



Post-translational modifications enhance NT-proBNP and BNP production in acute decompensated heart failure

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Background

Increases in plasma B-type natriuretic peptide (BNP) concentrations in those with acutely decompensated heart failure (ADHF) has been mainly attributed to an increase in *NPPB* gene transcription. Recently, proBNP glycosylation has emerged as a potential regulatory mechanism in the production of amino-terminal (NT)-proBNP and BNP. The aim of the present study was to investigate proBNP glycosylation, and corin and furin activities in ADHF patients.

Methods and results

Plasma levels of proBNP, NT-proBNP, BNP, as well as corin and furin concentration and activity were measured in a large cohort of 683 patients presenting with ADHF ($n = 468$), non-cardiac dyspnoea (non-ADHF: $n = 169$) and 46 patients with stable chronic heart failure (CHF); the degree of plasma proBNP glycosylation was assessed in a subset of these patients (ADHF: $n = 49$, non-ADHF: $n = 50$, CHF: $n = 46$). Our results showed a decrease in proBNP glycosylation in ADHF patients that paralleled NT-proBNP overproduction ($\rho = -0.62$, $P < 0.001$) but less so to BNP. In addition, we observed an increase in furin activity that is positively related to the plasma levels of proBNP, NT-proBNP and BNP overproduction (all $P < 0.001$, all $\rho > 0.88$), and negatively related to the degree of proBNP glycosylation ($\rho = -0.62$, $P < 0.001$).

Conclusion

These comprehensive results provide a paradigm for the post-translational modification of natriuretic peptides in ADHF: as proBNP glycosylation decreases, furin activity increases. This synergistically amplifies the processing of proBNP into BNP and NT-proBNP.

Clinical Trial Registration

<http://clinicaltrials.gov/>. Identifier: NCT01374880.

Keywords

Acute decompensated heart failure • ProBNP • BNP • NT-proBNP • Glycosylation • Furin activity

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Introduction

Plasma levels of B-type natriuretic peptide (BNP) and amino-terminal proBNP (NT-proBNP) are established biomarkers for the diagnosis of acutely decompensated heart failure (ADHF). Both peptides are rapidly released in the plasma of ADHF patients at the onset of the acute episode, and both are routinely used to discriminate dyspnoea of cardiac (ADHF) from non-cardiac origins (non-ADHF), according to international guidelines.^{1,2} Furthermore, in addition to their diagnostic value, elevated plasma levels of either BNP or NT-proBNP are predictive of adverse prognosis in HF³ and both have been used as a guide to HF therapy in stable chronic HF (CHF) patients.⁴

Upon mechanical cardiac stress with stretching of cardiomyocytes as occurs with ventricular overload or increase in wall tension, *NPPB* gene transcription is induced resulting in an increase in the production of the proBNP hormone precursor (pre-proBNP).⁵ After excision of the signal peptide and trafficking through the general secretory system, the remaining 108 amino acid protein, proBNP, is further cleaved between the Arginine 76 and the Serine 77 by the corin or the furin proteases into the bioactive BNP_{1–32} and biologically inactive NT-proBNP_{1–76}.⁶ It was recently shown that the proBNP is mainly secreted as an O-glycosylated protein.⁷ Among the seven glycosylated amino acids in proBNP that have been identified,⁸ the glycosylation at the Threonine 71 (T71) was shown to prevent proBNP cleavage by corin and furin into NT-proBNP and BNP.^{6,9}

It was previously postulated that NT-proBNP and BNP were the major secreted forms of B-type natriuretic peptides.¹⁰ However, this model was recently challenged, as human cardiomyocytes in primary culture mainly secreted glycosylated proBNP and very little bioactive BNP.^{6,7,9} These *in vitro* data support a model (Figure 1) whereby, under normal conditions, cardiomyocytes mainly secrete glycosylated proBNP that is resistant to processing by corin¹¹ and/or furin,¹² while non-glycosylated proBNP is processed by these enzymes to produce NT-proBNP and BNP.⁹ In humans with CHF, ~70% of circulating proBNP is glycosylated at T71.⁶ Considerable uncertainty about how BNP and NT-proBNP are 'liberated' from proBNP remains.

In this context, we hypothesized that the rapid overproduction of B-type natriuretic peptides in the plasma of ADHF patients is associated with post-translational modifications to facilitate cleavage of the parent proBNP peptide. The aim of the present study was therefore to investigate proBNP glycosylation and corin and furin activities in ADHF patients.

Materials and methods

Study population

The study population ($n = 683$) has been previously described,³ and consisted of 468 patients suffering from ADHF (*de novo* ADHF: $n = 224$; decompensation of CHF: $n = 244$), 169 suffering from non-cardiac

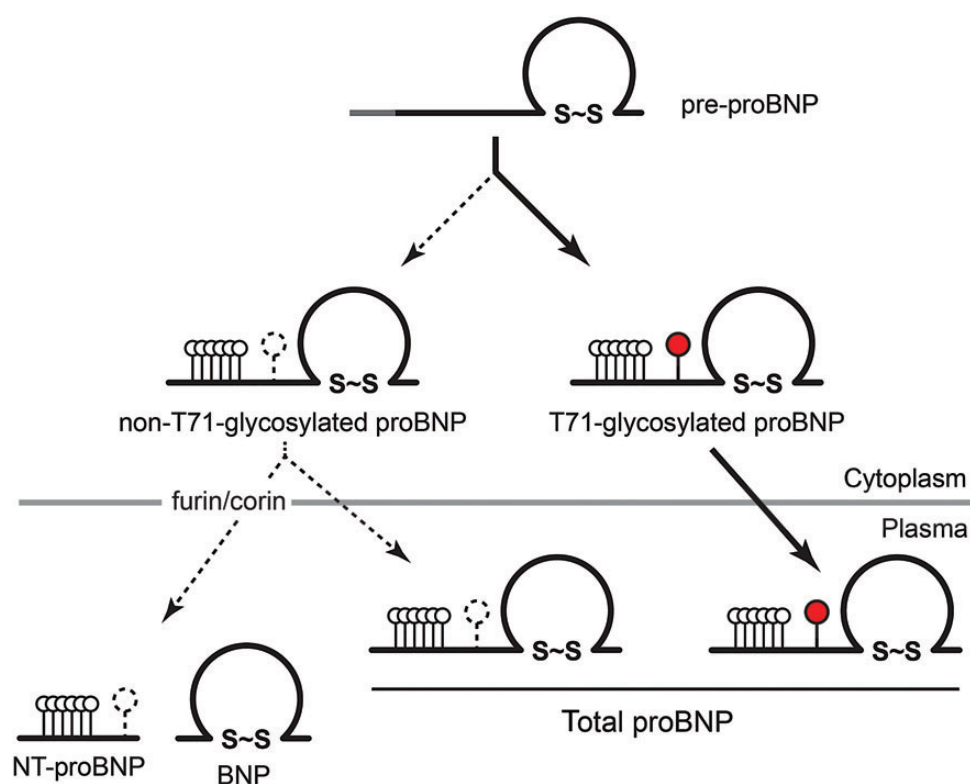


Figure 1 Schematic representation of the production and processing of the proBNP with respect to glycosylation at Threonine 71 (T71). Under normal or stable condition,^{6,7,26} most of the proBNP is secreted as a glycoprotein that is resistant to furin/corin processing. S~S, disulfide bonds; red circle, glycosylation at T71; white circle with dotted line, absence of glycosylation at T71; small solid-lined circles, other glycosylation sites than T71; proBNP, proB-type natriuretic peptide.

dyspnoea as controls (non-ADHF), and 46 patients with CHF (Supplementary material online, Table S1). The diagnosis of cardiac or non-cardiac dyspnoea was performed by the emergency physicians on clinical examination and based on BNP concentrations obtained at admission in the emergency department according to the current guidelines.¹ This study was approved by local ethics committees and written consent was obtained from patients or next of kin. This study using human samples was performed according to the current revision of the Helsinki Declaration and registered at clinicaltrials.gov under the NCT01374880 identifier. All the biomarkers studied were assayed in the entire cohort, except the degree of proBNP glycosylation at T71 that was assessed on a subset of 49 ADHF and 50 non-ADHF patients with characteristics representative of the entire cohort (Supplementary material online, Table S2). The degree of proBNP glycosylation was also assayed for the 46 CHF patients. Difference in patient numbers between this study and that of Seronde *et al.*³ was related to technical factors.

Natriuretic peptide measurements

All biomarker measurements in this study were performed on a single plasma EDTA aliquot. A maximum of one thaw/freezing cycle was permitted prior to biomarker measurements. ProBNP was measured with the monoclonal antibody mAb Hinge 76 from Bio-Rad, which binds the amino acids overlapping the cleavage site of proBNP (Arg76-Ser77), an epitope present only in the precursor form.¹³ NT-proBNP was measured using the Elecsys[®] proBNP II kit on a Roche Cobas analyser (Roche, Basel, Switzerland). B-type natriuretic peptide was measured using the BNP ARCHITECT assay on an Architect i2000 (Abbott Diagnostics, Abbott Park, IL, USA).

proB-type natriuretic peptide T71 O-glycosylation

Prior to tryptic mapping, each plasma sample was digested with either neuraminidase (NANase III) or O-glycosidase (both from Sigma or EMD Biosciences, Inc., San Diego, CA, USA) or neuraminidase alone in 250 mM sodium phosphate buffer, pH 6 at 37°C. Concentrated buffer was added to achieve a final concentration of 50 mM Tris-HCl, pH 8, and 1 µg trypsin was added. Digestion was allowed to proceed overnight at room temperature. Separation of the proBNP proteolytic fragments and characterization of O-glycosylated sites was performed by online nano-liquid chromatography coupled to electrospray ionization-linear ion trap-Fourier transform mass spectrometry in an LTQ-Orbitrap XL hybrid spectrometer (Thermo Scientific) equipped for electron transfer dissociation for peptide sequence analysis by MS/MS with retention of glycan site-specific fragments.¹⁴ Samples were dissolved in methanol/water (1:1) containing 1% formic acid and introduced by direct infusion via a TriVersa NanoMate ESI-Chip interface (Advion BioSystems, Ithaca, NY, USA) at a flow rate of 100 nL/min and 1.4 kV spray voltage. Mass spectra were acquired in a positive ion FT mode using parameters similar to previous studies⁹ except at a nominal resolving power of either 30 000 or 60 000. Electron transfer dissociation-MS/MS spectra were analysed by comparison with theoretical *c* and *z*. Fragment *m/z* values calculated for all positional combinations of one HexNAc residue distributed on the all potential S and T glycosylation sites in the sequence. Calculations were performed using the web-based Protein Prospector MS-Product software routine. The degree of plasma proBNP glycosylation is expressed as the glycosylated proBNP/total proBNP as measured by mass spectrometry (see Figure 1).

Furin and corin measurements

Plasma concentrations of corin protein were measured by the enzyme-linked immunosorbent assay (ELISA) according to Dong *et al.*¹¹ A

cell-based assay was used to examine corin-mediated proANP processing, as described previously.^{15,16} Briefly, recombinant proANP was expressed with a C-terminal V5 tag in HEK293 cells in serum-free-conditioned medium, lyophilized and dissolved in sterile PBS. Plasma samples were combined with pro-ANP medium containing 1 mM EDTA, 20 mM CaCl₂, 50 µM soybean trypsin inhibitor, 0.1% Triton X100, and incubated for 5.5 h at 37°C. The pH of the reaction mixture was adjusted by 3 M Tris-HCl, pH 8. ProANP and its derivatives were analysed by immunoprecipitation using a mouse monoclonal anti-V5 tag antibody (Invitrogen, Carlsbad, CA, USA) coupled to protein A-Sepharose (Pierce, Rockford, IL, USA). Immunoprecipitated proteins were solubilized in SDS-PAGE sample buffer and analysed by Western blotting under reducing conditions with an anti-V5 tag rabbit polyclonal antibody (Immunology Consultants Laboratory, Inc., Newberg, OR, USA) followed by incubation with an alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse antibody, and detection by ECF substrate (Amersham Biosciences, Piscataway, NJ, USA). On Western blots, recombinant human pro-ANP and ANP with the V5 tag appeared as bands of ~23 and ~11 kDa, respectively.

Plasma concentrations of furin protein were measured by ELISA (R&D Systems, DY1503, Abingdon, UK). Furin activity was measured using the fluorescence plate reader Safire 2 (Tecan, Männedorf, Switzerland) and the universal proprotein convertase substrate, the fluorogenic peptide pyroglutamyl-Arg-Thr-Lys-Arg-AMC (Bachem, Switzerland) in 100 mM HEPES buffer, pH 7, containing 0.2% Triton X-100, 2 mM CaCl₂, 0.02% sodium azide, and 1 mg/mL Bovine Serum Albumin, as described previously.¹⁷ The furin inhibitor nona-D-arginine (10 nM final concentration) was used to assess the specificity of the activity.

Statistics

Data are expressed as the median (inter-quartile range) or number and percentage as appropriate. Groups were compared with an independent sample *t*-test, Wilcoxon rank-sum test, χ^2 , or Fisher exact test as appropriate. Multiple groups were compared using the Kruskal-Wallis test, followed by the pairwise Wilcoxon rank-sum test. *P*-values for multiple comparisons were adjusted using Holm's method. The relationship of the three NPs with T71 proBNP glycosylation, furin, and corin concentration or activity was assessed using Spearman correlation coefficient. Statistical analyses were performed using the R-statistical software (<http://www.r-project.org/>). A two-sided *P*-value < 0.05 was considered statistically significant.

Results

All measured plasma natriuretic peptides were markedly elevated in ADHF patients at admission (Table 1) to well above levels measured in patients admitted for non-cardiac dyspnoea (non-ADHF) or in CHF patients (*P* < 0.001 for the three natriuretic peptides, Table 1).

Degree of proB-type natriuretic peptide glycosylation in the plasma of studied patients

The degree of proBNP glycosylation was lower in ADHF (20%) and non-ADHF (19%) than in CHF (31%, *P* < 0.001 vs. ADHF and non-ADHF; Table 1). Furthermore, we observed a strong negative correlation between the degree of proBNP glycosylation and plasma concentrations of NT-proBNP in both ADHF ($\rho = -0.62$, *P* < 0.001) and non-ADHF patients ($\rho = -0.62$, *P* < 0.001; Figure 2A): the lower the degree of proBNP glycosylation, the higher the plasma concentration of NT-proBNP. Figure 2A further shows

Table 1 Values of the biochemical variables measured in this work

	ADHF (n = 468)	non-ADHF (n = 169)	CHF (n = 46)	P-value
proBNP ^a	1339 (748, 2446)	96 (48, 258)	448 (101, 954)	<0.001
NT-proBNP ^a	5682 (1728, 11 020)	228 (79, 797)	1314 (159, 5302)	<0.001
BNP ^a	919 (480, 1806)	72 (30, 127)	210 (48, 469)	<0.001
Furin conc. ^a	19 (16, 24)	19 (16, 26)	19 (17, 23)	0.57
Furin act. ^b	3.8 (3.5, 4.3)	2.8 (2.4, 3.1)	3.3 (2.6, 3.6)	<0.001
Corin conc. ^a	458 (359, 563)	444 (315, 544)	473.5 (391, 546)	0.47
Corin act. ^c	1 (0.6, 1.3)	1.1 (0.7, 1.4)	0.9 (0.63, 1.3)	0.09
	n = 49	n = 50	n = 46	
proBNP glyc. ^d	20 (11,27)	19 (12.5,31)	31 (26.6, 45.7)	<0.001

^apg/mL.
^bpmol/mL/min.
^c% cleaved proANP.
^d% of proBNP glycosylation assayed by mass spectrometry.

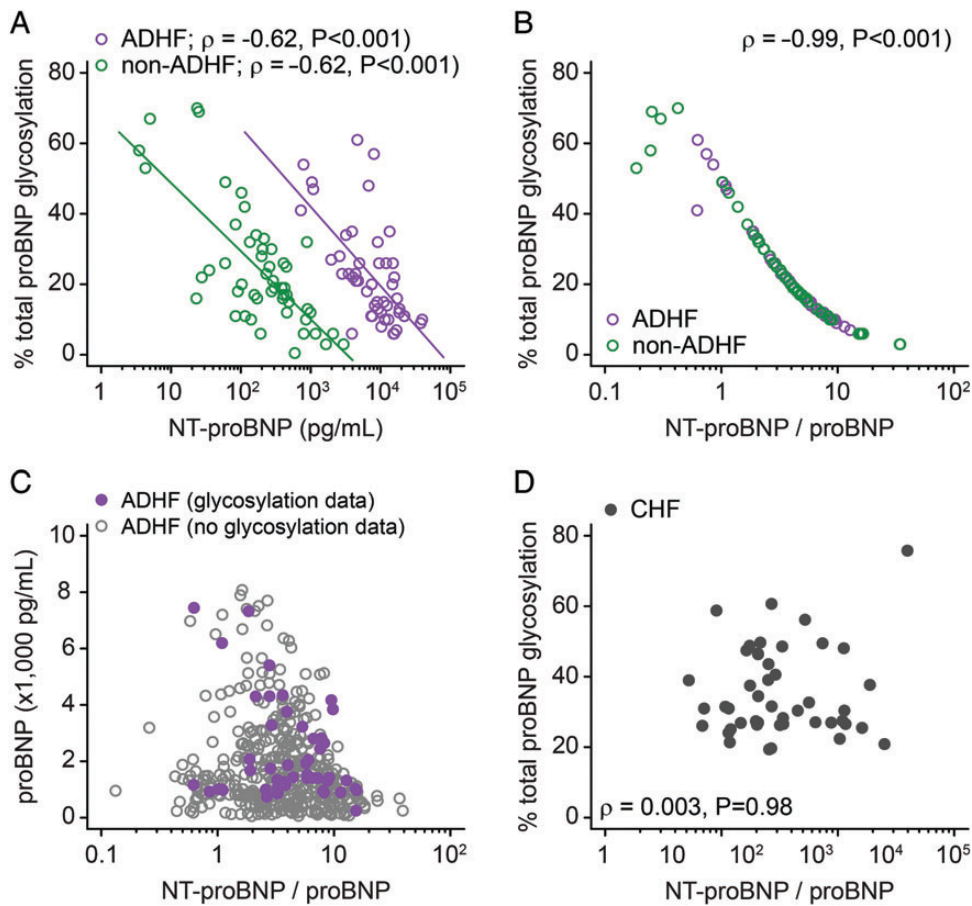


Figure 2 (A) Distribution of NT-proBNP plasma concentrations according to the degree of proBNP glycosylation expressed as the % of glycosylated proBNP/total proBNP measured by mass spectrometry in ADHF (n = 49, open purple circle) and non-ADHF (n = 50, open green circle) patients. (B) Distribution of the NT-proBNP/total proBNP ratio as an estimator of proBNP processing according to the level of proBNP glycosylation in ADHF and non-ADHF patients. (C) Distribution of the NT6proBNP/total proBNP ratio according to the plasma level of total proBNP in ADHF with (n = 49, purple circle) and without (n = 423, open grey circle) available proBNP glycosylation data. (D) Distribution of the NT-proBNP/total proBNP ratio according to the level of proBNP glycosylation in CHF patients (n = 46). ADHF, acutely decompensated heart failure; non-ADHF, non-acutely decompensated heart failure; CHF, chronic heart failure.

that the relationship was shifted to the right in ADHF patients compared with non-ADHF patients.

We next evaluated the impact of the degree of proBNP glycosylation on the rate of proBNP processing as estimated by the NT-proBNP/total proBNP ratio. We found that proBNP processing correlated extremely closely with the degree of proBNP glycosylation ($\rho = -0.99$, $P < 0.001$; Figure 2B); the lower the degree of proBNP glycosylation, the higher the degree of proBNP processing in both ADHF and non-ADHF patients. We extended this analysis to the ADHF patients for which the degree of proBNP glycosylation data were not available. We observed that the distribution of the proBNP processing rate as a function of the total proBNP was comparable in both sets of ADHF patients with ($n = 49$) or without ($n = 419$) available glycosylation data (Figure 2C).

In contrast to what we observed for NT-proBNP, no or moderate correlation was found between the degree of proBNP glycosylation and (i) the plasma concentrations of BNP (ADHF: $\rho = -0.09$, $P = 0.53$; non-ADHF: $\rho = -0.36$, $P = 0.008$; Supplementary material online, Figure S1A) or (ii) the proBNP processing estimated by the BNP/total proBNP ratio ($\rho = -0.52$, $P < 0.001$; Supplementary material online, Figure S1B). Furthermore, in CHF patients, there was no relationship between the degree of glycosylation of the proBNP and (i) NT-proBNP concentrations ($\rho = 0.09$, $P = 0.55$, Supplementary material online, Figure S1C), (ii) NT-proBNP/proBNP ratio ($\rho = 0.003$, $P = 0.98$, Figure 2D), (iii) BNP concentrations ($\rho = 0.12$, $P = 0.41$, Supplementary material online, Figure S1D), or (iv) BNP/proBNP ratio ($\rho = 0.08$, $P = 0.57$, Supplementary material online, Figure S1E).

There was no correlation between serum creatinine and the plasma levels of proBNP, NT-proBNP, the processing of proBNP, or the degree of proBNP glycosylation (Supplementary material online, Figure S2). Also, there was no relationship between the level of proBNP glycosylation, medications at admission, LVEF, or history of atrial fibrillation (data not shown).

Plasma concentration and activity of furin and corin

Plasma furin activity was the highest in ADHF patients among the three studied groups ($P < 0.001$ for all comparisons; Table 1 and Figure 3A). We observed strong positive correlations between circulating furin activity and plasma levels of total proBNP ($\rho = 0.82$, $P < 0.001$; Figure 3B), NT-proBNP ($\rho = 0.99$, $P < 0.001$; Figure 3C) and BNP ($\rho = 0.86$, $P < 0.001$; Figure 3D) over the entire study population. Activity of circulating furin was also negatively correlated with the degree of proBNP glycosylation in ADHF and non-ADHF patients ($\rho = -0.62$, $P < 0.001$; $\rho = -0.62$, $P < 0.001$, respectively; Figure 3E) but not in CHF patients ($\rho = 0.09$, $P = 0.54$; Figure 3F).

In contrast, no difference in furin concentration among the three groups was seen (Table 1 and Supplementary material online, Figure S3A). No correlation was found between furin concentration and (i) plasma levels of proBNP, (ii) NT-proBNP, (iii) BNP (Supplementary material online, Figure S3B–D, respectively), (iv) the processing of proBNP (NT-proBNP/proBNP and BNP/proBNP, Supplementary material online, Figure S4E and F, respectively), or (v) the degree of proBNP glycosylation (Supplementary material online, Figure S3G).

We also examined the circulating concentration (Supplementary material online, Figure S4) and activity (Supplementary material online, Figure S5) of the second proBNP convertase, corin. No

difference in corin concentration (Table 1 and Supplementary material online, Figure S4A) or activity (Table 1 and Supplementary material online, Figure S5A) was seen among studied groups. No correlation was found between corin concentration or activity and the plasma levels of proBNP, NT-proBNP, or BNP (Supplementary material online, Figure S4B–D and S5B–D, respectively), the processing of proBNP (Supplementary material online, Figure S4E and F and S5E and F respectively), or the degree of proBNP glycosylation (Supplementary material online, Figure S4G and S5G, respectively).

Discussion

Our study demonstrates two major post-translational modifications are pivotal to the increase in circulating B type natriuretic peptides (i.e. BNP and NT-proBNP) in those with ADHF: (i) reduction in proBNP glycosylation and (ii) increased furin activity. We found interesting differences between NT-proBNP and BNP relative to proBNP glycosylation, and also note that neither concentrations of furin/corin nor corin activity directly determine cleavage of proBNP. These results clarify the regulatory mechanisms through which plasma B type natriuretic peptides increase in acute conditions.

In ADHF patients, we showed that the degree of plasma proBNP glycosylation was reduced, compared with CHF patients. Furthermore, the degree of proBNP glycosylation was strongly inversely correlated to NT-proBNP plasma concentrations, and to the rate of proBNP processing (i.e. NT-pro-BNP/total proBNP ratio). Accordingly, the lower the degree of proBNP glycosylation, the higher the processing of proBNP and the plasma level of NT-proBNP. In parallel, we observed that circulating furin activity was increased in ADHF patients, and was highly correlated with plasma levels of NT-proBNP. Taken together, our results support a model (Figure 4) whereby in ADHF, besides the increase in *NPPB* gene expression, the decrease in proBNP glycosylation and the increase in circulating furin activity synergistically amplify the proBNP processing and hence the production of BNP and NT-proBNP. It is possible the factors (e.g. cardiomyocyte stretching or humoral factors) involved in the increase in *NPPB* transcription may also trigger these post-translational events.

In acute dyspnoea of non-cardiac origin (non-ADHF, $n = 169$), some cases displayed increased plasma levels of NT-proBNP (although at lower levels than seen in ADHF patients), likely in response to inflammation or infection rather than primarily due to myocardial dysfunction.^{18–21} Nevertheless, in those patients, the degree of proBNP glycosylation was similar to that of ADHF patients, and we observed the same correlation between the degree of proBNP glycosylation, plasma NT-proBNP and the NT-proBNP/total proBNP ratio as in ADHF patients. Likewise, plasma NT-proBNP level was highly correlated to circulating furin activity in non-ADHF patients. The results obtained in both ADHF and non-cardiac patients markedly contrast with those observed in CHF patients. Taken together, these data suggest that the post-translational cascade we describe above likely occurs in any case of acute overproduction of proBNP, rather than in more chronic disease.

Contrary to NT-proBNP, plasma levels of BNP were only moderately related to the degree of proBNP glycosylation and processing. Multiple non-exclusive hypotheses may account for the differences observed between BNP and NT-proBNP. Firstly, BNP assays have been reported to cross-react with proBNP up to 50%^{22,23} while

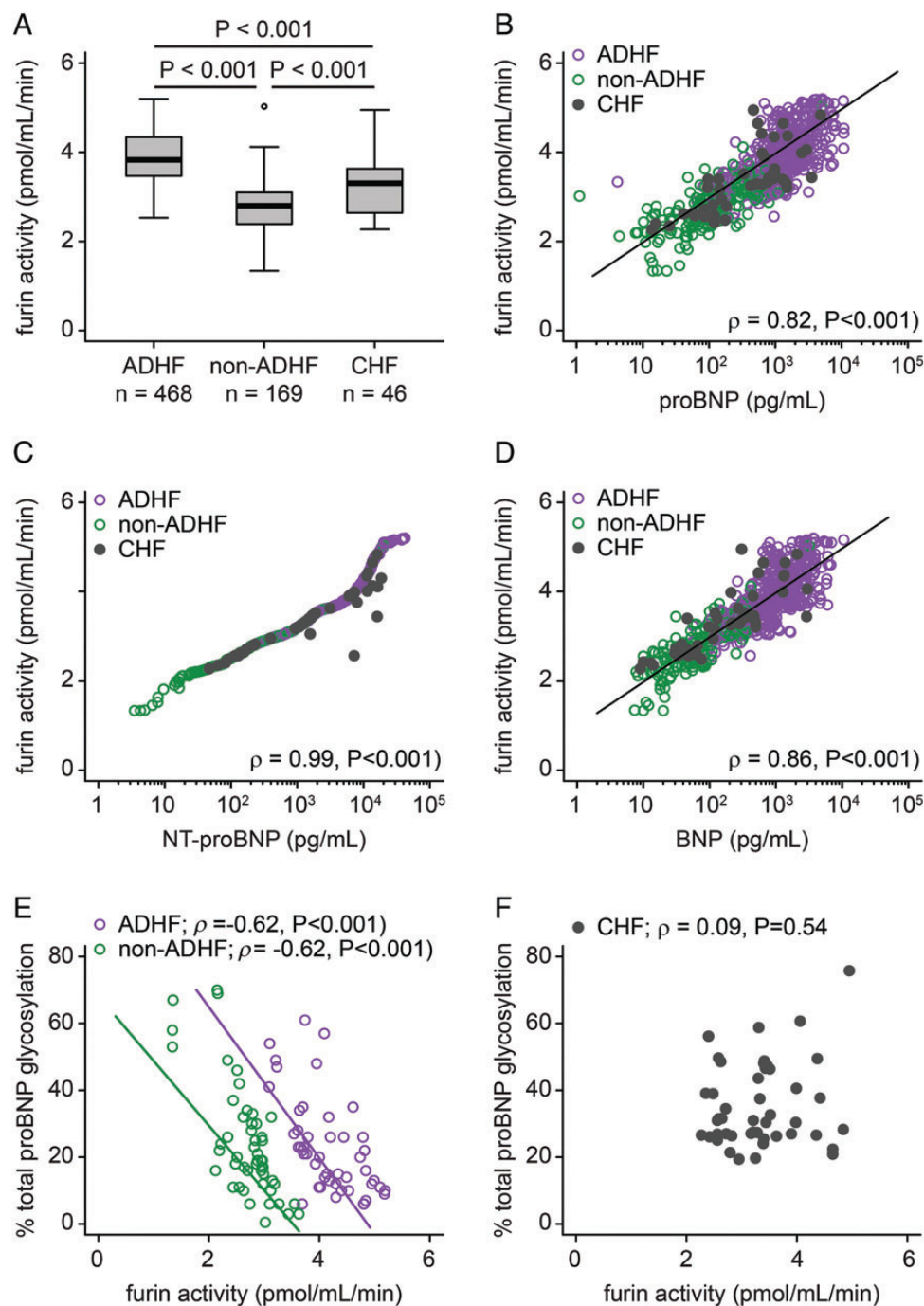


Figure 3 (A) Comparison of circulating furin concentration in ADHF ($n = 468$), non-ADHF ($n = 169$), and CHF ($n = 46$) patients. (B–D) Distribution of circulating furin activity according to proBNP (B), NT-proBNP (C), and BNP (D) plasma levels in ADHF (open purple circle), non-ADHF (open green circle) and CHF (grey circle) patients. (E and F) Distribution of circulating furin activity according to the degree of proBNP glycosylation in ADHF ($n = 49$, open purple circle) and non-ADHF ($n = 50$, open green circle) patients (E), and in CHF patients ($n = 46$). (F). ADHF, acutely decompensated heart failure; non-ADHF, non-acutely decompensated heart failure; CHF, chronic heart failure; proBNP, proB-type natriuretic peptide.

only 5²³ to 8%²⁴ of proBNP is detected by NT-proBNP assays. Secondly, metabolism of BNP and NT-proBNP differs²⁵ and the briefer half-life of BNP may contribute to the lack of correlation between

plasma BNP and degree of proBNP glycosylation. Thirdly, lack of sensitivity of the BNP assay used for detection of altered forms of BNP may also contribute to the lack of correlation observed. Differences

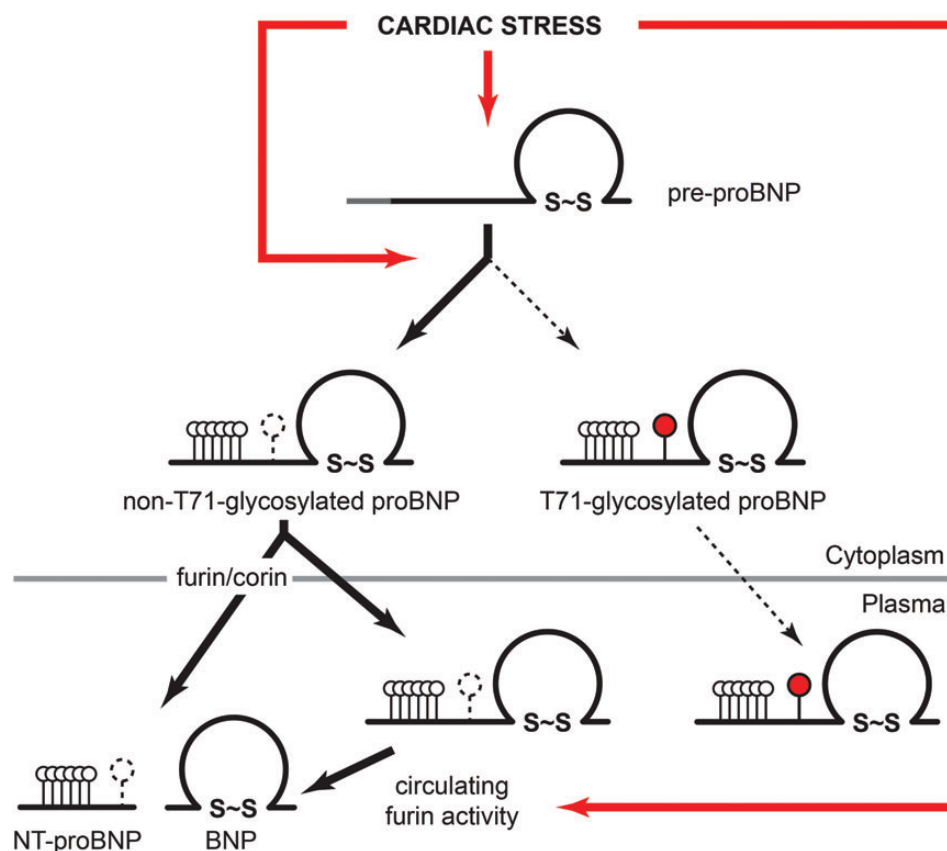


Figure 4 Schematic representation of the production and processing of the proBNP upon acute conditions. In this model, cardiac stress (red arrows) promotes a decrease in glycosylation of the proBNP and the increase in circulating furin activity, which synergistically enhanced proBNP processing, and natriuretic peptide bioavailability. S~S, disulfide bonds; red circle, glycosylation at T71; white circle with dotted line, absence of glycosylation at T71; small solid-lined circles, other glycosylation sites than T71; proBNP, proB-type natriuretic peptide.

between NT-proBNP vs. BNP relative to proBNP glycosylation require further studies. We found plasma levels of proBNP, BNP, and NT-proBNP were strongly correlated with increased furin activity but not furin concentration, corin activity, or corin concentration. Additionally, furin activity was inversely correlated with proBNP glycosylation. This suggests that the increase in furin activity is pivotally related to the overproduction of the B-type natriuretic peptides and may be triggered by reduction in glycosylation of T71. Of note, the degree of proBNP glycosylation measured in our patient was lower to those reported previously (~70%).⁶ Such a difference cannot be explained by a lack of sensitivity of the method we used to assay proBNP T71 glycosylation (maximum 75.8%) but rather may result from methodological differences (immunodetection of immunocaptured proBNP vs. mass spectrometry).

Our study has several limitations. First, the number of patients in which we measured the degree of proBNP glycosylation was relatively small compared with the size of the cohort. To run mass spectrometry on the whole cohort would be daunting. However, the results are compelling. Indeed, as seen in Figure 2C, the patients tested for proBNP glycosylation are representative of the whole cohort. Secondly, we did not assess the degree of NT-proBNP glycosylation in this study. It is known that commercial assays for NT-proBNP

only detect a fraction (~20%) of the total NT-proBNP, due to the heavy glycosylation of the NT-proBNP.²⁶ However, we chose to use a commercially available assay, when possible, in order to be able to transfer our findings to clinical practice. Thirdly, our study population lacks a healthy age-matched control group. Lastly, the mechanisms involved in the decrease in proBNP glycosylation remain unknown.

In summary, our study describes a post-translational cascade that includes a reduction in proBNP glycosylation and an increase in furin activity that synergistically lead to acute increase in circulating NT-proBNP and BNP. From a clinical standpoint, our data showed that, using the selected commercially available assay, NT-proBNP better reflects the degree of proBNP deglycosylation than the BNP. Furthermore, circulating furin activity appears as a promising companion biomarker for NT-proBNP to more precisely assess acuity of HF syndromes. Further studies should explore how increased myocardial stress in ADHF patients induces this post-translational cascade.

Supplementary material

Supplementary Material is available at *European Heart Journal* online.

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