

# NT-pro CNP Level Estimation; Its Experimental and Clinical Relevance

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**Abstract:** *C type natriuretic peptide is an important member of the natriuretic peptide family. A-type and B-type natriuretic peptides are established markers in chronic heart failure (HF). C-type natriuretic peptide (CNP) belongs to the same peptide family, but is predominantly localized in the endothelium. The prognostic role of CNP and its N terminal metabolite in various disorders has not been totally established. The widespread increase of CNP secretion in multiple tissues across the brain in response to dexamethasone implicates CNP and the NT-pro CNP in glucocorticoid actions possibly related to inflammation or local fluid dynamics mediated by glial cells. These findings pave the way for future studies designed to establish the role of CNP and NT-pro CNP in the CNS in normal health in vivo, and to establish a clinical application for this peptide in pathophysiological disorders in the CNS. The aim of the study is to evaluate the clinical correlates of the N-terminal part of pro CNP in various disease states and in experimental conditions.*

**Keywords:** CNP, NT-pro CNP, cerebrospinal fluid, vascular, memory, CNS, pituitary

## 1. Introduction

Natriuretic peptides (NPs) are endogenous hormones that are released by the heart in response to myocardial stretch and overload. Several types of NPs can be distinguished; atrial natriuretic peptide (ANP), brain or B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). NPs contain an obligatory C-terminal 17-residue disulphide ring structure (1), of which 11 residues are conserved across the family (Figure 1). These peptides are expressed in a broad range of tissues (2-4), although the main sites of NP production are the atria and ventricles for ANP and BNP, and vasculature and central nervous system for CNP (5). The CNP circulating levels are very low, and the half-life of CNP is short, due to rapid local and systemic degradation by the CNP clearance receptor-B and degradation through the widespread neutral endopeptidases. Gene expression of natriuretic pro peptide C was upregulated in several tissues anterior pituitary gland, posterior pituitary gland, hypothalamus, hippocampus and pons, which is supportive of increased synthesis of CNP following dexamethasone. The pituitary gland anterior and posterior, which is known for its enriched levels of CNP content had a similar concentration of NT proCNP when compared with most tissues sampled from the brain (6). Furthermore, the NT proCNP. CNP concentration ratio in pituitary gland (1:1) differed from brain (5:1 to 10:1), suggesting that little CNP degradation occurs in the pituitary gland however the expression of NPRC did not differ between pituitary and brain tissue (7-8). Gene expression of NPPC was upregulated in both the anterior and posterior pituitary gland following dexamethasone, despite no significant increase in CNP or NTproCNP concentration in these tissues. Studies conducted by this author suggested that CNP plays an important role in memory and cognition and the nuclear factor kappa B was involved in this process of memory and cognition (9). Thus it appears that NT-pro CNP may be possibly a marker for neurodegeneration and memory loss. This article specifically evaluates the role of NT-pro CNP level estimation and its role in various disease states.

## 2. NT-proCNP; a marker of CNP production

CNP has the shortest circulating half life due to its rapid receptor-mediated and enzymatic clearance, and has a half-life (2.6 min) of all the natriuretic peptides in humans (10) and a similarly short half-life (1.6 min) in sheep (11). The short half-life of CNP has made it a particularly difficult peptide to study, as circulating levels may not reflect tissue concentrations close to the site of production. Therefore, NT-proCNP may be considered as a long lived surrogate for CNP, so its levels in body fluids can provide an index of CNP secretion and activity (12). NT-proCNP has been established as a stable product of CNP gene expression and its concentration in plasma is highly correlated with CNP concentration. It is unlikely that NT-proCNP is hydrolysed by neprilysin or cleared from the circulation by NPR-C immediately (13), therefore its prolonged half-life makes it a more reliable indicator of CNP activity *in vivo* than measurement of CNP itself. Different clearance rates explain why the plasma concentration ratio of the two peptides (NT-proCNP:CNP) is consistently high across different species; 31:1 in adult humans (Schouten *et al.* 2011), 26:1 in 4-week-old lambs (14), and 28:1 in adult ewes. This ratio is even higher for CSF concentrations; i.e. 144:1 in human CSF. This may be due to the abundance of neprilysin enzyme (15) and the widespread distribution of NPR-C in the brain and spinal cord tissues which depletes the concentration of CNP relative to NTproCNP. There is one study which reported that the NT-proCNP : CNP ratio in human CSF increased with age.

**Synthesis of CNP and NT-pro CNP:** The C type natriuretic peptide, CNP is synthesized as a 103 amino residue peptide (proCNP 1-103, proCNP) and the sequence is highly conserved in mammals (16). The penultimate 22 amino acids form a ring structure at the carboxy terminal. Pro CNP is cleaved intracellularly by furin 11 yielding a 53-amino acid residue peptide (proCNP 51-103, CNP-53) and an inactive amino-terminal fragment (proCNP 1-50, NTproCNP) which are likely to be secreted in equimolar proportions. CNP-53 is

further cleaved – presumably extracellularly and at unknown sites to CNP-22 (proCNP 82-103) which retains the ring structure and full biological activity (17). Both biologically active forms (CNP-53 and CNP-22) are subject to rapid degradation at source whereas the inactive fragment NT-proCNP remains intact for longer periods and is detectable in plasma prior to its clearance by renal filtration and excretion.

### 3. Estimation of CNP and NT-pro CNP

The technique used was as described by earlier studies (27). Blood samples are collected using the 7.5 ml S-MonovetteW collection system (Sarstedt, Nümbrecht, Germany) containing either clotting activator for serum samples, sodium-citrate for plasma samples or Potassium-EDTA for full blood samples. All samples for baseline analyses are immediately centrifuged at  $2,000 \times g$  for 10 min at  $4^{\circ}\text{C}$  and supernatant is stored at  $-70^{\circ}\text{C}$  until analysis. For midterm analyses (30 minutes or 2 hours), plasma samples were centrifuged at  $2,000 \times g$  for 10 min at room temperature. Supernatant is removed and stored at room temperature for 30 min or 2 hours. Following blood clotting, serum samples were processed identically to plasma samples. Supernatant is separated and also stored at room temperature for 30 min or 2 hours. Full blood samples are stored at room temperature without centrifugation. After 30 min or 2 hours, full blood samples are centrifuged at  $2,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . Supernatant is removed and stored at  $-70^{\circ}\text{C}$  until analysis. The concentration of CNP are measured using the CNP-22 EIA Kit (Phoenix Pharmaceuticals, Karlsruhe, Germany) according to the manufacturer's protocol. NT-proCNP concentration is measured using the NT-proCNP EIA Kit (Biomedica, Vienna, Austria) according to the manufacturer's protocol. One-way ANOVA can be used for comparison between groups. For pairwise comparisons, the t-test was applied. Data are shown as means  $\pm$  standard deviation (SD) or 95% confidence interval. Statistical significance was at  $p < 0.05$  values. Data were analyzed using Med Calc W for Windows, Version 10.0.1.0 (Med Calc Software, Mariakerke, Belgium).

### 4. Importance of CNP in the Central Nervous System:

Research findings have shown that CNP increases the permeability of the cerebrovascular endothelium raise the possibility that systemic concentrations of the peptide may access the central circulation and contribute to concentrations of CNP in CSF (18, 19). This has not been studied previously but is an important issue as intraventricular administration of CNP affects food intake and energy regulation in rodents and lowers systemic blood pressure in sheep. In healthy adults, plasma CNP concentrations are low ( $< 1-2$  pmol/L), less than 30 % of CSF levels, making it unlikely that systemic CNP contributes significantly to CSF levels of CNP. Furthermore, more questions have been raised that are particularly important to resolve given the high amount of glucocorticoid use, the known detrimental effects of chronic glucocorticoid exposure on brain function including learning and memory (20). Thus it can be implicated with CNP, and the untapped

potential of CNP as a diagnostic and/or therapeutic target for cognitive impairment due to pathophysiological disorders of the CNS (21). Dexamethasone can be used as a 'tool' to answer these new fundamental questions which include: Which cell type(s) secretes CNP in response to dexamethasone stimulation and which of the signalling pathways induce dexamethasone-induced CNP secretion in the brain have to specifically delineated. Can the dexamethasone-induced increase in CNP concentration be attenuated. What are the downstream effects of such widespread increases in CNP secretion in the brain. The ultimate purpose for answering these questions is to determine whether there are neuropathological states that this is relevant to, and to establish how these new findings can be applied in clinical settings (22).

### 5. NT-pro CNP and Vascular Endothelial Growth Factor

Interestingly, we observed a strong negative correlation between vascular endothelial growth factor, VEGF and NT-proCNP (23). The exact role of VEGF in heart failure, HF is unknown. A Japanese study reported that VEGF levels are lower in HF patients compared with controls, and a large community-based study showed a clear positive correlation between serum VEGF levels and mean arterial pressure (24). There is strong evidence that microvascular dysfunction plays a role in HF. However, it is unknown whether VEGF is merely a marker of vascular disease or is being produced in response to increased haemodynamic load. A study with infarcted swine hearts with a preserved EF showed that increased fibrosis, metalloproteases, and capillary density were localized to the infarct border zone and were associated with increased expression of CNP (25). Interestingly, high concentrations of both CNP and VEGF-A were associated with a vasculogenic response. Table 1 depicts the levels of CNP analogues in rabbit plasma samples.

### 6. Clinical relevance in Heart Failure; Recent Evidence:

Heart failure is a chronic and deadly disorder. Levels of CNP and NT-proCNP are locally produced in order to counteract the activated renin-angiotensin system in the vascular tissue and ultimately cause smooth muscle cell relaxation and a drop in intravascular pressure, an effect elicited by local production and secretion of nitric oxide (26). Although speculative, these normal physiological steps could be less accurate or effective in patients with HFpEF and not in those with HFrEF. This altered response and subsequently higher levels of NT-proCNP might explain why higher NT-proCNP levels are more predictive in HFpEF compared with levels of NT-proBNP (27).

### 7. Sexual dimorphism and body mass index and levels of NT-pro CNP:

There is now more focus on the research on NT-pro CNP levels. The relationship between levels of plasma NT-proCNP and sex or parameters of renal function (e.g. creatinine) has been described in a recent study by Prickett and colleagues (12,28). In 257 healthy adults without

cardiovascular or renal disease, the researchers observed significantly lower levels of NT-proCNP in women as compared with men. In addition, multivariate linear regression revealed creatinine, height, and sex to remain independently associated with circulating levels of plasma NT-proCNP. This was not the case for NT-proBNP concentrations. Finally, a negative association between NT-proCNP and BMI was observed in univariate regression analysis. However, this significance was lost in multivariate analysis. A recent experimental research study with obese Zucker rats also showed a negative correlation between obesity and levels of NPs, although this observation was not confirmed in human adults (29). Still, the relationship between BMI and levels of NT-proCNP does merit further investigation.

## 8. NT-proCNP and evaluation of neurodegeneration and memory

CNP is produced mainly by endothelial cells, acts via the paracrine mechanism, mediates its actions through the natriuretic peptide receptor (NPR)-B and NPR-C, and is degraded by neutral endopeptidase (NEP) and clearance receptor (NPRC) binding. The widespread distribution of CNP in the rat and human brain and high concentration in cerebrospinal fluid (CSF), which exceeds that of other natriuretic peptides and its own concentration in the plasma (30-31), are suggestive of a homeostatic role in the brain. Infact, *in vitro* studies have shown a role for CNP in several neuroregulatory processes, including nervous system development (32) and neuroprotection. Schouten *et al.* (13) reported concurrent measurements of CNP and the stable amino-terminal fragment of proCNP (NTproCNP), where it was reported that CSF and plasma concentrations of the two peptides were independently regulated suggestive that central and peripheral sources of CNP were separate. For example, the high expression in the rodent brainstem indicates an involvement of CNP in autonomic control and its expression in the rat hippocampus suggests a role in learning and memory. CNP affects bidirectional plasticity in the hippocampus, by influencing hippocampal network oscillations in adult rats which raises the question as to whether CNP is involved with learning and memory (33). In support of this, central administration of CNP in rats has been shown to facilitate learning and consolidation of passive avoidance learning. Aside from memory and learning, CNP is involved with other neuroregulatory

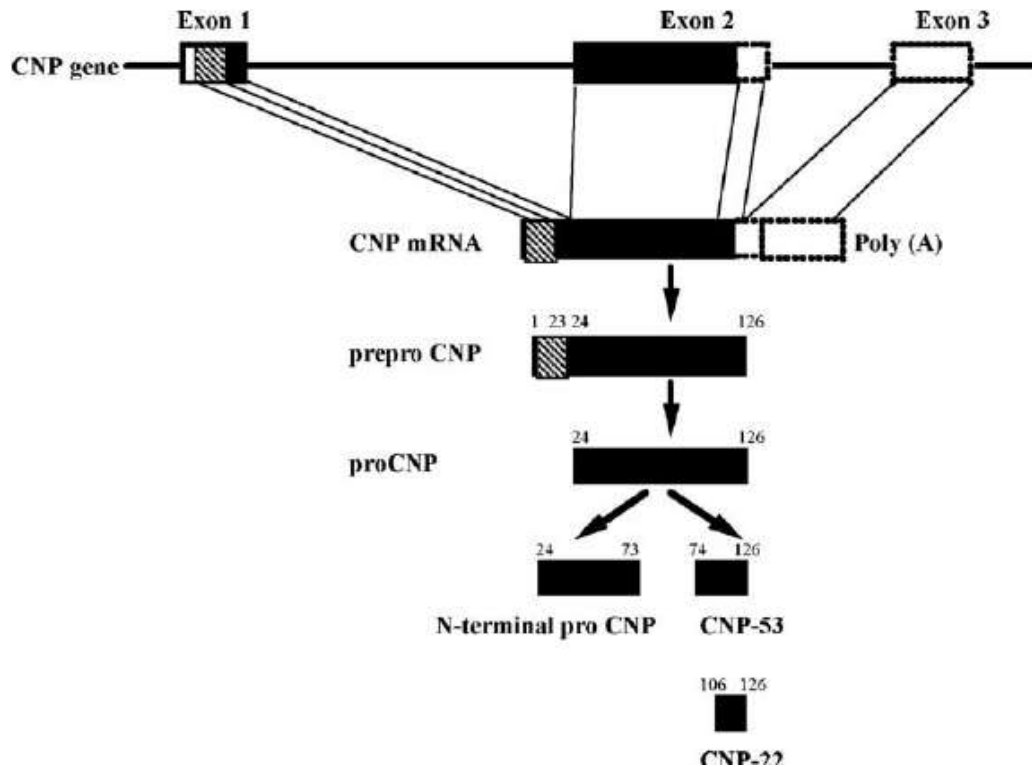
processes such as nervous system development (34). CNP stimulates axonal branch formation of DRG neurons in the spinal cord, and provides a cue that is necessary for bifurcation of central sensory afferents (35). In cultures of embryonic DRG neurons, CNP stimulates branch formation, induces axonal growth, and attracts growth cones. In addition, NPR-B is the most highly expressed natriuretic peptide receptor in the developing rat brain, and its expression is maximal around the first postnatal day of life, which coincides with an elevated expression of nestin a marker protein for stem/progenitor cells which indicates a role for NPR-B in perinatal neurogenesis. Other suggested roles include neuroprotection (6), regulation of blood-brain barrier permeability (36), involvement in pathways underlying pain hypersensitivity, and regulation of cocaine-induced changes in gene expression responsible for neuronal degenerative alterations.

## 9. Conclusion

C type natriuretic peptide, CNP has been shown to have anti-atherogenic actions and has been implicated in the pathophysiology of intimal plaque formation and vascular remodeling. The stable metabolite of CNP i.e NT-pro CNP is much more stable and is a prospective candidate for estimating the disease states. This peptide inhibits neointima formation in experimental models of atherosclerosis. CNP is expressed in human coronary arteries and has been shown to inhibit neointimal growth after percutaneous intervention in humans. Other supporting evidence for the anti-atherogenic actions of CNP include suppression of the expression of vascular adhesion molecules, suppression of plasminogen activator inhibitor-1 activity, and inhibition of the oxidized low-density lipoprotein (LDL)-induced migration of human coronary artery smooth-muscle cells in a concentration-dependent manner. Although it is presently a reasonable prospect, modulation of the CNP/GC-B system may represent a logical molecular target and this can be evaluated by measuring the NT-pro CNP levels in various tissues and body fluids..

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**Figure 1:** CNP and NT-pro CNP synthesis. (Courtesy: Toshio Nishikimi (MD, PhD, FJCC) , Koichiro Kuwahara (MD, PhD),Kazuwa Nakao (MD, PhD, Journal of Cardiology (2011) 57, 131—140)

**Table 1:** Plasma levels of NT-pro CNP, CNP-53 and CNP-22 per (n=5) from rabbits.

Protocol #	Treatment	NT-pro CNP pmol/L	CNP-53 pmol/L	CNP-22 pmol/L
1)	Saline (0.85%)	81.1 pmol/L	59.33 pmol/L	0.21 pmol/L
2)	Prednisolone (2.0 mg/kg i.m)	113.4 pmol/L	53.13 pmol/L	2.21 pmol/L
3)	Glibenclamide (2.5 mg/kg iv)	64.55 pmol/L	51.1 pmol/L	1.19 pmol/L

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