsutamoto *et al.* (1997, 1999) investigated patients with advanced heart failure as well as asymptomatic or minimally symptomatic left ventricular dysfunction and found that 2-year mortality was independently predicted by BNP, but not by ANP (Tsutamoto *et al.* 1997, Tsutamoto *et al.* 1999). However, it was later reported that both BNP and NT-proANP plasma levels were related to the two-year prognosis after the New York Heart Association (NYHA) classification (Selvais *et al.* 2000). Moreover, BNP and NT-proBNP, but not NT-proANP, were independently related to 4-year mortality in patients with severe heart failure receiving high-dose angiotensin-converting enzyme inhibitors and beta-blocker therapy (Stanek *et al.* 2001). In congestive heart failure the prognostic information obtained by

BNP assay is as powerful as that derived from the multivariate heart failure survival score (HFSS) (Koglin *et al.* 2001). More recent large trials have confirmed the prognostic usefulness of the BNP and NT-proBNP levels measured at baseline or predischarge in patients with moderate or severe heart failure (Anand *et al.* 2003, Koseki *et al.* 2003, Bettencourt *et al.* 2004, Hartmann *et al.* 2004, Logeart *et al.* 2004). Accordingly, a recent systematic review suggested that BNP is a strong prognostic indicator for patients with heart failure at all stages of disease and seems to be a better predictor of survival than many traditional factors, such as NYHA class, serum creatinine, and possibly even left ventricular ejection fraction (Doust *et al.* 2005). Furthermore, patients whose BNP values fail to fall in response to treatment tend to be at particularly high risk of death or a cardiovascular event. Thus the BNP level after stabilization of treatment may be an even more significant prognostic predictor than the baseline value (Maeda *et al.* 2000, Matsui *et al.* 2002, Anand *et al.* 2003, Ishii *et al.* 2003).

2.3.2.3 General population

The prognostic relevance of natriuretic peptides in the general population, mostly the elderly, has been evaluated by a few reports. In a pioneering study, ANP was demonstrated to be a specific and sensitive test for predicting heart failure in elderly subjects during the 1-year follow-up period (Davis *et al.* 1992). Later, in an elderly population sample NT-proANP predicted both total and cardiovascular mortality at 1.5 years of entry (Iivanainen *et al.* 1997). BNP was found to predict 5-year all-cause mortality better than ANP and NT-proANP in a cohort of 85-year-old individuals (Wallen *et al.* 1997). Furthermore, the prognostic value of BNP was evaluated in very elderly individuals (age ≥ 80 years) who had no history of hospitalization for cardiac reasons. Increasing BNP concentrations were associated with higher risk of cardiac events and total mortality (Ueda *et al.* 2003). In individuals older than 50 years NT-proBNP was the strongest independent predictor of mortality, hospital admissions for heart failure, and other cardiac admission (Groenning *et al.* 2004, Kistorp *et al.* 2005). McDonagh *et al.* (2001) investigated a cohort of general urban population and showed that BNP, but not NT-proANP, is an independent predictor of 4-year all-cause mortality (McDonagh *et al.* 2001). Wang *et al.* (2004) showed that BNP and to a lesser extent NT-proANP predicted the risk of death and cardiovascular events in the large Framingham community-based population (Wang *et al.* 2004). A recent report compared two distinct BNP assays and a NT-proBNP assay in a large community-based cohort, and the NT-proBNP assay was most predictive of mortality after adjustment for clinical phenotypes and echocardiographic abnormalities (McKie *et al.* 2006).

2.3.3 Natriuretic peptides in guiding the treatment of cardiovascular diseases

It has been suggested that natriuretic peptide assays may be useful in monitoring and tailoring medical therapy in patients with heart failure. The idea is supported by findings that treatment with e.g. angiotensin-converting enzyme (ACE) inhibitors, valsartan, diuretics and organic nitrates reduces concentrations of natriuretic peptides in parallel with hemodynamic and clinical improvement (Kettunen *et al.* 1994b, Yoshimura *et al.* 1994, Benedict *et al.* 1995, Missouris *et al.* 1998). Likewise, deterioration in cardiovascular function increases circulating concentrations (Koglin *et al.* 2001), and persistently high BNP levels despite aggressive treatment identify patients at especially high risk for adverse outcomes (Maeda *et al.* 2000). So far only few studies with limited patient populations have evaluated the clinical use of natriuretic peptides in the tailoring of heart failure therapy. It has been shown that BNP may be chronically reduced by tailored vasodilator therapy in patients with chronic heart failure (Murdoch *et al.* 1999). In addition, titration of vasodilator therapy according to plasma BNP associates with inhibition of the renin-angiotensin-aldosterone system and with a significant decrease in heart rate compared to empiric therapy (Murdoch *et al.* 1999). Later it was demonstrated with patients of left ventricular dysfunction and symptomatic heart failure that pharmacotherapy guided by plasma NT-proBNP reduces total cardiovascular events and delays the time to first event compared with standardized clinically guided treatment (Troughton *et al.* 2000). In agreement with these preliminary studies, BNP was reported to be a therapeutic indicator for risk stratification in patients with idiopathic dilated cardiomyopathy during beta-blocker therapy (Kawai *et al.* 2001). However, these small studies have still to be confirmed by larger trials. Furthermore, the high intraindividual biological variation of BNP and NT-proBNP in chronic heart failure might hamper the use of these assays in the follow-up and treatment optimization of heart failure patients (Wu *et al.* 2003a, Bruins *et al.* 2004).

3 Aims of the research

Circulating concentrations of natriuretic peptides and their profragments are markers of cardiac performance and heart failure. Due to their longer half-lives and better stability, the N-terminal peptides are considered to be better markers of prolonged cardiac overload compared to their biologically active forms. There are, however, serious analytical problems with assays of N-terminal fragments, relating especially to assay specificity and analyte stability. The exact circulating forms are also debated. Our intention was to develop reliable and novel immunoassays for N-terminal natriuretic peptides with clinical application, and to elucidate their molecular components and stability in human plasma and serum.

The specific aims of the present research were:

1. to set up reliable immunoassays for circulating aminoterminal fragments of natriuretic peptides (NT-proANP and NT-proBNP) based on full-length recombinant calibrators and characterized antisera, and to test the assays in healthy subjects and in cardiovascular patients.
2. to develop a single immunoassay (NT-proXNP) which could reflect the activation of either A- or B-type natriuretic peptide systems, and to test the assay in healthy subjects and cardiovascular patients.
3. to characterize the actual molecular components of NT-proANP and NT-proBNP in human circulation.
4. to elucidate the stability and degradation products of recombinant NT-proANP and NT-proBNP in human plasma and serum.
5. to find new applications for the natriuretic peptide assays.

4 Materials and methods

4.1 Expression of recombinant proteins in Escherichia coli (I-II)

In order to produce recombinant human NT-proANP, NT-proBNP, proBNP, BNP and combination epitope peptides (NT-proXNP) in *E. coli*, the corresponding cDNAs were cloned into a pGEX-4T-1 expression vector (Amersham Pharmacia Biotech, Uppsala, Sweden). These vectors allow cloning of recombinant cDNA in fusion with the coding sequence of glutathione S-transferase (GST). GST-fusion proteins can be easily purified from bacterial lysates by single-step glutathione affinity chromatography (Smith & Johnson 1988). The desired protein can be cleaved from GST-fusion partner using a site-specific protease (Chang 1985).

4.1.1 Construction of expression plasmids for NT-proANP, NT-proBNP, proBNP and Tyr_0-$proBNP_{77-108}$ (I)

The expression constructs were made by subcloning reverse transcripatese PCR (RT-PCR) fragments of ANP or BNP genes into pGEX-4T-1 vector. The constructs were designed to contain the amino acids 1-98 of the coding sequence of human ANP gene, or the amino acids 1-76, 1-108 or 77-108 of the coding sequence of human BNP gene. For the synthesis of cDNA total RNA was prepared from human atrial tissue by pulverizing in liquid N_2 and homogenizing in 9 volumes of 4 M guanidium thiocyanate, 8% 2-mercaptoethanol, 50 mM Tris-HCl, 10 mM EDTA, pH 7.5. RNA was isolated from the homogenates with the acidic phenol method (Chomczynski & Sacchi 1987).

The cDNA first strands were synthesized from human atrial RNA using sequence specific 3'- primers including the termination codon (TGA) and *Eco*RI linker (NT-proANP: 3'-GCGAATTCTCACCGAGGGGCAGTGAGC-5', NT-proBNP: 3'-GCGAATTCTCATCGTGGTGCCCGCAG -5', proBNP and Tyr-BNP: 3'-GCGAATTCTCATTAATGCCGCCTCAGCAC-5'). Subsequent PCR amplifications were done using the same 3'-primers and 5'-primers including *Bam*HI linkers (NT-proANP: 5'-GCGGATCCAATCCCATGTACAAT GCCG-3', NT-proBNP and proBNP: 5'-GCGGATCCCACCCGCTGGGCAGCCCCG -3'). The 5' primer for Tyr-BNP contained

a *Bam*HI linker and a codon for tyrosine (5'- GCGGATCCTACAGCCCCAA-GATGGTGCAA -3'). The PCR fragments were ligated into *Eco*RI/*Bam*HI site of pGEX-4T-1 vector.

All constructed expression plasmids were propagated and stored in *E. coli* strain DH5α and the cells were grown in Luria-Bertoni (LB) medium containing ampicillin (100 μg/ml). Plasmids were isolated either with alkaline lysis (Sambrook *et al.* 1989) or using Qiagen Midi columns (Qiagen, Hilden, Germany). The nucleotide sequences and reading frames of the constructs were confirmed by automated sequencing using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

4.1.2 Construction of expression plasmids for combination epitope peptides (II)

The expression constructs were produced as above by subcloning PCR fragments of both ANP and BNP genes end-to-end into pGEX-4T-1 vector. The nucleotide sequences encoding amino acids 1-98 of human NT-proANP and 1-76 of human NT-proBNP (a construct for GST-NT-proANP$_{1-98}$-NT-proBNP$_{1-76}$) were amplified by RT-PCR from human atrial RNA. The oligonucleotide 5'-primer for NT-proANP amplification contained the cleavage site for the restriction enzyme *Bam*HI (5'-GCGGATC-CAATCCCATGTACAATGC-3') and the 3'-primer for *Xba*I (5'-GCTCTAGA CCGAGGGGCAGTGAGC-3'). The 5'-primer for NT-proBNP had *Xba*I linker (5'-GCTCTAGACACCCGCTGGGCAGCCCCG-3') and the 3'-primer *Eco*RI linker and a termination codon (5'-GCGAATTCTCATCGTGGTGCCCGCAG-3'). Alternatively, the nucleotides encoding amino acids 1-76 or 1-37 of human NT-proBNP and those encoding amino acids 1-98 or 29-98 of human NT-proANP (constructs for GST-NT-proBNP$_{1-76}$-NT-proANP$_{1-98}$, GST-NT-proBNP$_{1-37}$-NT-proANP$_{29-98}$ and GST-Tyr$_0$-NT-proBNP$_{1-37}$-NT-proANP$_{29-98}$) were amplified. The 5'-primers for NT-proBNP amplification contained *Bam*HI linker (5'-GCGGATCCCACCCGCTGGGCAGCCCCG-3') or the linker followed by a codon for tyrosine (5'-GCGGATCCTCACACCCGCTGGGCAGCCCCG-3'). 3'-primers had *Xba*I linkers (1-76: 5'-GCTCTAGATCGTGGTGCCCGCAG-3', 1-37: 5'-GCTCTAGAGGATGTCTGCTCCACC-3'). The 5'-primers for NT-proANP amplification had *Xba*I linkers (1-98: 5'-GCTCTAGAAATCCCATGTACAATGC-3', 29-98: 5'-GCTCTAGAGAAGATGAGGTCGTGC-3') and the 3'-primer had *Eco*RI linker and a termination codon (5'-GCGAATTCTCACCGAGGGGCAGTGAGC-3'). The RT-PCR products were purified by agarose electrophoresis, cleaved with *Xba*I and *Bam*HI or *Eco*RI and subcloned end-to-end into *Bam*HI/*Eco*RI site of pGEX-4T-1 vector. All expression plasmids constructed in this study are summarized in Table 1.

Table 5. Escherichia coli expression plasmids constructed in this work.

Expression plasmid	cDNA
GST-NT-proANP$_{1-98}$	Human NT-proANP$_{1-98}$
GST-NT-proBNP$_{1-76}$	Human NT-proBNP$_{1-76}$
GST-proBNP$_{1-108}$	Human proBNP$_{1-108}$
GST-Tyr$_0$-proBNP$_{77-108}$	Human proBNP$_{77-108}$ (human BNP$_{1-32}$) with tyrosine
GST- NT-proANP$_{1-98}$-NT-proBNP$_{1-76}$	Human NT-proANP$_{1-98}$ and NT-proBNP$_{1-76}$
GST-NT-proBNP$_{1-76}$-NT-proANP$_{1-98}$	Human NT-proBNP$_{1-76}$ and NT-proANP$_{1-98}$
GST-NT-proBNP$_{1-37}$-NT-proANP$_{29-98}$ (GST-NT-proXNP)	Human NT-proBNP$_{1-37}$ and NT-proANP$_{29-98}$
GST-Tyr$_0$-NT-proBNP$_{1-37}$-NT-proANP$_{29-98}$ (GST-Tyr$_0$-NT-proXNP)	Human NT-proBNP$_{1-37}$ and NT-proANP$_{29-98}$ with tyrosine

GST, glutathione S-tranferase.

4.1.3 Production of GST-fusion proteins (I-II)

The expression constructs were transformed into the BL21 *Escherichia coli* host strain (Amershan Pharmacia Biotech, Uppsala, Sweden). An overnight culture of *E. coli* transformed with recombinant plasmid was diluted 1:100 in 2xYTA (Yeast extract, Tryptone, NaCl, pH7) containing ampicillin (100 μg/ml) and grown at 37°C until the OD_{660} nm reached 0.6. Isopropyl-1-thio-D-galactopyranoside (IPTG) was added to a final concentration of 0.1 mM and the culture was further incubated for 1-2 h. The bacterial cells were harvested by centrifugation at 7000g for 10 min. at +4°C, resuspended in PBS (0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3; 50 μl for ml culture) and sonicated. The cell lysate was cleared at 7000g for 15 min.

4.1.4 Purification and characterization of GST-fusion proteins (I-II)

GST-fusion proteins can be isolated from *E. coli* lysates using single-step affinity purification (Smith & Johnson 1988). Briefly, the supernatant was applied to a column containing either Glutathione Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) or Glutathione agarose (Sigma Aldrich, St. Louis, MO, USA) and washed three times with PBS. The fusion protein was eluted with 10 mM glutathione in 50 mM Tris-HCl, pH 8.0 and stored in aliquots at -20°C. The products were analyzed by 12% SDS-polyacryl gel electrophoresis (SDS-PAGE) under reducing conditions.

4.1.5 Purification and characterization of recombinant peptides (I-II)

Recombinant peptides were released from the GST fusion partner by treating with thrombin (Amersham Pharmacia Biotech, Uppsala, Sweden) at room temperature for 1h

(1U/100 μg protein). The peptides were purified by reverse-phase HPLC (RP-HPLC) using a 1 x 25 cm Vydac C_4 HPLC column (Separations Group, Hesperia, CA). The column was eluted with a linear 40 min gradient from 20-50% acetonitrile in aqueous trifluoroacetic acid. The elution rate was 2 ml/min and the absorbance at 220 nm was measured to monitor the purity of products. The peak fractions were collected manually. The peptides were quantified based on absorbance at 214 nm by gel-filtration HPLC (GF-HPLC) calibrated with bovine serum albumin. GF-HPLC was performed on Waters ProteinPak-125 (3.9 x 300 mm) column eluted with 40% ACN/0.1% trifluoroacetic acid at 1 ml/min. The peptides were also separated by 12% SDS-PAGE gel using Tris-tricine electrophoresis buffer. The amino-terminal sequences of the peptides were confirmed by 10 cycles of automated Edman degradation using an ABI 477A gas phase sequencer (Applied Biosystems, Foster City, CA). Micromass QToF I mass spectrometer (Micromass Ltd, Manchester, UK) was used for the assessment of the molecular masses of recombinant NT-proANP, NT-proBNP and NT-proXNP.

4.2 Synthetic peptides (I-II)

Synthetic peptides were made using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with an ABI 433A Peptide Synthesizer (Applied Biosystems, Foster City, CA). The following peptides were assembled: NT-proANP$_{1-20}$, NT-proANP$_{20-38}$, NT-proANP$_{26-46}$, NT-proANP$_{39-59}$, NT-proANP$_{60-80}$, NT-proANP$_{70-90}$, NT-proANP$_{88-98}$, NT-proBNP$_{1-22}$, NT-proBNP$_{4-16}$, NT-proBNP$_{5-24}$, NT-proBNP$_{10-29}$ NT-proBNP$_{22-40}$, NT-proBNP$_{41-56}$, NT-proBNP$_{52-70}$, Tyr$_0$-NT-proBNP$_{57-74}$ and Tyr$_0$-NT-proBNP$_{57-76}$.

4.3 Antisera (I-II)

The affinity-purified GST-NT-proANP and GST-NT-proBNP fusion proteins were used as such as immunogens. Prior to immunization the peptide immunogens NT-proANP$_{1-20}$, NT-proANP$_{39-59}$, NT-proANP$_{60-80}$, NT-proBNP$_{1-22}$, NT-proBNP$_{5-24}$, NT-proBNP$_{10-29}$, NT-proBNP$_{41-56}$, NT-proBNP$_{52-70}$ and Tyr$_0$-NT-proBNP$_{57-76}$, were coupled by water-soluble carbodiimide to bovine thyroglobulin or horseshoe crab hemocyanin. The conjugates were purified by dialysis against two changes of 2 liters of 0.9% NaCl as described previously (Vuolteenaho *et al.* 1981). Goats were injected subcutaneously at multiple sites in the back with 1.0 mg of immunogen emulsified in equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI). Boosters of 0.5 mg in Freund's incomplete adjuvant were given two to four times at 2- to 3-week intervals, and the goats were bled 14 days after the injections. The cross-reactivities of antisera were measured by assaying serial dilutions (0.001-1 μmol/L) of the following vasoactive peptides: human proANP$_{99-126}$ (ANP$_{1-28}$), rat proANP$_{99-126}$ (ANP$_{1-28}$), human proBNP$_{77-108}$ (BNP$_{1-32}$), human C-type natriuretic peptide (hCNP), rat adrenomedullin (rADM), human endothelin-1 (hET-1), human proBNP$_{1-108}$, human NT-proBNP and human NT-proANP. The epitope specificities of the antisera were tested using the synthetic peptides described

above. All the immunogens and the antisera produced in this work are summarized in Table 2.

Table 6. Immunogens and antisera used in this work.

Immunogen	Name of the antiserum	Antiserum titer
GST-NT-proANP$_{1-98}$	a-NT-proANP$_{46-79}$	1:40 000
bTG-NT-proANP$_{1-20}$	a-NT-proANP$_{1-20}$	1:20 000
HSC-HC-NT-proANP$_{39-59}$	a-NT-proANP$_{39-59}$	1:10 000
HSC-HC-NT-proANP$_{60-80}$	a-NT-proANP$_{60-80}$	1:10 000
GST-NT-proBNP$_{1-76}$	a-GST-NT-proBNP	1:40 000
HSC-HC-NT-proBNP$_{1-22}$	a-NT-proBNP$_{1-22}$	1:10 000
bTG-NT-proBNP$_{5-24}$	a-NT-proBNP$_{5-24}$	1:14 000
bTG-NT-proBNP$_{10-29}$	a-NT-proBNP$_{10-29}$	1:50 000
bTG-NT-proBNP$_{41-56}$	a-NT-proBNP$_{41-56}$	nd
HSC-HC-NT-proBNP$_{52-70}$	a-NT-proBNP$_{52-70}$	1:10 000
bTG-Tyr$_0$-NT-proBNP$_{57-76}$	a-NT-proBNP$_{57-76}$	1:50 000

GST, glutathione S-tranferase; bTG, bovine thyroglobulin; HSC-HC, horseshoe crab hemocyanin; Titer was determined as the antiserum dilution that bound 40% of the radioiodinated tracer; nd, not detectable.

4.4 Radioimmunoassays

4.4.1 Tracer preparations

4.4.1.1 Radioiodination of NT-proANP and NT-proBNP

Recombinant NT-proANP and NT-proBNP were radiolabeled at their tyrosine residues using the chloramine-T technique (Hunter & Greenwald 1962). In brief, iodination was performed with 0.5 mCi $Na^{125}I$ (Amersham Pharmacia Biotech, UK) in the presence of 5 µg chloramine-T in 0.5 M phosphate buffer, pH 7.5 for 15 s, followed by the addition of 10 µg Na-disulfite. The reaction mixture was purified by Sephadex G-25 (Amersham Pharmacia Biotech, Uppsala, Sweden) gel filtration followed by RP-HPLC in a Symmetry C_{18} column (4.6 x 150 mm, Waters, Milford, MA). The column was eluted with a 30-min 10% to 50% acetonitrile gradient in aqueous trifluoroacetic acid at a flow rate of 1 ml/min. Fractions of 1 ml were collected and monitored for radioactivity in a Multi-Gamma counter (Wallac, Turku, Finland). The iodinated peptides were usable for at least five weeks when stored in aliquots at -20°C.

4.4.1.2 Radioiodination of NT-proXNP

Recombinant NT-proXNP (2 µg) was iodinated at its histidine residue as described above with the exception that 10 µg of chloramine-T was used and the incubation was performed for 60 s. Recombinant Tyr$_0$-NT-proXNP was radiolabeled at its tyrosine

residue similarly as recombinant NT-proANP and NT-proBNP. Both tracers were usable for at least five weeks when stored in aliquots at -20°C.

4.4.2 Assay procedures

4.4.2.1 Radioimmunoassays for NT-proANP$_{1-20}$ and NT-proANP$_{46-79}$

Recombinant NT-proANP was used as a calibrator and tracer in both assays. The following assay buffer was used for all dilutions: 0.04 M sodium hydrogen phosphate, 0.01 M sodium dihydrogen phosphate, 0.1 M NaCl, 0.1% gelatin, 0.05% Triton X-100, pH 7.4. Calibrators (60-6000 pmol/L), or plasma or serum samples were pipetted in duplicates of 25 µl and incubated with 100 µl of antiserum solution at a dilution of 1:20000 (antiserum to NT-proANP$_{46-79}$) or 1:40000 (antiserum to NT-proANP$_{1-20}$) together with 100 µl of tracer solution containing approximately 8000 cpm of the appropriate radioiodinated peptide for 16-24 h at +4°C. Bound and free fractions were separated by precipitation with donkey anti-goat IgG (Scantibodies Laboratory, Inc., Santee, CA; Linco Research, Inc., St. Charles, MO) in 0.5 ml of 8% polyethylene glycol 6000 containing normal goat serum as a carrier. After centrifugation, the pellet was counted for radioactivity (Wallac Clinigamma 1272, Turku, Finland).

4.4.2.2 Radioimmunoassays for NT-proBNP$_{1-22}$, NT-proBNP$_{10-29}$, NT-proBNP$_{52-70}$ and NT-proBNP$_{57-76}$

Recombinant NT-proBNP was used as a calibrator and tracer in all the assays. Calibrators (40-4000 pmol/L), or plasma or serum samples were pipetted in duplicates of 25 µl and incubated with 100 µl of antiserum solution (final dilution of 1:10 000 for antiserum to NT-proBNP$_{1-22}$, 1:50 000 for antiserum to NT-proBNP$_{10-29}$, 1:10000 for antiserum to NT-proBNP$_{52-70}$ and antiserum to NT-proBNP$_{57-76}$) together with 100 µl of tracer solution containing approximately 8000 cpm of radioiodinated peptide for 16-24 h at +4°C. Bound and free fractions were separated with double antibody precipitation as described above. For sensitization of the assays we used delayed incubation, where standards (16-1600 pmol/L) or samples were incubated with antisera overnight. After the tracer was added the samples were further incubated for 16-24 h and precipitated.

4.4.2.3 Radioimmunoassay for NT-proBNP$_{5-24}$

Calibrators (62.5-1000 pmol/L of recombinant NT-proBNP) or plasma or serum samples were pipetted in duplicates of 50 µl and incubated for 16-24 h at +4°C with 100 µl of antiserum solution at a dilution of 1:14000. 100 µl of tracer solution containing approximately 8000 cpm of iodinated peptide was added, and the incubation was

continued for further 16-24 h. Bound and free fractions were separated with double antibody precipitation as described above.

4.4.2.4 Radioimmunoassay for NT-proXNP

Recombinant NT-proXNP (NT-proBNP$_{1-37}$-NT-proANP$_{29-98}$) was used as a calibrator and tracer in the assay. Calibrators (80-8000 pmol/L), or plasma or serum samples were pipetted in duplicates of 25 µl and incubated with 100 µl of antiserum solution (final dilution of 1:14000 for antiserum to NT-proANP$_{46-79}$ and 1:34000 for antiserum to NT-proBNP$_{10-29}$) together with 100 µl of tracer solution containing approximately 8000 cpm of radioiodinated NT-proXNP peptide for 16-24 h at +4°C. When radiolabeled Tyr$_0$-NT-proXNP was used as a tracer, the antiserum dilutions were 1:20000 for antiserum to NT-proANP$_{46-79}$ and 1:40000 for antiserum to NT-proBNP$_{10-29}$. Bound and free fractions were separated with double antibody precipitation as described above.

4.4.3 Radioimmunoassay validation (I-II)

Serial dilutions of human plasma and serum were assayed against the NT-proANP, NT-proBNP and NT-proXNP calibrators to test for parallelisms. The linearity of the NT-proANP and NT-proBNP assays was analyzed by linear regression analysis with normal human plasma spiked with the recombinant NT-proANP (100-5000 pmol/L) or NT-proBNP (50-3200 nmol/L, n = 4 each). The linearity of the NT-proXNP assay was studied similarly with normal plasma and serum spiked with the recombinant NT-proANP and NT-proBNP (100-7500 pmol/L). The within-assay and between-assay precisions were assessed by measuring 10 replicates of pooled plasma or serum with low, medium and high concentration of the peptides. The functional sensitivities were analyzed with normal plasma and serum samples as day-to-day coefficient of variation (CV) <20% (5 days, 5 replicates). Recoveries of the NT-proANP and NT-proBNP assays were determined by supplementing the normal plasma pool with 500, 2000 or 5000 pmol/L of NT-proANP or 200, 800 or 3200 pmol/L of NT-proBNP (n = 8 each). Recoveries of the NT-proXNP assay were assessed by measuring the serum pool with 200, 1500 or 4000 pmol/L of NT-proANP and/or NT-proBNP (n = 10 each).

4.4.4 Other immunoassays

NT-proANP$_{79-98}$ was determined according to the published radioimmunoassay method (Vuolteenaho *et al.* 1992). For the determination of the biologically active human BNP (proBNP$_{77-108}$) the plasma and serum samples were first extracted with SepPak C_{18} cartridges (Waters, Milford, MA). Standards (1-100 pmol/L of synthetic human BNP (proBNP$_{77-108}$) purchased from Bachem, Bubendorf, Switzerland) or plasma or serum extracts were pipetted in duplicates of 200 µl and incubated for 16-24 h at +4° C with

100 µl of rabbit antiserum against human $proBNP_{77-108}$ at a dilution 1:30 000. Radioiodinated recombinant Tyr_0-$proBNP_{77-108}$ (approx. 8000 cpm) was added and incubation was continued for a further 16-24 h. The bound and free fractions were separated as described above, with the exception that goat anti-rabbit IgG and normal rabbit serum were used.

C-reactive protein was measured with an immunometric assay (Immulite High Sensitivity C-reactive protein Assay, DPC, Los Angeles, USA). This C-reactive protein assay has been standardized against the WHO International Reference Standard for C-reactive protein Immunoassay. Fasting levels of serum lipids and creatinine were assessed as described previously (Salonen *et al.* 1991, Lakka *et al.* 1994).

4.5 Characterization of molecular forms of proANP and proBNP (I-III)

4.5.1 Incubation of NT-proANP and NT-proBNP in plasma and serum (III)

EDTA-plasma (n = 8) and serum samples (n = 8) were collected from healthy laboratory personnel into Vacutainer tubes. The whole blood (10 ml) was immediately spiked with recombinant NT-$proANP_{1-98}$ and NT-$proBNP_{1-76}$ (1 000 pmol/L of both analytes, Figure 1). The whole blood for plasma samples was immediately centrifuged at +4°C. The whole blood for serum samples was incubated at room temperature for 90 minutes and centrifuged at +4°C. As a control, RIA assay buffer was also spiked with recombinant NT-$proANP_{1-98}$ and NT-$proBNP_{1-76}$ (1 000 pmol/L of both analytes). Spiked serum, plasma and RIA assay buffer pools were then divided into aliquots of 0.6-mL and stored at room temperature, at +4°C and at -20°C for 0, 1, 2, 3, 4 and 8 weeks. After the incubation the immunoreactivities of the samples were determined and the samples were then stored at -70°C for further analysis by HPLC.

4.5.2 Reverse phase HPLC of plasma and serum samples (I-III)

For reverse phase high performance liquid chromatography (RP-HPLC), the human plasma or serum sample (300 µl) was mixed with 200 µl 0.25% trifluoroacetic acid in acetonitrile and cleared by centrifugation at 10000 g for 10 min. The supernatant was diluted two times with 0.1% TFA and passed through a 0.45 µm Millex HV filter (Millipore). The filtrate was applied into a Vydac C_4 (4.6 x 150 mm) HPLC column (Separation group, Hesperia, CA, USA). The column was eluted with a linear 40-min gradient from 16-40% acetonitrile in aqueous 0.1% trifluoroacetic acid. The elution rate was 1 ml/min and fractions of 1 ml were collected. Fractions were dried in a SpeedVac concentrator and dissolved in 0.5 ml of RIA assay buffer for use in the

radioimmunoassays. The column was calibrated with recombinant NT-proANP, recombinant NT-proBNP, recombinant proBNP and purified rat proANP.

4.5.3 Gel filtration HPLC of plasma and serum samples (I-III)

For gel filtration HPLC (GF-HPLC) analyses, the human plasma or serum sample was handled similarly as for RP-HPLC but omitting the dilution step. After that, the cleared filtrate was applied into a Proteinpak 125 GF-HPLC column (3.9 x 300 mm, Waters, Milford, MA), eluted with 40% acetonitrile in aqueous 0.1% trifluoroacetic acid. The flow rate was 1 ml/min and fractions of 0.5 ml were collected. Fractions were dried in a SpeedVac concentrator and dissolved in 0.5 ml of RIA assay buffer for use in the radioimmunoassays. The column was calibrated with bovine serum albumin (68 kD, void volume), purified rat proANP (14 kD), recombinant proBNP (12 kD), recombinant NT-proANP (11 kD), recombinant NT-proBNP (8.6 kD) and ^{125}I (total volume).

4.6 Collection of study samples

4.6.1 Normal samples (I-III)

Venous blood samples from 100 healthy blood donors (age range 20-65 years, 50 females, 50 males) were collected into vacutainer tubes. Serum fraction of the blood was separated. In addition, normal serum and EDTA-plasma samples were obtained from the staff of the Department of Physiology, University of Oulu (age range 24-63 years, 15 females, 7 males). Blood samples were stored at -20°C until analysis.

4.6.2 Patient samples (I, II, IV)

4.6.2.1 Study I (I)

EDTA-plasma samples were obtained from 86 patients with acute myocardial infarction (AMI, age range 39-90 years, 23% women), at entry to the emergency unit, at 96 hours and/or at discharge from the hospital, so that the total sample count was 206 (Puhakka *et al.* 2003). A few serum samples were also taken for use in the HPLC studies. All patients showed ST-segment elevation on electrocardiograms and were treated with thrombolytic therapy. Patients were using the following cardiovascular drugs on admission to hospital: beta-blockers (22%), nitrates (14%), aspirin (14%), angiotensin-converting enzyme inhibitors (13%), calcium channel blockers (14%), diuretics (5%), digitalis glycosides (1%), and hypolipidemic agents (8%). One week after admission to hospital the ejection fraction was ≤ 40% in 17 patients as determined by echocardiography. In addition, venous blood samples (serum) from five premature infants were utilized for the initial

characterization of the circulating forms of NT-proANP and NT-proBNP because of their extremely high peptide content. Plasma and serum samples were stored at -20°C until analysis.

4.6.2.2 Study II (II)

Serum samples were obtained from 111 patients with valvular heart disease (VHD, age range 29-81 years, 51% women). Forty-two (38%) of the VHD patients had aortic valvular disease, 57 (51%) mitral valvular disease, and 12 had both aortic and mitral valvular disease. Serum samples were also obtained from 367 patients with coronary artery disease (CAD, age range 33-88 years, 30% women). Twenty-eight (8%) of the CAD patients had postinfarction angina (1 week after acute myocardial infarction), and 160 (44%) had previous acute myocardial infarction. Of the 478 patients with VHD or CAD, 355 (74%) had hypertension, 146 (31%) had diabetes mellitus, and 304 (64%) had dyslipidemia. The medical therapies used before hospitalization included beta-blockers (76% of patients), angiotensin-converting enzyme inhibitors (69%), calcium channel blockers (21%), diuretics (24%), statins (53%), organic nitrates (53%), antithrombolytic drugs (64%), and digitalis glycosides (6%). EDTA-plasma for analytical studies was obtained from a subset of the patients and from healthy individuals. Ejection fractions were determined for 477 patients (111 VHD, 366 CAD) with M-mode echocardiography using the equation: EF = (SV / EDV) x 100, where SV is stroke volume and EDV is end-diastolic volume. The plasma and serum samples were stored at -20°C until analyses.

4.6.2.3 Study IV (IV)

Serum samples were collected from the participants of the Kuopio Ischemic Heart Disease Risk Factor Study (KIHD). The study population is a representative sample of men living in the city of Kuopio and its surrounding rural communities (age range 46-65 years). The baseline examinations were performed between 1991 and 1993. Of 1,229 potentially eligible and randomly selected men, 1,038 (83%) volunteered to participate in baseline examinations. Of the invited men, 107 declined, 52 could not participate because of death, severe illness or relocation, and 32 could not contacted. The 984 participants with no missing data were included and plasma samples were collected. Men with a history of heart failure (n=69) or atrial fibrillation (n=10) were excluded, leaving 905 men in the statistical analysis. Diagnosed diseases among these men were coronary heart disease (18.6%), cerebrovascular stroke (2.4%), diabetes (5%), hypertension (30.4%), claudication (2.4%), and chronic bronchitis (4.9%). 24.4% of the men used regularly anti-hypertensive medications, 6.2% used medication for hypercholesterolemia, 10.1% β-blockers and 9.5% aspirin.

4.6.3 Ethical approval

Informed consent was obtained from all volunteers and patients or their parents. The Ethics Review Boards of the Universities of Kuopio, Helsinki and Semmelweis Budapest approved the protocols. The investigations conform to the principles outlined in the Declaration of Helsinki.

4.7 Statistical analyses

Correlation coefficients were calculated by linear regression analysis. Comparison between two groups was performed using unpaired non-parametric analysis (Mann-Whitney). Data groups were analyzed by two-way analysis of variance (ANOVA) followed by Holm-Sidak post-hoc test. The associations of NT-proANP and NT-proBNP with the risk of death and its co-morbidities were analyzed using risk factor-adjusted Cox proportional hazards' model. The correlations between natriuretic peptides and risk factors were performed by Pearson's correlation test. Statistical significance was defined as $P < 0.05$.

5 Results

5.1 Immunoassays for NT-proANP and NT-proBNP (I)

5.1.1 Recombinant NT-proANP, NT-proBNP, proBNP and BNP (I)

Immunoassays for NT-proANP and NT-proBNP were set up based on full-length recombinant calibrators and well-characterized antisera. For this purpose human NT-proANP, NT-proBNP, $proBNP_{1\text{-}108}$ and $Tyr_0\text{-}proBNP_{77\text{-}108}$ (Tyr_0-BNP) were produced in bacterial cells as glutathione-S-tranferase (GST) fusion proteins. A yield of 1.5-2.4 mg of soluble affinity purified fusion protein was typically obtained from 1 L of bacterial culture. The yield of $GST\text{-}proBNP_{1\text{-}108}$ was somewhat lower: 0.75 mg from 1 L of culture. In SDS-PAGE the fusion proteins appeared as single bands without any major contaminants and the sizes of the fusion proteins were as expected (~40 kDa, ~37.5 kDa, ~41 kDa and ~32.5 kDa, respectively).

Recombinant peptides were released from their fusion partners by digestion with thrombin and purified by RP-HPLC with a yield of 0.1 - 0.5 mg/L of original bacterial culture. The sizes of the peptides as detected by SDS-PAGE and GF-HPLC, and the yielded N-terminal sequences were consistent with the native peptides sequences, including the vector-derived N-terminal Gly-Ser (GS) dipeptide (Table 7). The molecular identity of recombinant peptides was further ascertained with mass spectrometry. The obtained mass for NT-proANP corresponded to $GS\text{-}NT\text{-}proANP_{1\text{-}98}$, and the masses for NT-proBNP probably to $GS\text{-}NT\text{-}proBNP_{1\text{-}74}$ and $GS\text{-}NT\text{-}proBNP_{1\text{-}76}$.

Table 7. Recombinant peptides prepared in this work and their identities.

Recombinant peptide	Size by GF-HPLC (kDa)	Size by mass spectrometry (Da)	N-terminal sequence
NT-proANP$_{1-98}$	11	10767 ± 8.16	GSNPMYNAVS
NT-proBNP$_{1-76}$	8.6	8279 ± 0.43, 8603 ± 0.4	GSHPLGSPGS
proBNP$_{1-108}$	12	-	GSHPLGSPGS
Tyr$_0$-BNP	3.5	-	GSYSPKMVQG
NT-proXNP (NT-proBNP$_{1-37}$-NT-proANP$_{29-98}$)	12	11698 ± 1.03	GSHPLGSPGS

5.1.2 Antisera for NT-proANP and NT-proBNP (I, II)

To obtain highly specific antisera for propeptides, goats were immunized with GST-fusion proteins and several synthetic peptide fragments (NT-proANP$_{1-20}$, NT-proBNP$_{1-22}$, NT-proBNP$_{5-24}$, NT-proBNP$_{10-29}$, NT-proBNP$_{41-56}$ and Tyr$_0$-NT-proBNP$_{57-76}$) coupled to thyroglobulin or hemocyanin. After boosters (2-3), test bleeds showed 40% binding of the appropriate tracers at final dilutions 1/10000 - 1/50000 (Table 5). As an exception, an antiserum raised against NT-proBNP$_{41-56}$ bound NT-proBNP with very low titer. In addition, the assay utilizing antiserum to GST-NT-proBNP, which had the epitope located in the region of amino acids 57-76, did not show parallel dilution of plasma and serum samples with the NT-proBNP calibrator.

The epitopes and cross-reactivities of antisera were tested in detail by several peptide fragments. The epitope of the GST-NT-proANP antiserum was located broadly in the region of amino acids 46-79. The epitopes of antisera NT-proBNP$_{1-22}$, NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$ were sited in the regions of amino acids 5-20, 15-25 and 57-70, respectively. Cross-reactivities of the NT-proANP and NT-proBNP antisera relative to other vasoactive peptides human ANP (proANP$_{99-126}$), rat ANP (proANP$_{99-126}$), human BNP (proBNP$_{77-108}$), CNP, rat adrenomedullin and human endothelin-1 were < 0.03%. The NT-proANP antisera did not recognize recombinant human NT-proBNP, but fully cross-reacted with human proANP$_{1-126}$. None of the NT-proBNP antisera recognized recombinant human NT-proANP (< 0.03%), but they cross-reacted (90-100%) with recombinant proBNP$_{1-108}$.

5.1.3 Radioimmunoassays for NT-proANP and NT-proBNP (I)

Radioimmunoassays for the following epitopes of NT-proANP and NT-proBNP were set up: NT-proANP$_{1-20}$, NT-proANP$_{46-79}$, NT-proBNP$_{1-22}$, NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$. Recombinant NT-proANP and NT-proBNP were used as tracers and calibrators in the NT-proANP and NT-proBNP radioimmunoassays, respectively. Different amounts of samples from healthy and myocardial infarction subjects displaced in all assays the tracers similarly to the recombinant NT-proANP or NT-proBNP calibrators. The NT-proANP assays (NT-proANP$_{1-20}$ and NT-proANP$_{46-79}$) were linear in the range of 100 -

5000 pmol/L (r^2 = 0.999). The linearity range for the NT-proBNP assays (NT-proBNP$_{1-22}$, NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$) was 100 - 3200 pmol/L (r^2 = 0.994 - 0.9996) and for the NT-proBNP$_{5-24}$ assay 100 - 800 pmol/l (r^2= 0.995). The detection limit for the NT-proANP assays, as calculated at 95% displacement of the tracer, was 60 pmol/L. The detection limit for the NT-proBNP$_{1-22}$, NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$ assays was 40 pmol/L and for the NT-proBNP$_{5-24}$ assay 63 pmol/L. With the delayed addition of the tracer the detection limit for the NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$ assays was as low as 16 pmol/L. The functional sensitivities for the NT-proANP and NT-proBNP were 100 pmol/L and 70-80 pmol/L, respectively. The within-assay and the between-assay precisions were always < 15% and < 20%, respectively, and usually markedly better. The recoveries of the NT-proANP analyte varied between 79-97% and those of the NT-proBNP analyte between 58-104%. The analytes were recovered equally well from plasma and serum. All of our assays thus fulfill the basic requirements for usable immunoassays.

5.2 Immunoassay for NT-proXNP (II)

5.2.1 Recombinant NT-proXNP (II)

The currently available cardiac natriuretic peptide assays measure one analyte at a time. The biological effects of cardiac natriuretic peptides are, however, mediated by a single receptor, NPR-A. To mimic this, we developed a simple immunoassay which could reflect the activation of either A- or B-type natriuretic peptide systems. Because we utilized the N-terminal fragments of natriuretic peptides we call the new analyte 'NT-proXNP'. We prepared recombinant NT-proXNP molecule in bacterial cells for the use in assay development. In order to prepare recombinant NT-proXNP the cDNAs for the coding sequences of human NT-proBNP and NT-proANP were inserted end to end into the multiple cloning site of a bacterial expression vector pGEX-4T-1 (Table 5). Affinity-purified GST-NT-proANP$_{1-98}$-NT-proBNP$_{1-76}$ or GST-NT-proBNP$_{1-76}$-NT-proANP$_{1-98}$ fusion proteins were highly degraded as detected by SDS-PAGE. However, the affinity-purified GST-NT-proBNP$_{1-37}$-NT-proANP$_{29-98}$ fusion protein appeared as a single band in SDS-PAGE without any major contaminants and a yield of 2.5 mg was typically obtained from a 1-L bacterial culture. According to SDS-PAGE the size of the protein was ~41 kDa, which is consistent with that expected from the nucleotide sequence. Due to better yield and stability it was chosen for the assay development.

Recombinant human NT-proBNP$_{1-37}$-NT-proANP$_{29-98}$ (referred to as NT-proXNP) was released from its fusion partner and purified by RP-HPLC with a yield of 0.5 mg/L of original bacterial culture. The size of the peptide was ~12 kDa as detected by GF-HPLC, corresponding to that expected from the amino acid sequence (Table 7). The mass 11698 Da and the first 10 N-terminal amino acids GSHPLGSPGS were also consistent with the expected structure of NT-proXNP, including the vector-derived N-terminal Gly-Ser dipeptide and the Ser-Arg spacer between the NT-proBNP- and NT-proANP-derived sequences.

5.2.2 Radioimmunoassay for NT-proXNP (II)

Goat antisera prepared in the previous work (I, see chapter 5.1.2) were tested for the NT-proXNP assay. Antisera to NT-proBNP$_{10-29}$ and NT-proANP$_{46-79}$ (raised against the GST-fusion protein of NT-proANP) turned out to be optimal for the immunoassay development. Recombinant NT-proXNP was used as a tracer and calibrator. In the NT-proXNP assay, NT-proANP and NT-proBNP present in human blood compete with the NT-proXNP tracer for binding of specific antisera (Fig. 3). Different amounts of serum or plasma from healthy and heart failure subjects displaced the tracer in parallel with the recombinant NT-proXNP calibrator. The NT-proXNP assay was highly linear in the range of 150 - 7300 pmol/L ($r = 0.994$). A 5% displacement of the tracer, considered the detection limit of the assay, was obtained at 80 pmol/L. The within-assay precisions were < 6% and the between-assay precisions were < 12%. The recoveries of the analytes varied between 61-90%, depending on the ratio and the concentrations of NT-proANP and NT-proBNP. The affinity of NT-proXNP antiserum is chosen to favor NT-proBNP at the expense of NT-proANP at low peptide concentrations, resulting in varying recoveries. However, the NT-proXNP concentrations measured from serum samples of cardiovascular patients ($n = 478$) correlated well with both NT-proANP ($r^2 = 0.75$) and NT-proBNP ($r^2 = 0.85$). The correlation coefficient was even better between NT-proXNP and the arithmetic sum of NT-proANP and the NT-proBNP ($r^2 = 0.92$). Thus, the NT-proXNP assay can simultaneously detect the activation of ANP and BNP systems, regardless of whether they are activated alone or together.

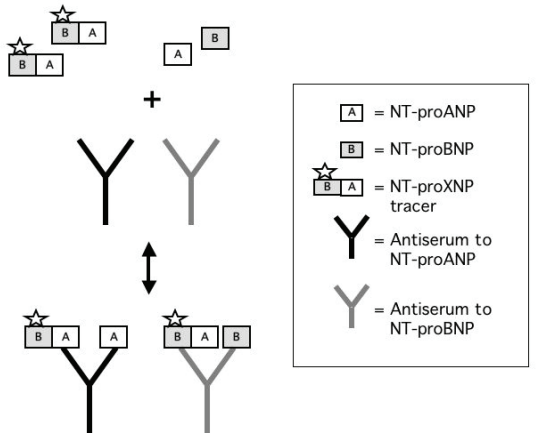

Fig. 3. Schematic presentation of the basic principle of the competitive NT-proXNP assay in a blood sample. Unlabeled antigen (NT-proANP and/or NT-proBNP) in a sample competes against labeled antigen (NT-proXNP tracer) for binding to antiserum.

5.3 Circulating forms of proANP and proBNP (I-III)

5.3.1 Molecular heterogeneity of NT-proANP and NT-proBNP in patients with acute myocardial infarction (I, II)

Despite many attempts to clarify the issue, the actual circulating forms of proANP-, and especially, proBNP-derived peptides are partly unknown. Thus, we wanted to determine the nature of immunoreactive NT-proANP and NT-proBNP in human blood using chromatographic methods and different sequence-specific immunoassays. The plasma and serum samples of patients with acute myocardial infarction (AMI) were separated by high-performance liquid chromatography (HPLC) and the collected fractions were analyzed using the NT-proANP and NT-proBNP antisera with epitope specificities in different parts of the peptides. A single, fairly broad area of immunoreactivity was detected with the NT-proANP$_{1-20}$, NT-proANP$_{46-79}$ and NT-proANP$_{79-98}$ assays in the reverse phase-HPLC (RP-HPLC, Fig. 4A) and gel-filtration HPLC (GF-HPLC) analyses of human plasma, peaking at the elution position of the recombinant NT-proANP calibrator. The N- and C-terminal antisera, however, consistently detected 30-50% lower concentrations of immunoreactive NT-proANP in plasma samples compared to the NT-proANP$_{46-79}$ antiserum. The difference was even greater (30-70%) with serum. Furthermore, the NT-proANP$_{46-79}$ antiserum detected immunoreactivity in the serum samples eluting after the main peak in GF-HPLC and thus having lower molecular weight.

The major peak of immunoreactive NT-proBNP in RP-HPLC of human plasma was detected by all the three different NT-proBNP assays (NT-proBNP$_{1-22}$, NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$, Fig. 4C). The broad peak eluted before the NT-proBNP calibrator, showing that the immunoreactivity was due to material which is less hydrophobic, and thus probably smaller in size, than NT-proBNP$_{1-76}$. RP-HPLC of serum revealed the presence of even less hydrophobic immunoreactivity, which was recognized with the NT-proBNP$_{10-29}$ antiserum only (Fig. 4D). The results from the plasma GF-HPLC analyses were consistent with the RP-HPLC data. NT-proBNP immunoreactivity eluted as a broad region peaking later than the NT-proBNP calibrator, indicating a molecular weight of approximately 6-8 kDa. The GF-HPLC of immunoreactive NT-proBNP in human serum revealed an even broader peak and indicated the presence of immunoreactive material with molecular weight as low as 3.5 kDa. The chromatographic results obtained by the NT-proXNP assay confirmed these findings.

The heterogeneity of NT-proANP and NT-proBNP was also seen in concentrations of plasma and serum samples obtained from patients with AMI. Although the results of the NT-proANP$_{1-20}$ and NT-proANP$_{46-79}$ assays showed a significant correlation with each other ($r^2 = 0.82$), the absolute values varied between these two assays, so that the NT-proANP$_{1-20}$ assay consistently yielded about 30% lower concentrations (Fig. 7). The NT-proBNP$_{5-24}$, NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$ concentrations also correlated significantly with each other ($r^2 = 0.77$-0.85), but the absolute values varied again greatly between the assays. A reduction of about 30% in concentrations measured by the NT-proBNP$_{57-76}$ assay compared to the NT-proBNP$_{10-29}$ and the NT-proBNP$_{5-24}$ assays was

detected in the raw plasma samples. Taken together, there is structural heterogeneity in circulating immunoreactive NT-proANP and NT-proBNP due to N- and C-terminal truncation. Especially NT-proBNP is strongly heterogeneous, and the smallest components can be detected with antisera directed at the central region of NT-proBNP only.

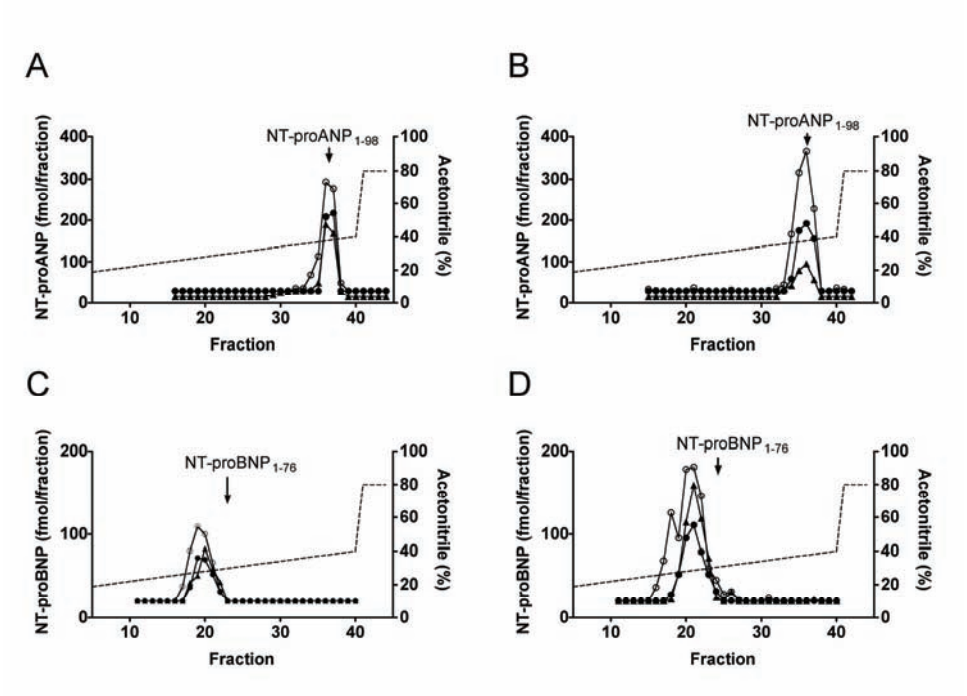

Fig. 4. RP-HPLC analysis of plasma and serum samples from patients with AMI. RP-HPLC fractions of (A) plasma and (B) serum samples were assayed for NT-proANP (NT-proANP$_{1-20}$, closed circles; NT-proANP$_{46-79}$, open circles; NT-proANP$_{79-98}$, triangles). The fractions in (C) plasma and (D) serum were assayed for NT-proBNP (NT-proBNP$_{1-22}$, closed circles; NT-proBNP$_{10-29}$, open circles; NT-proBNP$_{57-76}$, triangles).

5.3.2 Truncation of recombinant NT-proANP and NT-proBNP in plasma and serum (III)

To investigate further the degradation and stability of the terminal and central epitopes of NT-proANP and NT-proBNP, we spiked fresh whole blood samples with recombinant NT-proANP and NT-proBNP peptides. After incubation of separated plasma and serum fractions at room temperature, at +4°C and at -20°C, immunoreactivities of the samples were analyzed by different antisera. The starting concentrations of NT-proANP$_{1-20}$ and NT-proANP$_{46-79}$ in plasma and serum did not differ significantly from each other. After one week of storage at room temperature NT-proANP$_{46-79}$ immunoreactivity was

unchanged, but NT-proANP$_{1-20}$ immunoreactivity in plasma and serum was only 60-75% of the original concentration. At room temperature the NT-proANP$_{46-79}$ concentration did not decrease significantly in plasma during four weeks and in serum during two weeks. However, after two weeks of storage in plasma and serum the terminal concentration had decreased to 40-50% of the starting level. After eight weeks NT-proANP$_{1-20}$ was about 10% and NT-proANP$_{46-79}$ 63-79% of the initial immunoreactivity. At +4°C the rate of decrease was slightly slower and at -20°C NT-proANP immunoreactivity did not show any significant change.

NT-proBNP showed more extensive degradation at the termini compared to NT-proANP. The NT-proBNP$_{1-22}$ concentration in serum was significantly lower than the NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$ concentrations even at the starting point before incubations ($P < 0.001$, Fig. 5E-F). The starting plasma concentrations differed less, but NT-proBNP$_{1-22}$ was still significantly lower compared to NT-proBNP$_{10-29}$ ($P < 0.01$, Fig. 5C-D). The starting concentrations were similar in assay buffer (Fig. 5A-B). Not more than 30% of the original amount of NT-proBNP$_{1-22}$ and NT-proBNP$_{57-76}$ immunoreactivity in plasma and serum was detectable after one week of storage at room temperature, whereas 80% of NT-proBNP$_{10-29}$ immunoreactivity was preserved (Fig. 5C, E). At eight weeks the immunoreactivity detected by the terminal antisera was decreased to about 15% of the original level and NT-proBNP$_{10-29}$ to about 50%. In assay buffer the truncation of the terminal epitopes started more slowly, reaching 20-30% at two weeks (Fig. 5A).

At +4°C the terminal NT-proBNP immunoreactivity in plasma and serum was about 50-60% of the original level after one week of storage, thereafter decreasing to about 30% at two weeks and 10-15% at eight weeks (Fig. 5D, F). NT-proBNP$_{10-29}$ immunoreactivity was at about the original level until 4 weeks, decreasing to 80% at eight weeks. In assay buffer the NT-proBNP immunoreactivity remained unchanged at +4°C for eight weeks, except that NT-proBNP$_{1-22}$ immunoreactivity had decreased to ≈80% of the original level (Fig. 5B). At -20°C there were no significant changes of the NT-proBNP immunoreactivities during the two-month storage period. However, antiserum to NT-proBNP$_{1-22}$ detected a significantly lower level of NT-proBNP in serum compared to antiserum to NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$ ($P < 0.001$). In conclusion, the central parts of aminoterminal ANP and BNP are stable even at harsh storage conditions.

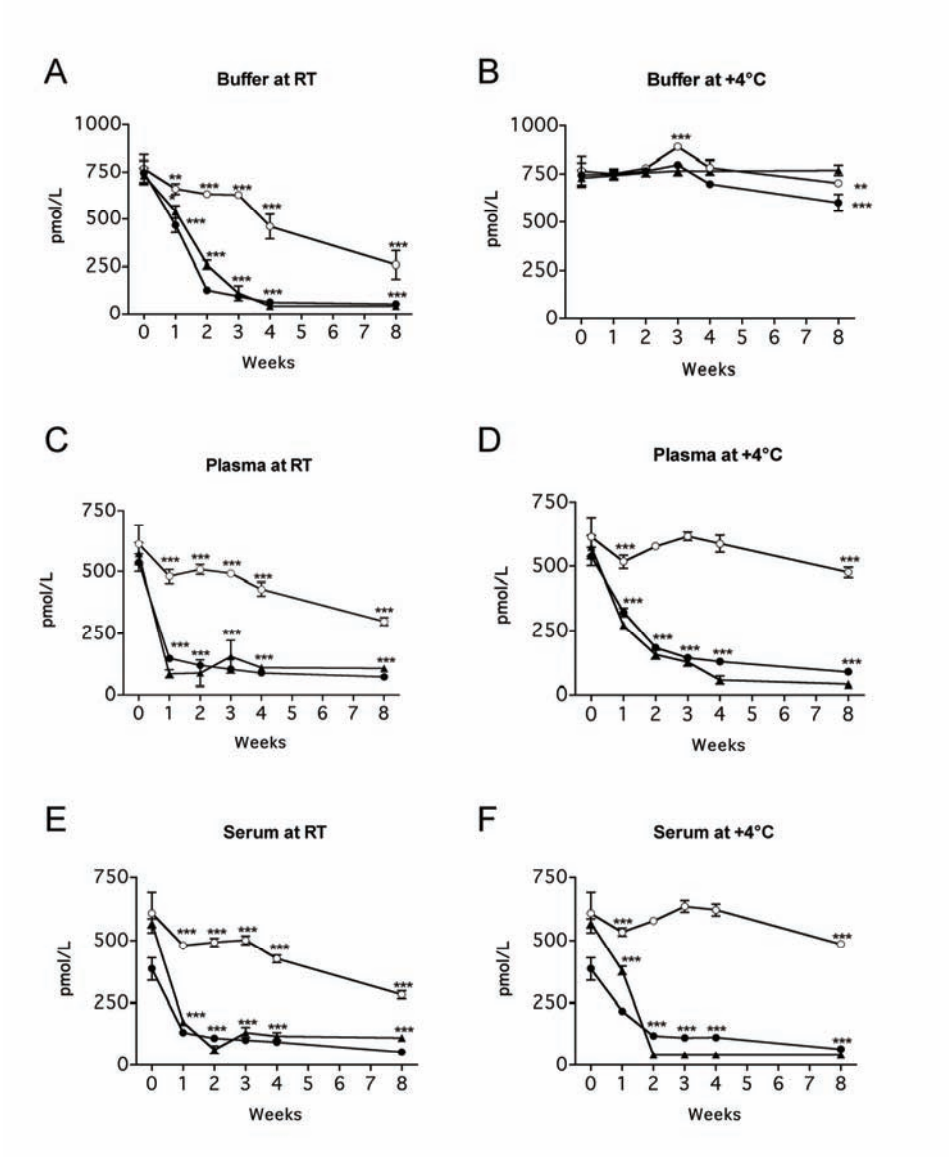

Fig. 5. Time course of truncation of recombinant NT-proBNP in RIA assay buffer, plasma and serum at different storage conditions. Assay buffer and whole blood were spiked with recombinant NT-proBNP (1000 pmol/L). The buffer, plasma and serum samples were stored at room temperature (A, C, E) and at +4°C (B, D, F). Samples were analyzed by assays specific to NT-proBNP$_{1-22}$ (closed circles, n = 4 for each time point), NT-proBNP$_{10-29}$ (open circles) and NT-proBNP$_{57-76}$ (triangles). Results are expressed as mean ± SD, $P < 0.05$ compared to the starting point was considered significant, ** $P < 0.01$, *** $P < 0.001$.

5.3.3 Degradation products of recombinant NT-proBNP in plasma and serum (III)

The degradation products of NT-proBNP were analyzed in more detail by RP-HPLC (Fig. 6). For this purpose we used spiked plasma and serum samples incubated at room temperature for a week. In plasma three NT-proBNP$_{10-29}$ immunoreactive species were detected, two eluting before and one after the recombinant calibrator (Fig. 6A). The terminal antisera detected immunoreactivity in only one of the species, eluting near or right after the recombinant calibrator. The NT-proBNP$_{1-22}$ antiserum showed less immunoreactivity than the NT-proBNP$_{57-76}$ antiserum. In serum there were also three NT-proBNP$_{10-29}$ immunoreactive species, two before and one at the position of the recombinant NT-proBNP calibrator (Fig. 6B). The terminal antisera detected only the third area, but very weakly.

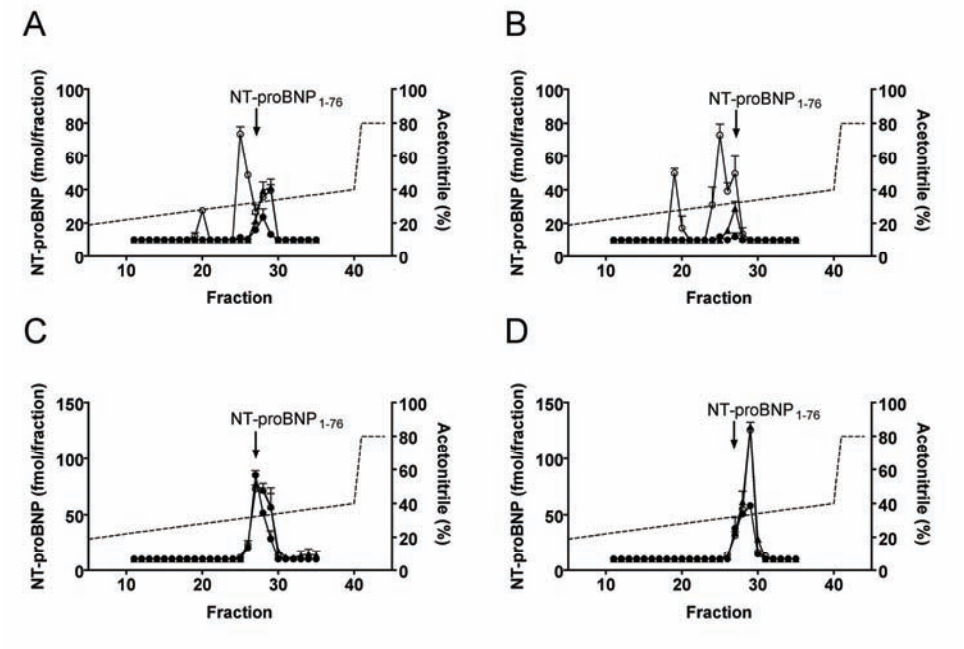

Fig. 6. RP-HPLC analysis of NT-proBNP stability in plasma and serum at room temperature and at -20°C. Fresh whole blood was spiked with recombinant NT-proBNP (1 000 pmol/L) and aliquots of separated plasma and serum were stored at room temperature (A, B) and at -20°C (C, D) for a week. Chromatography fractions (1-mL) were analyzed by assays specific to NT-proBNP$_{1-22}$ (closed circles, n = 3), NT-proBNP$_{10-29}$ (open circles) and NT-proBNP$_{57-76}$ (triangles). Results are expressed as mean ± SEM.

We also analyzed a few spiked plasma and serum samples stored at -20°C by RP-HPLC. These samples were freeze-thawed two to three times before analysis. In plasma samples there was an immunoreactive area at the position of the NT-proBNP calibrator, which

was equally detected by all three antisera (Fig. 6C). The peak was slightly extended and these fractions eluting after the main peak were best detected by the NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$ antisera. However, in serum the whole immunoreactivity eluted after the position of the recombinant calibrator and was equally recognized by the central and C-terminal antisera, but the N-terminal antiserum detected only about 50% of that immunoreactivity (Fig. 6D). The N-terminus of NT-proBNP is thus most vulnerable to degradation.

5.4 NT-proANP, NT-proBNP and NT-proXNP in health and disease (I, II, IV)

5.4.1 NT-proANP, NT-proBNP and NT-proXNP in healthy subjects (I, II)

To obtain reference values for the assays, serum samples from healthy subjects of both sexes, aged 20-65 years, were measured with the different NT-proANP and NT-proBNP immunoassays and with the NT-proXNP assay (Fig. 7). The median concentrations with 95% confidence intervals (CI) in healthy persons measured with assays specific to NT-proANP$_{1-20}$ and NT-proANP$_{46-79}$ were 132 and 246 pmol/L (130-151 and 236-274 pmol/L, n = 100, 50% men), respectively. The corresponding concentrations measured with assays specific to NT-proBNP$_{5-24}$, NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$ were 67 (78-94), 84 (79-92) and 48 (51-66) pmol/L, respectively. Notably, different assays gave different absolute values depending on the antiserum used. No gender differences were detected in either NT-proANP or NT-proBNP values. However, the median NT-proXNP concentration 148 (<80-295) pmol/L in women (median and 2.5-97.5th percentiles, n=50) was significantly higher than that in men, 114 (<80-298) pmol/L (n=50, P=0.015). The reference NT-proXNP concentrations both in women and men were significantly lower (P <0.0001) than the reference NT-proANP$_{47-79}$ concentrations but significantly higher than the NT-proBNP$_{10-29}$ (P <0.0001) concentrations.

5.4.2 NT-proANP and NT-proBNP in patients with acute myocardial infarction (I)

In order to test the functionality of our NT-proANP and NT-proBNP assays we measured natriuretic peptide concentrations in plasma samples obtained from patients after acute myocardial infarction (AMI). All the amino-terminal assays revealed significantly increased levels in the AMI patients at 96 h (n = 45) compared to healthy subjects (P < 0.001, Fig. 7). The mean concentrations of NT-proANP$_{1-20}$ and NT-proANP$_{46-79}$ were increased 2.3- and 1.8-fold, respectively. The increases of NT-proBNP$_{5-24}$, NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$ concentrations were more pronounced: 4.2-, 4.5- and 4.5-fold, respectively. The development of heart failure (ejection fraction ≤ 40%, n = 16) was

associated with a further 1.4- to 1.7-fold increase of NT-proANP$_{1-20}$ and NT-proANP$_{46-79}$ concentrations ($P < 0.05$). The corresponding heart failure-associated increases for NT-proBNP$_{5-24}$, NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$ were 1.6- to 1.7-fold. These results indicate that our assays are applicable to clinical samples.

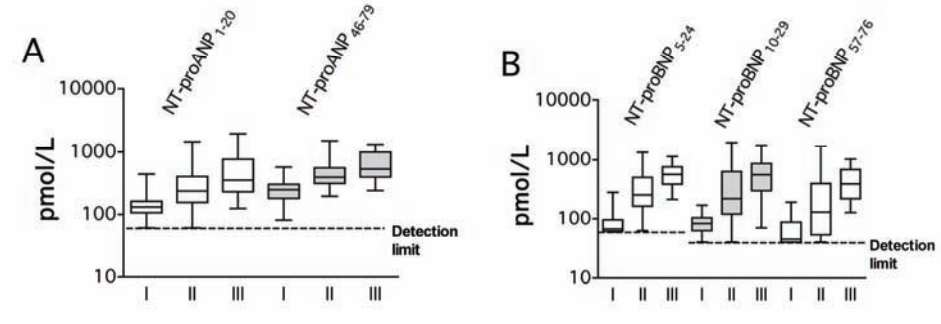

Fig. 7. Circulating concentrations of NT-proANP (A) and NT-proBNP (B) in healthy individuals (group I, n = 100), AMI patients 96 h after admission to the emergency room (group II, n = 45), and AMI patients with heart failure at 96 h (group III, n = 16). (A), NT-proANP was measured with immunoassays specific for NT-proANP$_{1-20}$ and NT-proANP$_{46-79}$. (B), NT-proBNP was measured with immunoassays specific for NT-proBNP$_{5-24}$, NT-proBNP$_{10-29}$ and NT-proBNP$_{57-76}$. The plots display the median values (lines inside boxes), 25th and 75th percentiles (lower and upper limits of boxes), and the minimum and 99th percentiles (error bars). The dashed lines correspond to the detection limits of the assays.

5.4.3 NT-proXNP, NT-proANP and NT-proBNP in patients with valvular heart disease or coronary artery disease (II)

To test the applicability and differences in clinical utility of NT-proXNP, NT-proANP and NT-proBNP assays we analyzed 478 serum samples from patients with valvular heart disease (VHD, n = 111) or coronary artery disease (CAD, n = 367). The median concentration of NT-proXNP was significantly higher in the VHD patients [1113 (553-1963) pmol/L, median (25^{th}-75^{th} percentiles), $P > 0.0001$] than in the CAD patients [445 (262-782) pmol/L]. No gender differences were detected in either the VHD or the CAD group.

To test the usefulness of increased serum concentration of NT-proXNP as an indicator of cardiac disease, receiver operating characteristic (ROC) analyses were performed. The mean area under the curve (AUC) for detecting patients with VHD was 0.961, and statistical comparison showed that it was significantly larger than that of NT-proBNP (0.913, $P = 0.004$, Table 8). The AUC for detecting patients with CAD was 0.924 and it was larger than those of either NT-proANP (0.872) or NT-proBNP (0.782) in CAD ($p<0.0001$). We also performed ROC curve analysis for the sum of the NT-proANP and the NT-proBNP concentrations. The AUC for the VHD patients was 0.947 and for the CAD patients 0.875. The optimal cutoff values were chosen by ROC analysis. The best cutoffs for NT-proXNP, NT-proANP and NT-proBNP in VHD, as defined as the

concentration with the largest sum of sensitivity and specificity, were 303, 416 and 169 pmol/L, respectively (Table 8). The best cutoff values for detecting CAD were 248, 408 and 127 pmol/L, respectively.

We also examined whether our assays can detect impaired systolic ventricular function characterized by ejection fraction ≤ 50%. The AUC values for NT-proXNP (0.689), NT-proANP (0.672), NT-proBNP (0.684), and the arithmetic sum of NT-proANP and NT-proBNP (0.686) did not differ significantly from each other ($p>0.05$). Altogether, these results suggest that NT-proXNP has diagnostic efficiency that is equal or better compared to that of NT-proANP or NT-proBNP alone.

Table 8. Comparison of NT-proXNP, NT-proANP and NT-proBNP assays in detecting valvular heart disease or coronary artery disease. AUCs, sensitivities and specificities are expressed as mean values (95% confidence intervals).

Parameter	NT-proXNP	NT-proANP	NT-proBNP
AUC for VHD	0.961 (0.933-0.989)	0.947 (0.913-0.981)	0.913 (0.870-0.956)
Best VHD cutoff, pmol/L	303	416	169
Sensitivity, %	88 (81-94)	89 (82-94)	79 (71-86)
Specificity, %	100 (96-100)	94 (87-98)	100 (96-100)
AUC for CAD	0.924 (0.900-0.947)	0.872 (0.840-0.904)	0.782 (0.742-0.823)
Best CAD cutoff, pmol/L	248	408	127
Sensitivity, %	78 (73-82)	69 (64-74)	64 (59-69)
Specificity, %	95 (89-98)	93 (86-97)	91 (84-96)

AUC, area under the curve; VHD, valvular heart disease; CAD, coronary artery disease

5.4.4 NT-proANP and NT-proBNP as prognostic markers in general population (IV)

Scarce data are available on the prognostic value of natriuretic peptides in general population. Therefore, we investigated whether NT-proANP and NT-proBNP are predictive for overall death, cardiovascular events, and atrial fibrillation (AF) among middle-aged men without heart failure or AF. Plasma NT-proANP and NT-proBNP were measured at baseline in a representative population-based sample of 905 men (age range 46-65 years) from eastern Finland. Median concentrations for NT-proANP and NT-proBNP were 231 and 56 pmol/L, respectively. Both NT-proANP and NT-proBNP correlated weakly with age ($r = 0.389$ and $r = 0.181$, $P < 0.001$) and C-reactive protein ($r = 0.126$ and $r = 0.139$, $P < 0.001$). There were 110 deaths during the median follow-up of 9.8 years (range 0.1-11.8 years). Half of these deaths were due to cardiovascular causes (n = 58), 40 were due to coronary heart disease (CHD), and 52 were due to non-cardiovascular reasons. In addition, there were 105 acute coronary events, 59 cases of AF, and 19 cases of heart failure.

The predictive value of NT-proANP and NT-proBNP for overall and CHD death was evaluated by multivariable adjusted risk model including age, smoking, diabetes, systolic blood pressure, family history of CHD, presence or absence of CHD, body mass index,

serum LDL- and HDL-cholesterol, C-reactive protein, serum creatinine, and the use of anti-hypertensive medications. The risk of overall death and CHD death was 1.35 and 1.52 for each SD (160.8 pmol/L) increment in NT-proANP, and 1.26 and 1.44 for each SD (58.9 pmol/L) increment in NT-proBNP, respectively (Table 9). There was no association between propeptides and non-cardiovascular death. Furthermore, men with the highest 90th percentile of NT-proANP (> 455.0 pmol/L) had a 1.98-fold (95% CI 1.18-3.32, $P = 0.009$) risk of overall death and a 2.11-fold (1.07-4.19, $P = 0.032$) risk of cardiovascular death after adjustment of risk factors. The corresponding risk among men with highest 90th percentile of NT-proBNP (> 133 pmol/L) was 2.01 (1.23-3.29, $P = 0.005$) and 2.30 (1.23-4.23, $P = 0.009$). After adjustment for risk factors, NT-proANP and NT-proBNP were associated with the risk of death in several risk groups including smokers, men with low and high (> 26.98 kg/m^2) body mass index and men with low or high (>132.7 mmHg) systolic blood pressure. However, NT-proANP was not significantly related to the risk of death in men with good lipid profile and low C-reactive protein (< 1.39 mmol/L), whereas NT-proBNP was not clearly predictive in men with low LDL-cholesterol levels (< 3.87 mmol/L).

One SD increment in NT-proANP was related to a 72% increase in multivariable adjusted risk of AF and one SD increment in NT-proBNP amounted to a 46% increase in risk (Table 9). Men above the highest 90th percentile of NT-proANP and NT-proBNP had a 2.59-fold (1.38-5.00, $P < 0.0001$) and 3.22-fold (1.79-5.80, $P < 0.0001$) risk of AF, respectively. Only a few new heart failure cases (n = 19) were observed during the follow-up. However, the association between NT-proANP, NT-proBNP and the risk of heart failure was significant. The risk for heart failure was 1.76 for NT-proANP and 1.61 for NT-proBNP. Moreover, no statistically significant relationship between propeptides and the risk of acute coronary events was detected in the multivariable model. Altogether, these results show that NT-proANP and NT-proBNP are strong predictors of death and its co-morbidities in middle-aged men.

Table 9. Relative risks of main outcomes per 1 unit (SD) change in NT-proANP and NT-proBNP in 905 men from the eastern Finnish population study. Risk ratios are adjusted for age, smoking, diabetes, systolic blood pressure, family history of CHD, presence or absence of CHD, body mass index, serum LDL- and HDL cholesterol, C-reactive protein, serum creatinine, and the use of anti-hypertensive medications.

Outcome (no. of events)	Relative risk per 160.8 pmol/L increment in NT-proANP		*P*-value	Relative risk per 59.9 pmol/L increment in NT-proBNP		*P*-value
	Risk ratio	95% CI		Risk ratio	95% CI	
Death (110)	1.35	1.15-1.57	<0.001	1.26	1.12-1.42	<0.001
CVD death (58)	1.48	1.21-1.81	<0.001	1.41	1.21-1.65	<0.001
CHD death (40)	1.52	1.21-1.91	<0.001	1.44	1.22-1.70	<0.001
AF (59)	1.72	1.41-2.09	<0.001	1.46	1.27-1.68	<0.001
Heart failure (19)	1.76	1.24-2.48	0.001	1.61	1.18-2.18	0.003

CVD, cardiovascular; CHD, coronary heart disease; AF, atrial fibrillation; CI, confidence interval

6 Discussion

6.1 Reliable immunoassays for NT-proANP and NT-proBNP

Measurement of natriuretic peptides has been shown to be a useful tool in the diagnosis of cardiac diseases. Due to their longer half-lives and better stability the N-terminal peptides are considered potentially better markers of prolonged cardiac overload compared to their biologically active counterparts (Ruskoaho 2003, Apple *et al.* 2005). Both competitive and sandwich immunoassays have been developed for measuring circulating concentrations of NT-proANP and NT-proBNP (see Table 2 and Table 3); however, in most cases they utilize peptide calibrators. In addition, the antisera used are produced against peptide fragments without knowledge of how well they recognize the corresponding full-length peptide. This may cause the great variation seen in circulating concentrations measured by different assays, especially in the case of NT-proBNP (Clerico & Emdin 2004, Apple *et al.* 2005, Vuolteenaho *et al.* 2005).

In the present study several immunoassays for NT-proANP and NT-proBNP were developed based on well-characterized antisera and full-length recombinant calibrators. The identity of prepared recombinant NT-proANP corresponded with the full-length NT-proANP$_{1\text{-}98}$ as detected by N-terminal sequencing and mass analyses. The mass spectrum of recombinant NT-proBNP revealed two species probably corresponding to NT-proBNP$_{1\text{-}76}$ and NT-proBNP$_{1\text{-}74}$. This slight C-terminal truncation did not have any effect on the quantitative analyses or the calibration of the assays because our antisera did not bind to this region of the peptide. The recombinant calibrator for at least NT-proBNP assay has been difficult to prepare (Apple *et al.* 2005). In addition to us there are only two reports of the utilization of recombinant NT-proANP or NT-proBNP as a calibrator in the immunoassay (Missbichler *et al.* 2001, Karl *et al.* 1999). However, in these publications the production or the identities of the peptides were not reported. Most commonly used calibrators in the NT-proANP and NT-proBNP assays are short peptide fragments which do not correspond to native material circulating in blood (Table 2, Table 3). The antibodies used are also often against these same fragments. Detection by immunoassays has to be equimolar for the fragments that exist in blood; therefore, the best calibrators for NT-proANP and NT-proBNP assays should resemble these native circulating peptides as closely as possible (Apple *et al.* 2005). Furthermore, because of

different-sized peptide calibrators the results expressed as mass values (pg/ml) are difficult to compare.

Two homologous radioimmunoassays for NT-proANP and four for NT-proBNP were developed using high titer antisera. Each assay utilized the appropriate recombinant full-length calibrator but had different epitope specificity. Because of molecular heterogeneity most reliable assays use antisera directed at the central portions of NT-proANP and NT-proBNP. Special care has to be taken with NT-proBNP. However, many published assays for NT-proBNP utilize antibodies or antisera directed at the N- or C-terminus (Hunt *et al.* 1995, Hunt *et al.* 1997b, Hughes *et al.* 1999, Karl *et al.* 1999, Campbell *et al.* 2000, Schulz *et al.* 2001, Goetze *et al.* 2002). Assays with stricter epitope requirements are more prone to preanalytical errors, caused by differences in sampling and storage, for example. Most if not all circulating NT-proANP and NT-proBNP is of cardiac origin, regardless of the degree of fragmentation. Therefore, the assays, such as our NT-proANP$_{46-79}$ and NT-proBNP$_{10-29}$ assays, that are capable of picking the largest proportion of the fragments are likely to reflect best the functional status of the heart.

6.2 Simultaneous measurement of NT-proANP and NT-proBNP

Currently available cardiac natriuretic peptide assays measure one analyte at a time. However, ANP and BNP are divergently expressed, produced and secreted, and thus their plasma concentrations differ markedly in different pathophysiological conditions (Sagnella 1998, Ruskoaho 2003). Despite this, their functions are targeted by a common receptor, NPR-A. Therefore, to mimic the natural signal transduction at the receptor level a simple immunoassay for the simultaneous measurement of circulating NT-proANP and NT-proBNP (referred to as NT-proXNP) was developed, and its clinical utility was tested.

The novel assay was based on a hybrid combination epitope peptide, NT-proXNP, containing amino acid sequences from both NT-proANP and NT-proBNP in a single molecule. The GST-fusion proteins prepared containing the full-length NT-proBNP and NT-proANP sequences showed major degradation during the expression procedure. Moreover, NT-proBNP antisera bound recombinant NT-proANP$_{1-98}$ - NT-proBNP$_{1-76}$ only weakly. This may be related to the fact that the sequences of proANP and proBNP contain leucine zipper-like motifs which might induce oligomerization (Seidler *et al.* 1999). Therefore a construct encoding NT-proBNP$_{1-37}$ - NT-proANP$_{29-98}$ was designed in which the leucine zipper-like motif regions are not fully present. Indeed, this peptide turned out to be more stable and easier to express. It was also recognized by the NT-proANP and NT-proBNP antisera with similar avidity as recombinant NT-proANP$_{1-98}$ and NT-proBNP$_{1-76}$, respectively.

An immunoassay for the detection of both NT-proANP and NT-proBNP was developed using this novel NT-proXNP peptide and well-characterized antisera for stable parts of NT-proANP and NT-proBNP. The concentrations of both propeptides can be measured directly in a small amount of plasma or serum without prior extraction. The assay was designed, by the selection of antisera affinity, to give more weight to NT-proBNP over NT-proANP at low peptide concentrations. This compensates for the potential diagnostic disadvantage of the relatively high basal secretion of NT-proANP

(Buckley *et al.* 1990b). Nevertheless, the results of the novel NT-proXNP assay correlated well with the results obtained by individual NT-proANP and NT-proBNP assays and even better with the sum of the NT-proANP and NT-proBNP. Furthermore, the chromatographic results showed that the novel NT-proXNP assay recognized the same material in blood as individual NT-proANP and NT-proBNP assays. Thus we were successful in preparing an assay that gives a single laboratory value reflecting the activation or inactivation of the ANP or BNP systems, or both.

6.3 Heterogeneity of NT-proANP and NT-proBNP in cardiac patients

The actual circulating forms of proANP-, and especially, proBNP-derived peptides are still unknown and debated (Goetze 2004, Apple *et al.* 2005). In the present study the nature of immunoreactive NT-proANP and NT-proBNP in the blood of patients with acute myocardial infarction was clarified. The HPLC results of circulating NT-proANP appeared to be consistent with previous finding indicating that it appears in human blood as a single peptide, NT-proANP$_{1\text{-}98}$ (Sundsfjord *et al.* 1988, Meleagros *et al.* 1989, Buckley *et al.* 1990a, Azizi *et al.* 1996). We did, however, find some heterogeneity at the N- and C-termini of NT-proANP which showed up in markedly lower concentrations of immunoreactive NT-proANP in the NT-proANP$_{1\text{-}20}$ and the NT-proANP$_{79\text{-}98}$ assay compared to the NT-proANP$_{46\text{-}79}$ assay. The difference was especially clear with serum samples. Nevertheless, according to the present results major part of NT-proANP in human plasma (60-70%) is likely due to intact NT-proANP$_{1\text{-}98}$.

Studies of proBNP have indicated the presence of high-molecular weight proBNP (Tateyama *et al.* 1992, Yandle *et al.* 1993b) together with a shorter NT-proBNP peptide (Hunt *et al.* 1995, Schulz *et al.* 2001). The present results did not reveal any high-molecular-mass proBNP peptide in our samples of AMI patients. We did, however, detect a shorter NT-proBNP peptide in human plasma and serum that was recognized by both terminal and more central antisera and appeared to be near to the size of previously reported NT-proBNP$_{1\text{-}76}$ (Fig. 4). However, this main immunoreactive species was less hydrophobic, having a smaller molecular weight than NT-proBNP, and thus it probably represented NT-proBNP$_{1\text{-}76}$ derived peptide(s) truncated at both termini. In line with this, the NT-proBNP$_{1\text{-}22}$ and NT-proBNP$_{57\text{-}76}$ assays also detected 20-40% less immuno-reactivity in this peak compared to the NT-proBNP$_{10\text{-}29}$ assay immunoreactivity. The smaller molecular weight NT-proBNP immunoreactivity in RP-HPLC of serum samples was recognized with antisera directed at the NT-proBNP$_{10\text{-}29}$ only. Altogether, these findings suggest that NT-proBNP is fragmented into smaller components in the blood, and very little, if any, of the immunoreactive material is due to intact NT-proBNP$_{1\text{-}76}$. The fragmentation is more pronounced in serum compared to plasma.

6.4 The central parts of NT-proANP and NT-proBNP are stable

It has been reported that NT-proANP and NT-proBNP are relatively stable in routine clinical practice (Hall *et al.* 1995a, Hunt *et al.* 1997b, Numata *et al.* 1998, Downie *et al.*

1999, Karl *et al.* 1999). We studied the epitope specific stability of NT-proANP and NT-proBNP at different storage conditions. In accordance with our HPLC studies of molecular forms in cardiac patients, the central parts of recombinant NT-proBNP and NT-proANP were found to be stable even at harsh storage conditions, whereas the termini were labile and sensitive to preanalytical degradation. This clearly contradicts previous studies in which NT-proBNP was reported to be stable in whole blood at room temperature up to 72 hours as detected by terminal antisera (Downie *et al.* 1999, Karl *et al.* 1999). It has previously only been demonstrated that circulating proBNP may lack a few N-terminal amino acids (Hunt *et al.* 1997a). We found that the N-terminus of NT-proBNP was most vulnerable to degradation, especially in serum. N-terminal levels right after serum separation were markedly lower compared to those of central and C-terminal antisera. Our serum samples were prepared by centrifugation after 90-minute incubation of whole blood at room temperature. This was sufficient to cause degradation at the N-terminus of NT-proBNP but not NT-proANP. In clinical laboratories the incubation times for obtaining serum samples can be very variable, potentially resulting in severe damage to terminal epitopes of NT-proBNP. Furthermore, it is completely possible that degradation takes place while the peptides are still circulating. The mechanism of degradation cannot be deduced from the present study, but the termini of NT-proBNP have, for example, several proline residues which are susceptible to exopeptidases (Rawlings & Barrett 1993).

The degradation products of NT-proBNP were analyzed in detail by HPLC. After incubation of spiked plasma at room temperature for a week there were at least three separate NT-proBNP species as detected by the central antisera (Fig. 6). The third immunoreactive area peaking after the recombinant calibrator was fully detected by central and C-terminal antisera and partially by N-terminal antiserum. It is probably due to full-length and N-terminally truncated NT-proBNP. The N-terminus of NT-proBNP is hydrophilic (Sudoh *et al.* 1989), and the removal of N-terminal amino acids increases the hydrophobicity and retention time in RP-HPLC column. The first two species are most likely composed of NT-proBNP fragments which are truncated at both termini. The truncation must be at least about 10-15 amino acids because the epitopes of our N- and C-terminal antisera are located between amino acids 5-20 and 57-70, respectively. In the HPLC profiles of serum the first two immunoreactive areas were at the same position as in plasma samples, probably presenting the same kind of terminally truncated material as in plasma. The third immunoreactive area, however, was at the same position as the calibrator, but the terminal antisera did not identify it as efficiently as the central antiserum. One can assume that this material was due to slightly N- and C-terminally degraded NT-proBNP with hydrophobicity at the level of that of the intact peptide.

Interestingly, HPLC profiles of spiked plasma and serum samples stored at -20°C and freeze-thawed two or three times also showed some heterogeneity. In plasma samples some minor degradation was detected at the N-terminus of NT-proBNP only. The N-terminal truncation was more obvious in serum. Thus it can be concluded that the freeze-thawing of plasma and serum causes at least N-terminal degradation of NT-proBNP. This finding contradicts previous studies which indicated that NT-proBNP remains nearly 100% immunoreactive despite several freeze-thaw cycles (Hunt *et al.* 1997b, Nowatzke & Cole 2003). This contradiction may be due to slight differences in epitope specificities although all these assays utilized N-terminal antisera.

6.5 Applicability of NT-proANP, NT-proBNP and NT-proXNP in cardiac disease

Natriuretic peptides have been shown to be useful in the diagnosis of heart failure (Sagnella 1998, Ruskoaho 2003). Furthermore, high concentrations correlate with poor prognosis in myocardial infarction or heart failure (de Lemos *et al.* 2001, Omland *et al.* 2002, Doust *et al.* 2005). Therefore, the developed immunoassays were applied for cardiac patient samples to confirm that the conclusions on the assays apply both in physiological and pathophysiological situations. In the patients with acute myocardial infarction (AMI), all of our NT-proANP (1-20, 46-79) and NT-proBNP assays (5-24, 10-29, 57-76) were able to identify the postinfarction patients who had reduced cardiac function and heart failure. The fold changes between healthy and AMI subjects found in this study were in accordance with previous studies. Despite the varying absolute levels obtained by different assay methods a 2- to 3-fold increase of NT-proANP (Omland *et al.* 1996, Uusimaa *et al.* 1999, Kettunen *et al.* 1994, Richards *et al.* 1998, White *et al.* 2001) and a 3- to 4-fold increase of NT-proBNP (Omland *et al.* 2002, Luchner *et al.* 2002, Talwar *et al.* 2000, Richards *et al.* 2003, Jernberg *et al.* 2002, Nilsson *et al.* 2002) has been typical in earlier studies as well.

In the patients with vascular heart disease (VHD) the NT-proXNP, NT-proANP$_{46-79}$ and NT-proBNP$_{10-29}$ concentrations were significantly higher than in patients with coronary artery disease (CAD). This is consistent with previous reports according to which ANP concentrations are higher in VHD than in CAD, presumably due to more pronounced pressure overload in the heart (Soos *et al.* 2002), and in which NT-proANP concentrations increase only slightly in CAD (Ärnlöv *et al.* 2000). Acute coronary syndromes cause only moderate increase in BNP and NT-proBNP concentrations compared to that seen in e.g. heart failure (Jesse *et al.* 2003). Altogether, despite the varying absolute levels obtained by different assay methods all of our assays are applicable to clinical samples.

6.6 NT-proANP and NT-proBNP predict the risk of cardiovascular events and mortality

Previous studies have shown that natriuretic peptides predict the presence of heart failure, other cardiovascular diseases, and their prognosis (de Lemos *et al.* 2001, Davis *et al.* 1992). However, the prognostic significance of NT-proANP and NT-proBNP is not well documented in population-based prospective studies. A few studies have suggested that elevated natriuretic peptides may be related to the risk of death and cardiovascular events among elderly subjects from urban population (Wallen *et al.* 1997, McDonagh *et al.* 2001) or apparently asymptomatic subjects (Wang *et al.* 2004, Kistrop *et al.* 2005). In our study the prognostic value of NT-proANP and NT-proBNP was investigated in a population-based sample, and it was found that both peptides were strong predictors of mortality and its co-morbidities in middle-aged men. These results are in accordance with the Framingham study, which has shown the predictive value of BNP and NT-proANP

with respect to cardiovascular events and death (Wang *et al.* 2004). In line with our results, they showed that natriuretic peptides were predictive of atrial fibrillation and heart failure but not coronary heart disease. The lack of an independent association between the natriuretic peptides and acute coronary events may be explained by the fact that BNP and NT-proBNP levels are not related to the formation of unstable plaque in the coronary arteries (Jernberg *et al.* 2004). On the other hand, a recent report from the BELSTRESS study indicated that NT-proBNP predicts coronary events in men at work (De Sutter *et al.* 2005). This contradiction can be due to differences in study populations, length of follow-up, or other factors. Moreover, recent studies have shown that natriuretic peptides are powerful determinants of cardiovascular events in patients with acute coronary syndromes, stable coronary heart disease and cerebrovascular disease (Heeschen *et al.* 2004, Schnabel *et al.* 2005, Cambell *et al.* 2005).

Natriuretic peptides may reflect the functional status of the heart, and elevated levels may be due to a sustained elevation in left ventricle filling pressure also among population-based sample. The activation of the neurohumoral system is a unifying feature in patients at high risk for death after acute coronary syndrome (de Lemos *et al.* 2001). Natriuretic peptide levels increase after myocardial infraction and unstable angina pectoris (James *et al.* 2003, Hunt *et al.* 1997), but only some prospective studies have shown the predictive value of natriuretic peptides for atrial fibrillation in patients with congestive heart failure and coronary heart disease (Yamada *et al.* 2005, Hakala *et al.* 2002). However, it is suggested that among patients with prevalent cardiac disease or lone atrial fibrillation, natriuretic peptides are markers of left atrium and left ventricular stretch and diastolic dysfunction (Schirmer *et al.* 1999, Lubien *et al.* 2002, Ellinor *et al.* 2005). Taken together, the pathophysiological mechanisms and causality responsible for the independent association between natriuretic peptides and mortality and its comorbidities cannot be deduced from the present study. The main conclusion is that NT-proANP and NT-proBNP added to the prognostic value of conventional risk factors, providing a non-invasive measure for identifying men with a high risk.

6.7 Comparison of NT-proANP, NT-proBNP and NT-proXNP as cardiac markers

The quantitative results of our amino-terminal ANP, BNP and XNP assays can be compared because the assays use equivalent recombinant calibrators. In the present study the normal NT-proANP$_{46-79}$ level was about 3-fold higher than the normal NT-proBNP$_{10-29}$ level. This resembles the ratios of biologically active ANP and BNP in circulation (Clerico *et al.* 1998). The amino-terminal counterparts of these peptides have longer half-lives and thus higher concentrations in the blood (Thibault *et al.* 1988, Pemberton *et al.* 2000). The NT-proBNP concentrations in some patients groups with cardiovascular diseases are almost as high as those of NT-proANP, resulting in more pronounced fold changes between normal and patient samples compared to NT-proANP (Sagnella 1998). In the present study this was the case with patients having acute myocardial infarction. They had 4.5 times higher concentrations of NT-proBNP$_{10-29}$ compared to healthy subjects, whereas the increase in NT-proANP$_{46-79}$ was no more than 1.8-fold. However, in

patients with valvular heart disease (VHD) or coronary artery disease (CAD), the fold change compared to healthy individuals was almost similar for NT-proANP and NT-proBNP (2- to 5-fold), but clearly more pronounced for NT-proXNP (3- to 7-fold).

Compared to NT-proBNP, NT-proANP has not received as much attention as a cardiac marker. However, there are several publications in which NT-proANP appears to have clinical utility approaching or even exceeding that of NT-proBNP (Wang *et al.* 2004, Jarai *et al.* 2005, Gegenhuber *et al.* 2006). Our results support these findings, especially in the case of diagnosis of VHD and CAD patients and in risk stratification of middle-aged men. In addition, according to the results of the present study, NT-proANP has the advantage of being a clearly more stable analyte and its assays could be expected to be considerably less prone to preanalytical errors than NT-proBNP. However, our novel NT-proXNP method can combine the advantages of both the N-terminal peptides, making futile the dispute over which one has the higher diagnostic and prognostic value. Furthermore, in the present study it showed diagnostic efficiency that is equal or slightly better compared to NT-proANP and NT-proBNP. It must be noticed, nonetheless, that there might be clinical conditions in which the individual NT-proANP or NT-proBNP value is still a more powerful diagnostic indicator than the combined level.

6.8 Future perspectives

Findings of the present study show that the N- and C-terminal heterogeneity can hamper the measurement of NT-proANP, and to a greater extent that of NT-proBNP. Thus it is essential to evaluate further the actual circulating components and degradation products of these peptides in human plasma and serum, and characterize in detail at the molecular level the stable central epitopes of NT-proANP and NT-proBNP. New insights into biochemistry could pave the way for more sensitive and robust assays that are not sensitive to preanalytical handling. In addition, this study indicated that NT-proANP has diagnostic utility that is equal to or even better than NT-proBNP. Furthermore, it is clearly a more stable analyte compared to NT-proBNP. BNP and NT-proBNP have dominated the diagnostic field of natriuretic peptides because their immaterial property rights are in the possession of large commercial companies. This fact has led to the bias that marketing efforts are merely concentrated on proBNP-derived peptides. Therefore, the diagnostic utility of NT-proANP should be reassessed in large clinical trials, and compared properly to that of BNP and NT-proBNP. Finally, we were able to develop a novel NT-proXNP assay for measurement of both circulating NT-proANP and NT-proBNP. This innovative assay proved to be clinically promising. Its diagnostic utility has to be further ascertained by larger clinical trials together with individual natriuretic peptide assays. The NT-proXNP assay format also needs further refinement.

7 Summary and conclusions

The aim of the present study was to elucidate circulating molecular components of NT-proANP and NT-proBNP in human blood, and to develop reliable and novel assays for their measurement with clinical applications.

1. In order to set up reliable immunoassays for NT-proANP and NT-proBNP recombinant peptides were prepared in bacterial cells and antisera against different epitopes of NT-proANP and NT-proBNP were produced in goats. Several assays for NT-proANP and NT-proBNP with different epitope specificities were developed based on well-characterized antisera and full-length recombinant calibrators. All of the assays fulfilled basic requirements for an immunoassay and were applicable to clinical samples.
2. An immunoassay for measurement of both NT-proANP and NT-proBNP, referred to as NT-proXNP assay, was set up. In this innovative assay format NT-proANP and NT-proBNP peptides present in human blood sample compete with the NT-proXNP tracer for binding to the specific antisera to NT-proANP and NT-proBNP. A single laboratory value reflects the activation or inactivation of the ANP or BNP systems, or both. In patients with valvular heart disease or coronary artery disease NT-proXNP showed diagnostic efficiency that is equal or slightly better compared to individual NT-proANP or NT-proBNP assays.
3. Circulating molecular forms of NT-proANP and NT-proBNP were investigated in blood samples of patients with acute myocardial infarction by chromatographic and immunoassay methods. The results revealed that NT-proANP and especially NT-proBNP are heterogeneous in plasma and serum. They are truncated at both termini causing a serious risk of preanalytical errors. The major part of NT-proANP in human plasma (60-70%), however, is likely due to intact NT-proANP$_{1\text{-}98}$, whereas the majority of NT-proBNP is fragmented into fragments of 6-8.5 kDa (Fig. 8). The heterogeneity is even more pronounced in serum than in plasma.
4. The time course of epitope specific NT-proANP and NT-proBNP truncation was studied in plasma and serum samples. NT-proANP and NT-proBNP immunoreactivities in plasma and serum detected by antisera directed to central epitopes were retained at high level for three weeks even at room temperature, whereas the terminal immunoreactivities decreased rapidly. HPLC analysis of these samples showed at least three separate NT-proBNP$_{10\text{-}29}$ immunoreactive species. In

addition, the freeze-thawing of the samples caused N-terminal truncation of NT-proBNP. Thus the central, but not terminal, epitopes of recombinant NT-proBNP and NT-proANP are stable even at harsh storage conditions. The most reliable assays for NT-proANP and NT-proBNP are directed at the central portions of the molecule only (Fig. 8). Moreover, NT-proANP is a clearly more stable analyte and its assays are less prone to preanalytical errors than those of NT-proBNP.

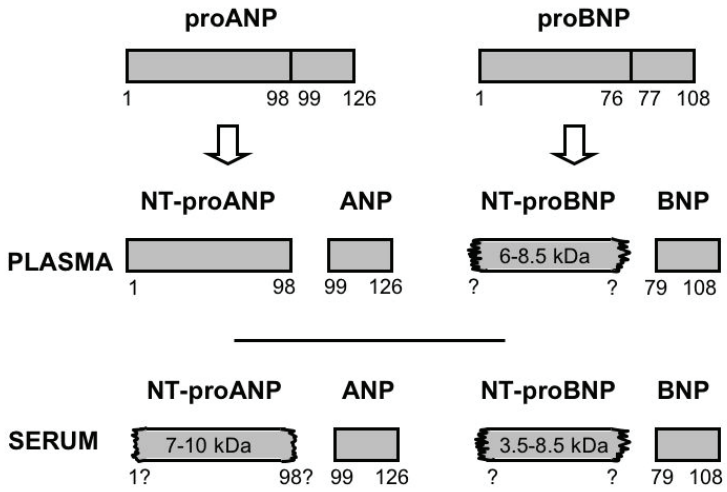

Fig. 8. Postulated molecular forms of NT-proANP and NT-proBNP in human plasma and serum.

5. The prognostic value of NT-proANP and NT-proBNP was investigated in a population-based sample of men. The main finding was that both NT-proANP and NT-proBNP are strong predictors of mortality and its co-morbidities in middle-aged men. They add to the prognostic value of conventional risk factors, providing a non-invasive measure for identifying men with a high risk.

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Original papers

I Ala-Kopsala M, Magga J, Peuhkurinen K, Leipälä J, Ruskoaho H, Leppäluoto J & Vuolteenaho O (2004) Molecular heterogeneity has a major impact on the measurement of circulating N-terminal fragments of A- and B-type natriuretic peptides. Clin Chem 50: 1576-1588.

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III Ala-Kopsala M, Ruskoaho H & Vuolteenaho O (2006) Measurement of circulating N-terminal fragments of A- and B-type natriuretic peptides: the central but not terminal epitopes are stable. Manuscript.

IV Laukkanen J, Sudhir K, Ala-Kopsala M, Vuolteenaho O, Ruskoaho H, Nyyssönen K & Salonen JT (2006) Plasma N-terminal fragments of natriuretic propeptides predict the risk of cardiovascular events and mortality in men. Eur Heart J 27: 1230-1237.

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