Clinical Chemistry 63:1
359–368 (2017)

Unraveling the Molecular Complexity of O-Glycosylated Endogenous (N-Terminal) pro–B-Type Natriuretic Peptide Forms in Blood Plasma of Patients with Severe Heart Failure

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BACKGROUND: Currently, N-terminal pro–B-type natriuretic peptide (NT-proBNP) and its physiologically active counterpart, BNP, are most frequently used as biomarkers for diagnosis, prognosis, and disease monitoring of heart failure (HF). Commercial NT-proBNP and BNP immunoassays cross-react to varying degrees with unprocessed proBNP, which is also found in the circulation. ProBNP processing and immunoassay response are related to O-linked glycosylation of NT-proBNP and proBNP. There is a clear and urgent need to identify the glycosylation sites in the endogenously circulating peptides requested by the community to gain further insights into the different naturally occurring forms.

METHODS: The glycosylation sites of (NT-) proBNP (NT-proBNP and/or proBNP) were characterized in leftovers of heparinized plasma samples of severe HF patients (NT-proBNP: >10 000 ng/L) by using tandem immunoaffinity purification, sequential exoglycosidase treatment for glycan trimming, β-elimination and Michael addition chemistry, as well as high-resolution nanoflow liquid chromatography electrospray multistage mass spectrometry.

RESULTS: We describe 9 distinct glycosylation sites on circulating (NT-) proBNP in HF patients. Differentially glycosylated variants were detected based on highly accurate mass determination and multistage mass spectrometry. Remarkably, for each of the identified proteolytic glycopeptides, a nonglycosylated form also was detectable.

CONCLUSIONS: Our results directly demonstrate for the first time a rather complex distribution of the endogenously circulating glycoforms by mass spectrometric analysis in HF patients, and show 9 glycosites in human (NT-) proBNP. This information may also have an impact on commercial immunoassays applying antibodies specific for the central region of (NT-) proBNP, which detect mostly nonglycosylated forms.

N-terminal pro–B-type natriuretic peptide (NT-proBNP)[3] and its C-terminal physiologically active counterpart, B-type natriuretic peptide (BNP), have become well-accepted heart failure (HF) biomarkers and were implemented into the guidelines for HF-management more than 10 years ago *(1)*. However, several factors, such as biological and individual variability as well as the structural microheterogeneity of these polypeptides, are far from being completely understood, and differences in epitope recognition have not revealed clear differences in clinical performance as evaluated by ROC analyses *(2)*.

BNP, NT-proBNP, and the precursor proBNP (for structural details see Supplemental Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue1) have been demonstrated to circulate as various forms (e.g., truncated and O-glycosylated) in severely ill HF patients *(3–7)*. Moreover, a recent study reported that the BNP signal peptide derived from preproBNP also circulates in human blood and represents a potential bio-

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Received August 13, 2016; accepted October 14, 2016.
Previously published online at DOI: 10.1373/clinchem.2016.265397

[3] Nonstandard abbreviations: NT-proBNP, N-terminal pro–B-type natriuretic peptide (1–76); BNP, B-type natriuretic peptide (1–32); HF, heart failure; proBNP, pro–B-type natriuretic peptide (1–108); (NT-) proBNP, N-terminal pro–B-type natriuretic peptide (1–76) or proBNP (1–108); mAbs, monoclonal antibodies; IP, immunoprecipitation; pAb, polyclonal antibody; aa, amino acids; TIP, tandem immunoaffinity purification; μ-IA-HPLC, μ-immunoaffinity-HPLC; BEMAD, β-elimination and Michael addition; nano-LC ESI-MS^n, nano-flow liquid chromatography electrospray ionization multistage mass spectrometry; nano-LC MALDI-TOF MS/MS, nano-flow liquid chromatography MALDI TOF tandem mass spectrometry; XICs, extracted ion chromatograms.

marker for cardiac ischemia and myocardial infarction *(8)*. Further, we and others demonstrated that the prohormone proBNP itself also is present in the bloodstream *(3, 4, 9, 10)*. However, the biologically active form is BNP, particularly full-length BNP1–32, which promotes physiological functions such as diuresis, natriuresis, vasodilation, inhibitory action on inflammatory processes through binding to its natriuretic receptor, and activation.

The diverse glycosylated or truncated forms of the prohormone are differently recognized by the numerous immunoassays established for the measurement of BNP, NT-proBNP, and proBNP *(11)*. Particularly in HF it was found that proBNP is the predominant NP and not the active form BNP, and the proBNP:total BNP ratio was suggested to increase in pathophysiological conditions such as ventricular overload *(12)*. This BNP paradox was elucidated by the observation of reduced concentrations of biologically active BNP forms in severe HF patients although high amounts of measurable BNP were present *(13–15)*. The explanation for this paradox is the inaccuracy of the present commercially available immunoassays for measurement of intact biologically active BNP, because these assays cross-react to a variable but significant degree with proBNP1–108. Thus the assays yield high results derived by their measurement of a mixture of at least BNP and proBNP not reflecting the correct concentration of active BNP *(16–18)*. Therefore, a better understanding of the structural attributes and molecular heterogeneity of the different natriuretic peptide B forms is a highly desirable prerequisite for improved design of assays as well as their clinical application in the diverse settings of HF.

In earlier studies we observed proBNP and NT-proBNP circulating as O-glycosylated forms in human blood of severe HF patients *(3)*. In the following, we will use the term "(NT-) proBNP" (NT-proBNP and/or proBNP) for better reading purposes, since the glycosylation positions are true for both peptides. To date, the specific O-glycosylation sites in human endogenous (NT-) proBNP have not been determined. There are a few reports in which indirect methods utilizing monoclonal antibodies (mAbs) were applied to reveal that mainly the central region of human NT-proBNP is affected by O-glycosylation *(5, 19, 20)*. First evidence of O-glycosylation was shown on recombinant proBNP expressed in Chinese hamster ovary cells *(4)*. Semenov et al. demonstrated a biological role of O-glycosylation near the cleavage site in proBNP at Thr71 *(21, 22)*. Although some of N-terminally as well as C-terminally truncated BNP variants could be detected at very low levels by a mass spectrometry immunoassay in patients with HF *(15)*, proBNP was not detectable, most probably due to O-glycosylation. Thus, one can speculate that O-glycosylation has a major impact on biological proBNP processing and/or analytical detectability. Currently, no data exist regarding the specific O-glycosylation sites of endogenous proBNP forms circulating in human blood, highlighting the need to address this crucial question.

In this study we set out to define the specific O-glycosylation sites of human endogenous (NT-) proBNP in HF patients. We applied our previously reported affinity-chromatography method in combination with a bottom-up proteomics approach comprising an enzymatic deglycosylation scheme to retain a small glycan moiety attached to the glycopeptides as direct proof of glycosylation, single as well as combined enzymatic protein digestion, β-elimination with concomitant Michael addition chemistry, and high-resolution nano–LC-MS *(3, 23, 24)*. These methods allow a sufficiently high enrichment of this low-abundance protein to directly and unambiguously characterize the O-glycosylation sites of (NT-) proBNP peptides in HF patients. An overview of the experimental setup is shown in Fig. 1A.

Materials and Methods

CHEMICALS

Chemicals and other materials, unless stated in the text, are listed in the online Supplemental Methods section.

PATIENT SAMPLES

This study was conducted according to the current revision of the Declaration of Helsinki and with approval from the local ethics committee of the Medical University of Innsbruck (UN3852). After routine measurement, leftovers of heparinized plasma samples with concentrations >10 ng/L as assayed with the E 170 module of the Roche MODULAR ANALYTICS system were anonymized and used in this study.

IMMUNOPRECIPITATION OF ENDOGENOUS (NT-) proBNP

Plasma pooled from 9 patients and, additionally, 3 individual patient plasma samples were analyzed. For immunoprecipitation (IP), either a biotin-conjugated polyclonal antibody (pAb) directed against amino acids (aa) 1–21 or biotin-conjugated F(ab′)2 fragments of an mAb specific to aa 42–46 of (NT-) proBNP were bound to streptavidin-coated magnetic microparticles (Roche Diagnostics).

MINIATURIZED IMMUNOAFFINITY CHROMATOGRAPHY

For further purification of (NT-) proBNP, 2 miniaturized immunoaffinity chromatography (μ-immunoaffinity-HPLC) methods were set up similarly to our previous approach *(3)*.

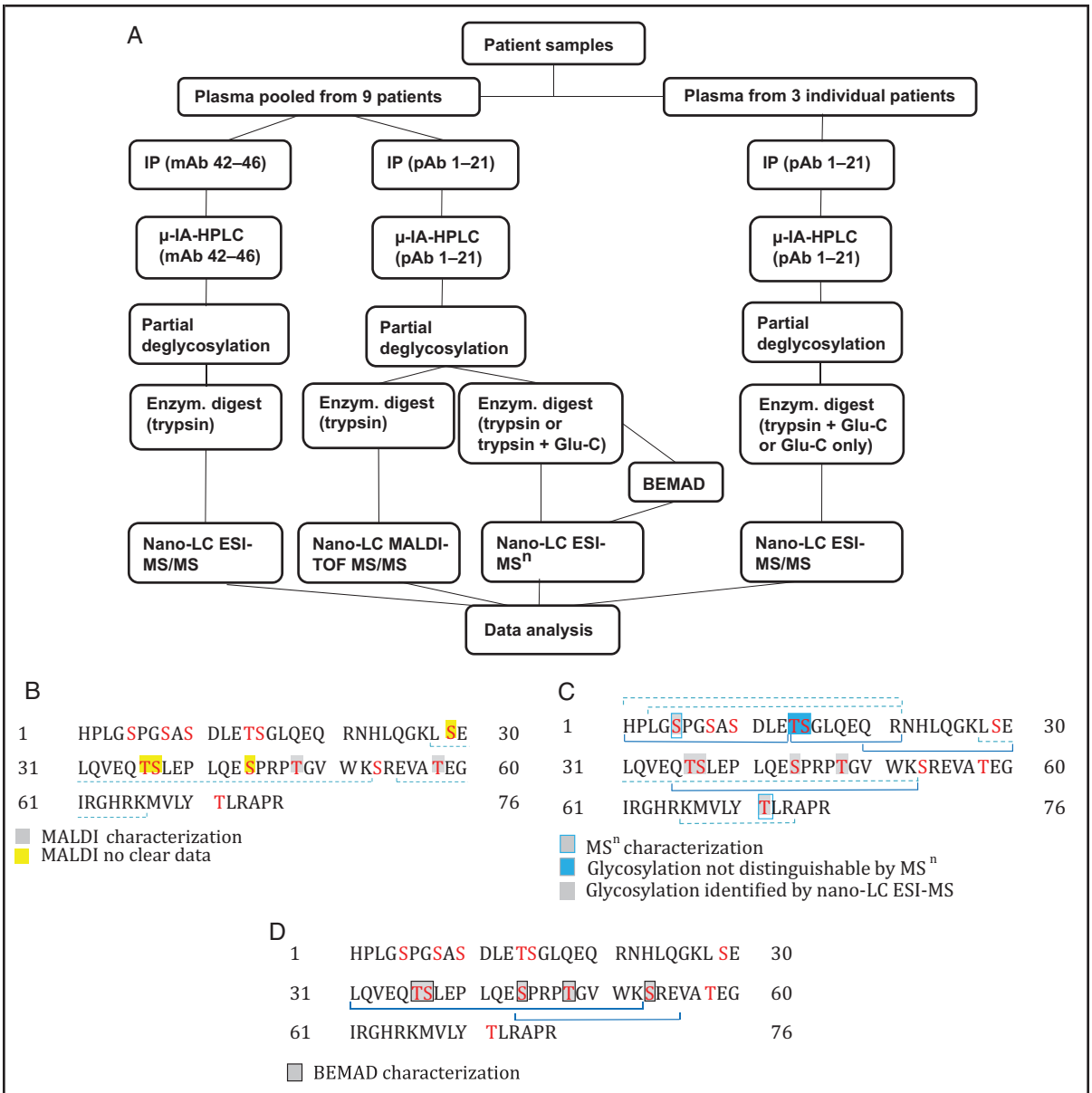

Fig. 1. Overview of experiments performed in this study (A) and herein characterized O-glycosylation sites (B–D).

Flow chart of this study (A); glycosites characterized by nano-LC MALDI-TOF-MS (B), nano-LC ESI-MS (C), and BEMAD (D) and corresponding peptides. Potential O-glycosylation positions are indicated by aa residues marked in red and the obtained peptides as lines. Peptides gained by tryptic cleavage are indicated by turquoise dashed lines. Peptides received from endoproteinase Glu-C digestion or obtained by combined digestion using both enzymes sequentially are marked with blue lines. Enzym., enzymatic.

PARTIAL DEGLYCOSYLATION OF (NT-) proBNP

(NT-) proBNP samples derived from tandem immunoaffinity purification (TIP) [IP followed μ-IA-HPLC = TIP] were incubated with an exoglycosidase cocktail (see online Supplemental Data).

PROTEOLYTIC IN-SOLUTION DIGESTION

Partially deglycosylated immunoaffinity-purified samples were digested with trypsin or endoproteinase Glu-C or by a combination of both enzymes in 50 mmol/L ammonium bicarbonate containing 1 mmol/L calcium dichloride, pH 8.0.

β-ELIMINATION AND MICHAEL ADDITION OF PARTIALLY DEGLYCOSYLATED GLYCOPEPTIDES AND SYNTHETIC GLYCOPEPTIDES

Synthetic glycopeptides and glycopeptides obtained from double proteolytic digestion were mixed with 1 μL 2-mercaptoethanol and dimethylamine to give a final concentration of 30% and incubated at 55 °C for 6 h. Excess reagents were removed by evaporation *(24)*.

MASS SPECTROMETRIC ANALYSIS OF SYNTHETIC GLYCOPEPTIDES

Synthetic glycopeptides before and after β-elimination and Michael addition (BEMAD) treatment were analyzed by static nano-electrospray mass spectrometry on an LTQ Velos dual-pressure linear ion trap mass spectrometer (Thermo Fisher Scientific).

MASS SPECTROMETRIC ANALYSIS OF IMMUNOPURIFIED ENDOGENOUS (NT-) proBNP

Proteolytically digested samples were analyzed by nano-flow liquid chromatography electrospray ionization multistage mass spectrometry (nano-LC ESI-MS^n) on an LTQ Orbitrap XL (Thermo Fisher Scientific). Alternatively, nano-flow liquid chromatography MALDI-TOF tandem mass spectrometry (nano-LC MALDI-TOF MS/MS) on a 4800 MALDI TOF/TOF Plus mass spectrometer (AB Sciex) was applied.

Detailed protocols are available in the online Supplemental Methods section.

Results

SITE-SPECIFIC MAPPING OF ENDOGENOUS (NT-) proBNP GLYCOSYLATION FORMS IN HUMAN PLASMA BY NANO-LC MALDI-TOF MS/MS

In a first attempt to characterize circulating glycosylated forms, we enriched (NT-) proBNP by TIP using a pAb (pAb 1–21) in both steps of TIP (IP and μ-IA-HPLC), partially deglycosylated the purified fractions, and analyzed the samples after protein digestion with trypsin by nano-LC MALDI-TOF MS/MS. Owing to exoglycosidase treatment, a single monosaccharide residue or a small glycan on the glycopeptides (e.g., HexNAc) could be retained, which facilitated the direct and unambiguous proof of glycosylation. By applying strict criteria (99% peptide identification confidence), we could elucidate Thr58 to be glycosylated within the peptide 55–66 (Fig. 1B; online Supplemental Fig. 2A) and Thr48 within the peptide 28–52 (see online Supplemental Fig. 2B). Importantly, both peptides also coexisted unmodified (data not shown). The analyses gave evidence for a possible glycosylation of Ser29, Thr36, Ser37, and Ser44 in the peptide 28–52; however, owing to insufficient spectral quality, further confirmation was required.

CHARACTERIZATION OF GLYCOSYLATED ENDOGENOUS (NT-) proBNP FORMS IN HUMAN PLASMA BY NANO-LC ESI-MS^n

To identify additional glycosylation sites, single (trypsin) and double (trypsin combined with endoproteinase Glu-C) protease digestions of partially deglycosylated TIP samples were analyzed by nano-LC ESI-MS^n. The tryptic peptide 28–52, which has 5 potential glycosylation sites, was found in nonglycosylated form, but also mono-, di-, tri-, and tetraglycosylated in different abundances. These glycoforms were well resolved in nano-flow reversed-phase chromatography according to the number of attached glycans (Figs. 1C and 2A–E). Because a pentaglycosylated form could not be detected in any of the samples analyzed, it was still unclear which of the 5 potential sites were modified. By further protease digestion of the tryptic peptide 28–52 with endoproteinase Glu-C, a shorter peptide (aa 35–52) was obtained that was equally glycosylated as peptide 28–52 (tetraglycosylated). Therefore, we concluded that Ser29 is not significantly glycosylated, whereas Thr36, Ser37, Ser44, and Thr48 are modified by glycans. Fig. 3 shows an estimation of the relative amounts of different glycoforms of peptide 35–52 determined from extracted ion chromatograms (XICs) of 3 individual and a pooled patient plasma sample (for more details see Spectral Analysis and Data Interpretation section in the online Supplemental Data). Similar glycan distribution patterns were observed in the individual as well as in the pooled samples, revealing the triglycosylated form with a mean (SD) of 72.97% (4.98%), as the most abundant one. To further demonstrate that Ser29 is not glycosylated TIP (pAb 1–21) enriched samples were digested with Glu-C only, which among others produced the peptide 20–30 without any hint for Ser29 glycosylation.

To investigate the influence of antibodies in the recognition of glycosylated (NT-) proBNP, an mAb (mAb 42–46) was used for affinity enrichment instead of the pAb (pAb 1–21). After tryptic digestion, the nonglycosylated form of peptide 28–52 was the most abundant one, followed by the di- and mono-, and to a minor extent tri- and tetraglycosylated form, which implies that the antibody recognition is indeed affected by glycosylation (Fig. 4, A–E).

We further aimed to assess the glycosylation status of Thr71, which is close to the proBNP cleavage site (Arg76 ↓ Ser77) and has been shown to suppress proBNP processing *(21)*. Applying the TIP protocol with pAb 1–21, subsequent tryptic digestion and partial deglycosylation the peptide 66–73 was investigated. We identified unmodified, glycosylated, and oxidized forms (see online Supplemental Fig. 3, A–D). According to the peak areas, the ratio between the nonglycosylated and glycosylated form was 13.2, very similar to the ratio of 11.2 observed between the oxidized and oxidized plus glycosylated forms (see online Supplemental Table 1).

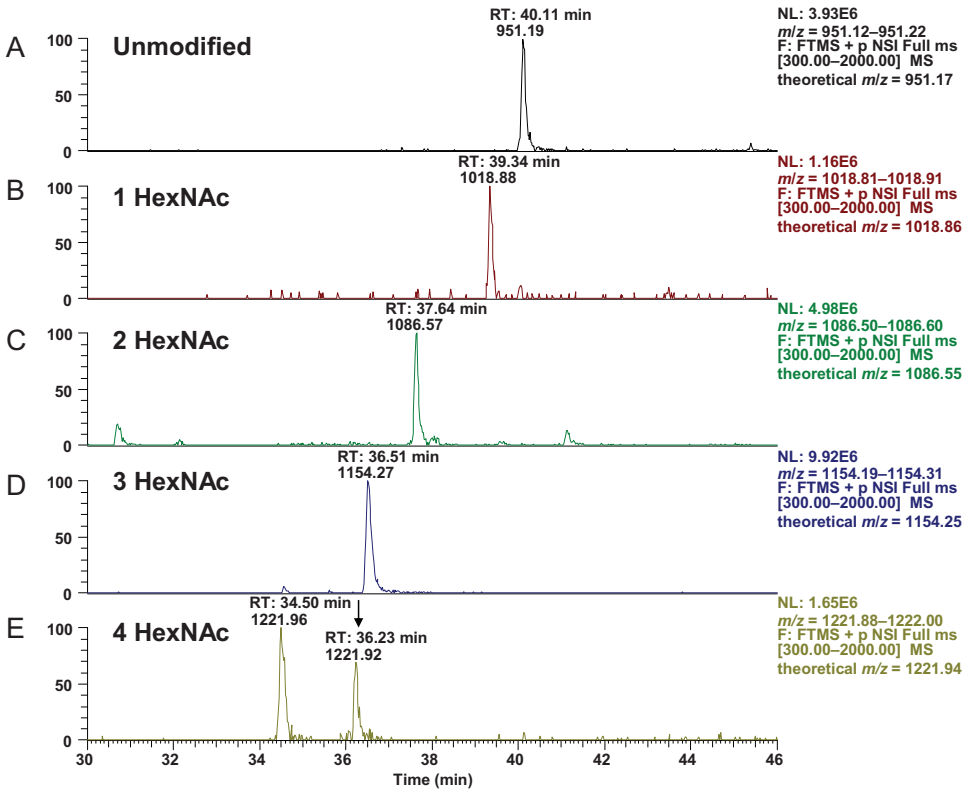

Fig. 2. Nano-LC ESI-MS of a tryptic digest of a TIP-enriched plasma fraction pooled from 9 patients after limited exoglycosidase treatment.
(A–E), XICs of glycoforms of the peptide 28–52. Peaks represent the monoisotopic *m/z* ratios ($z = 3+$) with ±50 ppm mass tolerance. The arrow in (E) indicates an interfering peptide. RT, retention time; NL, normalization level; FTMS, Fourier transform mass spectrometer; NSI, nanospray ionization.

The most abundant form was the unmodified one [77.8% (4.7%)]. The calculated ratio of 10.9 (nonglycosylated:glycosylated) most probably reflects the endogenous NT-proBNP:proBNP ratio, because the pAb 1–21 used for TIP also isolated total (NT-) proBNP.

In a further nano-LC ESI-MS^3 experiment of the trypsin/endoproteinase Glu-C digested pool sample peptide 14–21 carrying 2 possible glycosylation sites was found to be substoichiometrically glycosylated on either Thr14 or Ser15 (see online Supplemental Fig. 4, A–C). Neither site has been described to be glycosylated previously. However, it was impossible to determine which aa residue, Thr14 or Ser15, was glycosylated owing to the fact that these aa residues are adjacent and the glycosylation cannot be allocated after the loss of HexNAc during MS/MS fragmentation. Moreover, only a few spectra of the glycosylated peptide could be detected and the XICs were of very low signal intensity (data not shown).

Another new glycosylation site was discovered in this study, corresponding to Ser5 in the peptide 1–13 which has 3 potential glycosylation sites (Fig. 1C; online Supplemental Fig. 4D). Higher modified forms apart from the monoglycosylated variant could not be detected. This also was true for peptides 1–21 and the truncated peptide 3–21 showing unmodified or monoglycosylated forms only (see online Supplemental Fig. 5, A and B). Since there were no multiglycosylated variants of these latter peptides detected, it suggests that the glycosylation on Thr14 or Ser15, respectively, is of very low abundance. In our experiments, the unmodified peptide 3–21 appeared to be most abundant (see online Supplemental Fig. 5C). Further, nano-LC MALDI-TOF MS/MS analyses showed additional N-terminally truncated variants (see online Supplemental Table 2).

CHARACTERIZATION OF MULTIPLY GLYCOSYLATED (NT-) proBNP DERIVED PEPTIDES BY BEMAD AND NANO-LC ESI-MS^n

Ideally, BEMAD results in an exchange of the glycan of formerly glycosylated amino acids by a chemically more stable surrogate in collision-activated dissociation mass spectrometry. Following our previous work *(24)*, β-elimination with a mild alkylamine base and concomitant Michael-type addition of 2-mercaptoethanol as nu-

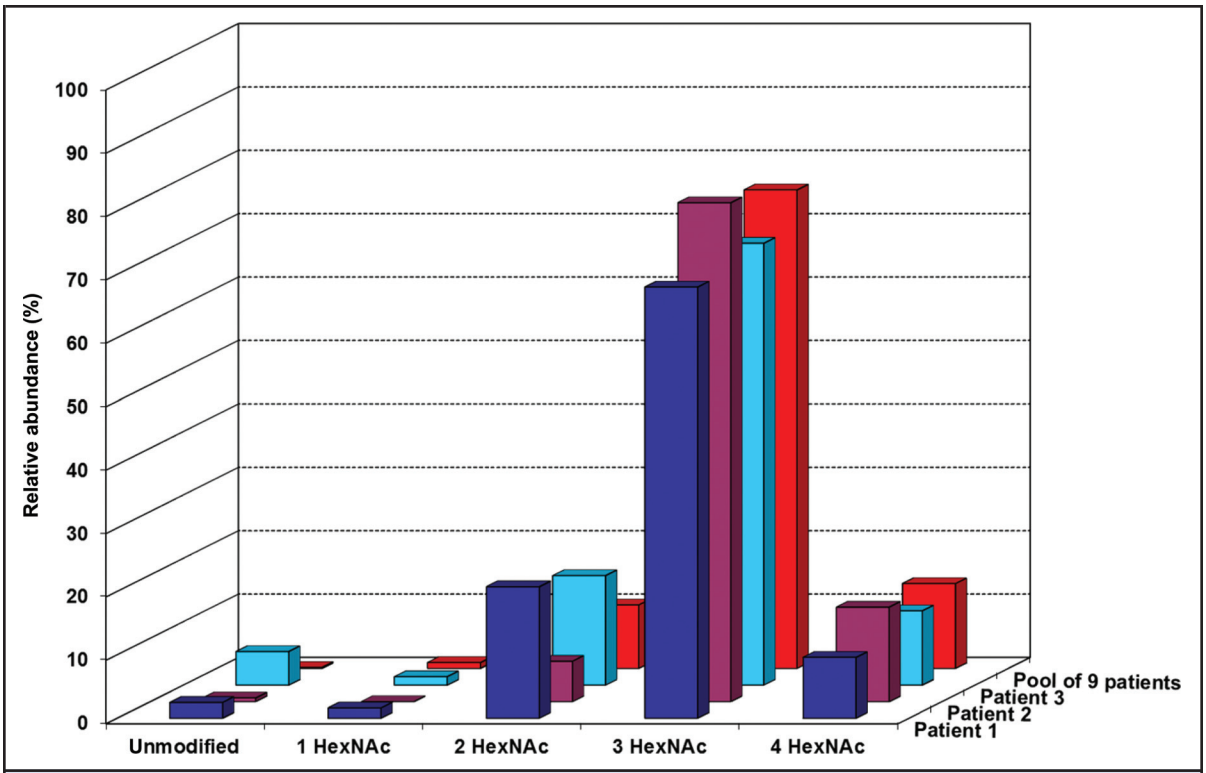

Fig. 3. Relative proportion of glycan residues on peptide 35–52 of secreted (NT-) proBNP glycoforms separated by nano-LC and detected by ESI-MS survey scans represented by a 3D histogram.

TIP-enriched samples were treated with exoglycosidases, trypsin, and endoproteinase Glu-C. Proportional glycan contents were determined on the basis of the peak area of the mean masses with ±0.5 Da mass tolerance for each glycoform and are presented as percentage of total residues within each sample.

cleophile was applied. First, BEMAD was tested on the diglycosylated synthetic peptide 44–55 modified at Thr48 and Ser53 mimicking the endogenous form. The specificity of the method was confirmed by leaving Ser44 unaffected. As a further cross-check, nonglycosylated peptide 44–55 was unambiguously identified by the MS/MS spectrum, whereas the MS/MS spectrum of the diglycosylated synthetic peptide was insufficient for glycosylation site assignment (see online Supplemental Fig. 6, A and B). Since endogenous peptide 44–55 showed the similar fragmentation pattern after BEMAD treatment as the synthetic glycosylated one, glycosylation of Thr48 and Ser53 could be assigned (Fig. 1D; online Supplemental Fig. 6, C and D).

With the proposed method, we were further able to unambiguously confirm the glycosylation occupancy of the endogenous tetraglycosylated form of the peptide 31–52 obtained from double proteolytic digested samples in the central region of (NT-) proBNP carrying 4 possible sites (see online Supplemental Fig. 7). Although the BEMAD treatment was incomplete at Ser44, 3 of the 4 sites revealed sequence-specific ions related to 2-mercaptoethanol adducts at positions Thr36, Ser37, and Thr48. The presence of O-glycosylated Ser44 was confirmed by prominent peaks caused by neutral losses HexNAc in collision-induced dissociation and the accurately determined precursor mass (Δ mass = 1.43 ppm). Online Supplemental Table 3 provides an overview of the glycosylated amino acids and peptide variants confirmed in this study. A scheme of identified glycosylation sites in (NT-) proBNP is shown in Fig. 5.

Discussion

In this study, we characterized 9 specific O-glycosylation sites on human (NT-) proBNP circulating in plasma of patients with severe HF. None of them had been characterized in humans, but only in Chinese hamster ovary cells, and, to the best of our knowledge, 2 of them were not observed in any investigations until now. Most strikingly, all peptides characterized coexist also in an unmodified form.

Previous studies on O-glycosylation of endogenous proBNP were based on indirect detection aided by im-

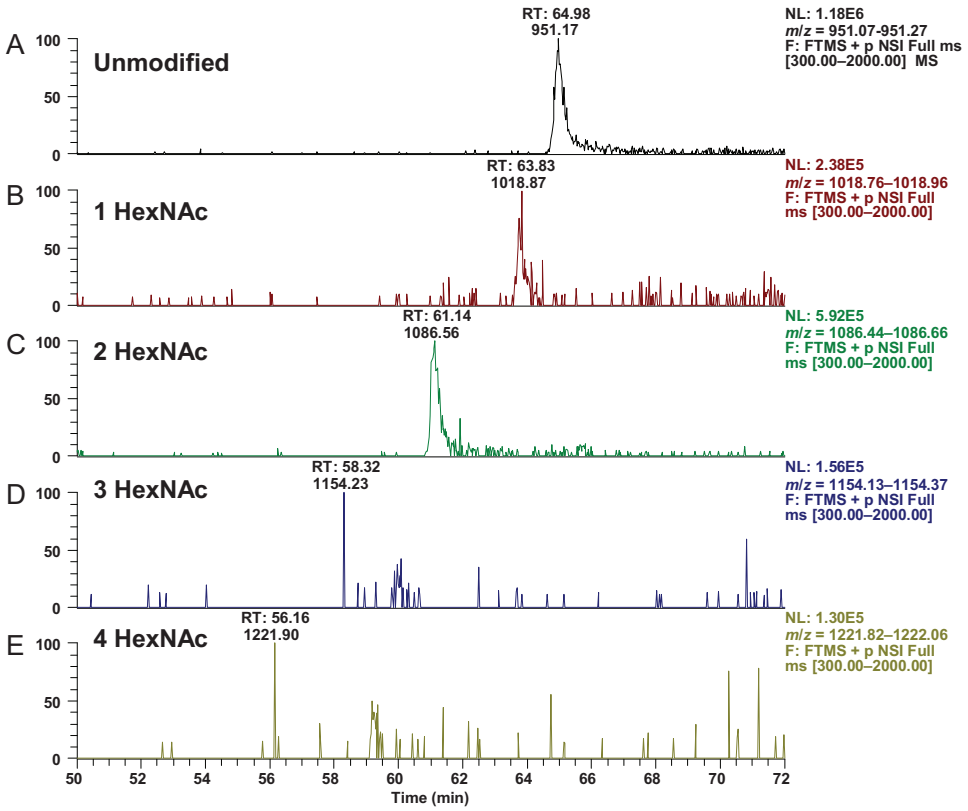

Fig. 4. Nano-LC ESI-MS of a representative plasma fraction from a single patient TIP-enriched with mAb 42–46 after limited exoglycosidase treatment and trypsin digestion.

XICs of glycoforms of the peptide 28–52. (A), Nonglycosylated form; (B–E), 1–4 HexNAc modified forms. Peaks represent the monoisotopic *m/z* ratios ($z = 3+$) with ±100 ppm mass tolerance. RT, retention time; NL, normalization level; FTMS, Fourier transform mass spectrometer; NSI, nanospray ionization.

munoassays applying specific mAbs *(5, 19, 20)*. Antibodies directed against the midregion of NT-proBNP (aa residues 28–56) poorly recognized endogenous NT-proBNP, whereas after deglycosylation, the reactivity increased by 5.8–41-fold with a mean of 19 and 4-fold in another study based on 186 HF patients *(20, 25)*. Antibodies raised against aa residues 13–24 and 63–76 detected glycosylated and deglycosylated endogenous NT-proBNP similarly well, indicating that these regions are probably not modified or are O-glycosylated to a much lower extent *(20)*. Our samples were immunopurified with the pAb 1–21 that has 3 epitope specificities within the N-terminal region of (NT-) proBNP (Roche Diagnostics, personal communication), which means that the

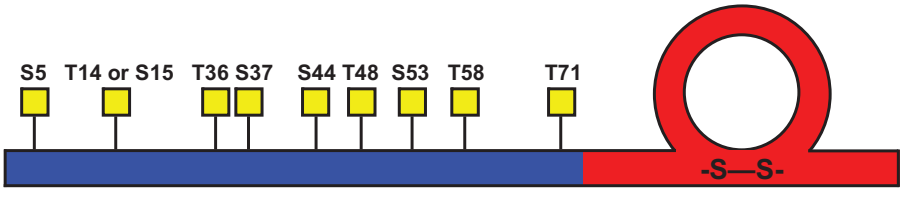

Fig. 5. Scheme of identified glycosylation sites in (NT-) proBNP.

Glycosylated positions are indicated by small boxes.

antibody is capable of detecting N-terminally glycosylated (NT-) proBNP variants.

More than a decade ago, a poor recognition of antibodies specific to the region 37–49 in endogenous NT-proBNP was shown *(19)*. In the current study, we were able to confirm that the midregion of human (NT-) proBNP is O-glycosylated at aa residues Thr36, Ser37, Ser44, Thr48, Ser53, and Thr58. Glycosylation on residue 58 was not considered in former studies applying immunoassays before and after deglycosylation *(20, 21)*. Moreover, we could validate 2 previously unknown low abundant O-glycosylation sites, Ser5 and Thr14/Ser15. Additionally, very preliminary data also suggest Ser8 and Ser10 to be O-glycosylated in very low abundances (data not shown). However, as the evidence for O-glycosylation of either of these aa residues is not definitive, both these sites need to be confirmed in further experiments.

Further, we proved aa residue Thr71 in (NT-) proBNP occurs in O-glycosylated form. Since it was shown that proBNP1–108 is barely processed into BNP and NT-proBNP if this residue is glycosylated *(21, 22, 26)*, and glycosylated proBNP was detected in human blood, we speculate that this verified glycosylation site may at least partly originate from unprocessed proBNP.

Single O-GalNac-glycosylation of Thr71 results in partial blocking of proBNP procession by furin in vitro *(26)*. On the other hand, recombinant proBNP expressed in HEK293 cells (chosen due to their high glycosylation profile at the desired site) failed to be processed by furin in vitro *(21)*. One might speculate that blockage is complete only when glycosylation is of a more complex nature, e.g., a disialyl-T–type glycosylation *(27)*. The authors of this study suggest that remodeled O-glycosylation machinery observed in progressive cardiac hypertrophy and HF could explain the impeded binding of processing proteases. The described altered O-glycosylation machinery in heart tissue causes an increased synthesis of mucin-type O-glycan forms, especially disialyl-T, and, moreover, the downregulation of other glycan structures. For example, cysteine and glycine-rich protein 3, a sensor and mediator of stretch signaling and, thereby, a key regulator of cardiac muscle function, is suggested to alter its monomer-to-oligomer ratio and thereby also its cellular localization and function through atypical O-glycosylation. However, the increase of O-glycosylation is not limited to specific proteins but affects a variety of proteins in heart tissue proteome. Whether this altered O-glycosylation machinery also affects glycosylation of proBNP, and, as a possible consequence, its biological activity, remains to be observed. Glycoprofiling of proBNP is therefore one important future step to understanding the complex entirety of proBNP forms.

Our data on endogenous human proBNP extend the results of prior investigations on recombinant proBNP expressed in Chinese hamster ovary cells that proposed complete glycosylation of Ser37, Ser44, Thr48, Ser53, and Thr71, as well as partial glycosylation of Thr36 and Thr58 *(4)*. Recombinant human proBNP probably does not reflect the endogenous circulating forms of human proBNP, and, for the latter, we observed all of the listed residues to coexist variably glycosylated as well as nonglycosylated.

Commercial immunoassays use nonglycosylated calibrator material and mostly antibodies directed against epitopes with potential O-glycosylation site occupancy. Thus, these assays probably do not recognize all glycosylated (NT-)proBNP forms and eventually might underestimate the amount in individual samples, although clinical significance is given. Until now, except for the described negative influence of O-glycosylated Thr71 on proBNP processing by furin, the biological significance of O-glycosylated proBNP forms has not been very well understood.

In patients suffering from severe HF, increased concentrations of NT-proBNP, BNP, and proBNP have been observed, but these high concentrations, caused by various less-active forms of BNP *(9, 13, 28)*, do not represent the biological active state of this hormone. Patients with chronic HF had the highest percentage of glycosylated proBNP as compared to patients with acute decompensated HF and nonacute decompensated HF *(29)*.

Thus it can be assumed that in chronic HF increased release of glycosylated proBNP that is not sufficiently processed into BNP will lead to a comparably low amount of the active hormone, despite high measured concentrations of proBNP (endocrine paradox of the heart). On the other hand, in the acute state increased nonglycosylated proBNP that is rapidly processed into the active hormone may be an attempt of the heart to maintain blood pressure and volume homeostasis *(30)*. Consequently, it would be helpful to generate immunoassays that were able to recognize the specific forms of BNP, NT-proBNP, and proBNP to generate more clinically relevant information about the disease state, because the current assays cannot adequately differentiate between these forms *(31)*. In this study, we showed which sites of human proBNP are prone for O-glycosylation, thereby providing a basis for future immunoassay development. Future immunoassays should be able to distinguish between HF patients with or without a deficiency of mature BNP and to help select patients who, for example, could possibly profit from therapies employing BNP *(32)*. Choosing the right epitope for antibody generation matters, as glycosylated and nonglycosylated proBNP forms will be detected differentially. When EDTA samples were pretreated with deglycosylation enzymes, NT-proBNP values were markedly

higher, and the diagnostic and long-term prognostic accuracy improved modestly (approximately 2%), but significantly in patients with acute dyspnea *(33)*.

We found a ratio of nonglycosylated to glycosylated Thr71 of 10.9 by nano-LC ESI-MS^n in the plasma of severe HF patients. This is in good agreement with 2 studies revealing a median ratio of 10.0 (range: 2.48–12.5) and a percentage of 8% proBNP as determined by immunoassays *(5, 20)*. However, a recent study on 23 plasma samples from HF patients discovered a considerable amount (about 30%) of circulating nonglycosylated proBNP that could be processed further *(22)*. This challenges the previous conclusion to some extent, but may be explained by the use of a different immunoassay applying different antibodies than the previous ones. Nevertheless, the existence of circulating nonglycosylated proBNP in HF patients that is capable of being processed into the active hormone in peripheral blood could open new pharmacological strategies, e.g., with drugs modulating the maturation of prohormone into active hormone *(16, 18)*. Further, HF patients, particularly chronic HF patients, suffer from a hormone deficiency that can partly be rescued by exogenous recombinant BNP administration *(34, 14)*.

In conclusion, we characterized 9 distinct glycosylation sites on human (NT-) proBNP derived from patients with severe HF, which gives new insight into the molecular heterogeneity of this peptide. Although it was shown that NT-proBNP, BNP, and proBNP concentrations increase with the severity of HF stages, it is not clear which molecular forms of BNP are present in different HF stages, and to what extent. These will be topics to investigate in further studies.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: Research grant from Roche Diagnostics GmbH, Penzberg, Germany (D150820-011-012).
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, and final approval of manuscript.

Acknowledgments: We are very grateful to Dr. Klaus Hallermayer, Dr. Uwe Kobold, and Dr. André Ziegler from Roche Diagnostics for material transfer and supporting this project with fruitful discussions, as well as Prof. Andreas Huber from Kantonsspital Aarau for his help with the manuscript.

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