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In this issue

Foreword of the editor

Editor in Chief: Gábor L. Kovács, MD, PhD, DSc

This issue is devoted to recent development of cardiac markers. The guest editor is Allan S. Jaffe, M.D., Consultant and Chair of the Division of Clinical Core Laboratory Services at Mayo Clinic in Rochester, Minnesota, US, with a joint appointment in the Division of Cardiovascular Diseases. He holds the academic rank of Professor of Laboratory Medicine and Pathology and also Professor of Medicine.

Dr. Jaffe's research interests include a long academic career investigating the use of biomarkers to characterize the pathobiology of acute cardiovascular disease. With investigators at Washington University in St. Louis, he helped develop and was responsible for the validation studies of the first cardiac troponin I assay. His cutting-edge research explores questions surrounding many commonly used cardiac biomarkers.

Dr. Jaffe is a highly esteemed national and international presenter who has co-authored five books and written more than 500 peer-reviewed articles, book chapters, and abstracts. His writings particularly focus on the use of both cardiac troponin and natriuretic peptides to characterize patients with both acute and chronic heart failure. Dr. Jaffe has received many awards and honors throughout his career, including the Citation of International Service from the American Heart Association. He is simply the ultimate authority on the use of many of these markers in the clinical arena and for that reason serves on many of the national and international groups who make guidelines in this area.

Dr. Jaffe completed his undergraduate studies at University of Maryland where he also earned his medical degree. He trained at Washington University School of Medicine in St. Louis, Missouri for house staff, Chief Residency and fellowship training and was on the faculty there for 22 years, rising to the rank of Professor of Medicine. He left in 1995 to become Chief to Cardiology and Associate Chair of Medicine for Academic Affairs at the State University of New York at Syracuse in 1995. In 1999, he was recruited to the Mayo Clinic in Cardiology and Laboratory Medicine.

As editor of eJIFCC I am glad that Professor Jaffe accepted to be the guest editor of this issue.

The world is changing – are we ready?

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EDITORIAL

It is clear that there is an ongoing revolution in medicine as cost and regulatory pressures begin to intersect with our clinical responsibilities for the care of patients. In the past, particularly in the area of in vitro diagnostics, all that was required for approval for clinical use of biomarkers was an analytically robust measuring system, a reasonable analytic validation, a modicum of clinical validation and resources to market the testing. It was then often left to clinicians to figure out how and where any particular assay happened to fit. Publications indicating a rationale or enthusiasm for those markers were often more than was necessary to get clinicians to utilize these assays. From the point of view of developers this was a very facile process that was lucrative because even if an assay failed to work it took a large amount of time and multiple test runs for the field to understand the difficulties.

Our present environment challenges this previous paradigm. We have now progressed to the point where assays can no longer be used without some understanding of their clinical utility (1,2). Thus, clinical validation has become an essential part of assay validation in addition to a reasonable analytic validation of the accuracy of the assay. Unfortunately,

or fortunately, as the case may be, it used to be that a speculative utilization plan was adequate. One would argue that a given new marker probably should work based on interesting data about the biomarker. Often, the incremental prognostic value of the marker could be shown, which was a way of acknowledging that the marker was capable of identifying something that had important pathophysiologic relationship. However, it is no longer adequate to show incremental prognostic risk stratification. Knowing that a patient is at higher or lower risk than originally thought is often not helpful clinically. If one is high risk, knowing he/she is at still higher risk often does not result in a change in therapeutic response (2). And if the marker suggests the patient is at lower than low risk, are we willing to not treat as we had intended. Often that is now what occurs (2).

It now is deemed important and this author would suggest correct that the proper use of a given biomarker requires an answer to what one might do to respond to any given marker value and the efficacy of that response should be understood. Specifically, one needs to understand whether or not one has a specific action to implement in response to a given elevation of a biomarker. Absent that information, even if there are prognostic implications, the uptake in the use of the biomarker is unlikely to be extensive because in a cost sensitive environment having actionable data that informs clinicians about something important about his or her patient has become important criteria for test implementation. This is the evolving nature of the biomarker field. Thus, one ought to be cynical about using biomarkers when one does not know what to do in response to the data. High sensitivity troponin is a good example of this. We have exciting information about the possible utility of this marker and many are ready to implement this long before there is robust clinical validation of how one might proceed to use

the data associated with the applications (2,3). This has the potential to put patients at risk because although most often the suggestions for use are reasonable, that does not always mean they are correct, nor does it imply that they are generically or consistently cost effective. Therefore, the bar is much higher today than it was in the past.

Thus, we take the opportunity in this addition of the Journal to review the analytic and clinical substrate for some attractive biomarkers. Some such as natriuretic peptides have already been approved for clinical use for a variety of indications but not necessarily the ones we will discuss. Similarly, high sensitivity troponin is in use throughout much of the world save the United States and is being used to great advantage. However, the ability to implement their use in an optimal manner that will improve patients care has been problematic. Indeed, some of the algorithms proposed have been criticized because of an inadequate data substrate (4,5). In addition, we will discuss new more novel markers as well, both analytically and clinically. These markers have potential to substantially improve our ability to triage patients who have heart failure in particularly. They carry tremendous promise because of the way in which they interdigitate with the pathophysiology of heart failure.

However, in order to use these markers intelligently, one must understand the analytic issues related to the assays. There are often problem areas or areas of that are unknown to clinicians where eventually refinements are very likely to change our understanding of their clinic use. It is one thing to speculate how they might work and another to prove it. As indicated above, we are in a time when proving value and not just speculating about it is the mantra. From that perspective the articles included on natriuretic peptides, ST2 and Glaectin-3 are of particular importance. The articles on the use

of these markers and how to implement high sensitivity cardiac troponin begin to probe what is necessary from the evidence base and from the implementation perspective before we can initiate specific interventions based of the results from these assays. Some will require more clinical data predominately to define actionable information that tells clinicians what to do as opposed to simply recapitulating the idea of increased risk. On the other hand, some assays such as high sensitivity troponin have an adequate data sense to start implementation but it is the steps to optimize operationalization that are key. That does not mean that all the answers are in or that there are no controversies. That is far from the case. However, as when one starts a new major paradigm, coordination of those efforts become key and how we coordinate these efforts such that all members of the care teams responsible for patients are pulling together to implement this successfully is not clear. This desperately needs to be emphasized, followed, appreciated, and finally in the interest of patient care.

Finally, there are new insights into some of the older markers whose use we understand but they could influence markedly our ability to utilize these markers in a way that will intelligently allow for appropriate utilizing and improvements in patient care. What is critical to appreciate about all of these articles is that they present the state-of-the-art as it is today and that state-of-the-art no longer recapitulates what it used to be. It is a new era with new metrics that as you read the articles in this themed issue, you will be clearly sensitized to.

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Analytical issues with natriuretic peptides – has this been overly simplified?

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ABSTRACT

Natriuretic peptides (NPs) were first described as cardiac biomarkers more than two decades ago. Since that time, numerous studies have confirmed NPs' diagnostic and prognostic utilities as biomarkers of myocardial function. However, we must now admit that despite the NPs' relatively long period of use in clinical practice, our understanding of the biochemistry and the variety of circulating forms of NPs, as well as of their potential as biomarkers, remains far from being complete and comprehensive. The highly complex nature and wide diversity of circulating forms of NPs make their accurate measurements in plasma far more complex than initially believed. A highly simplistic view of the NPs' use is that elevated values of NPs indicate the severity of heart failure and thus reflect the prognosis. However, as shown by a variety of studies, deep understanding of how the NP system works will be required for correct interpretation of test results in routine practice of cardiovascular disease. In this review, we summarize the recent advances in understanding of the complexity of the NP system and discuss related analytical issues, which open new horizons, as well as challenges for clinical diagnostics.

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BACKGROUND

Natriuretic peptides (NPs) belong to a family of structurally and functionally related circulating peptides involved in maintaining cardio-renal homeostasis. The finding that the heart not only reacts to the humoral stimulus but actively participates in maintaining the fluid and salt balance by producing physiologically active compounds emerged with the discovery of atrial natriuretic peptide (ANP) and later of B-type natriuretic peptide (BNP) (1, 2). This new concept of the heart as an endocrine organ was introduced in 1981, when de Bold et al. showed that the intravenous injection of atrial extracts into rats led to an increase in sodium chloride and fluid excretion and a decrease in

blood pressure, showing involvement in the maintenance of blood pressure, water, and electrolyte balance in organisms (3). The two peptides, ANP and BNP, have been shown to display very similar physiological activities and under normal conditions are mainly produced in the atria. Both peptides are known to reduce vascular resistance and increase both diuresis and sodium excretion, reducing systemic blood pressure (4-6).

The third member of the NP family, C-type natriuretic peptide (CNP), was isolated shortly after the discovery of ANP and BNP (7). However, in contrast to ANP and BNP, which are mainly produced in the myocardium, CNP is predominantly produced in the central nervous system and vascular endothelium and acts as a paracrine factor (8). This member of the NP family was not shown to have much value as biomarker of cardiovascular complications. As a result, CNP did not attract much interest and was not introduced to routine clinical practice.

All 3 members of the NP family share a common structural feature – a ring structure consisting in humans of 17 amino acid residues (aar) and formed by an intramolecular disulfide bond. It is thought to be essential for mediating receptor binding and the biological activity of NPs (Fig. 1).

Two types of natriuretic peptide receptors (NPRs), NPR-A and NPR-B, are responsible for most of the physiological effects of NPs. The binding of NPs to these guanylyl cyclase-coupled receptors leads to an increase in cGMP, resulting in natriuresis, vasorelaxation, diuresis, inhibition of the renin-angiotensin-aldosterone system, enhanced myocardial relaxation, inhibition of fibrosis and hypertrophy, promotion of cell survival, and inhibition of inflammation (reviewed in (9)). Another type of NPR, NPR-C, lacking the guanylyl cyclase domain, is responsible for clearance and possibly involved in regulation of the cell proliferation (reviewed in (10)).

Figure 1   **The primary structures of human ANP, BNP, and CNP**
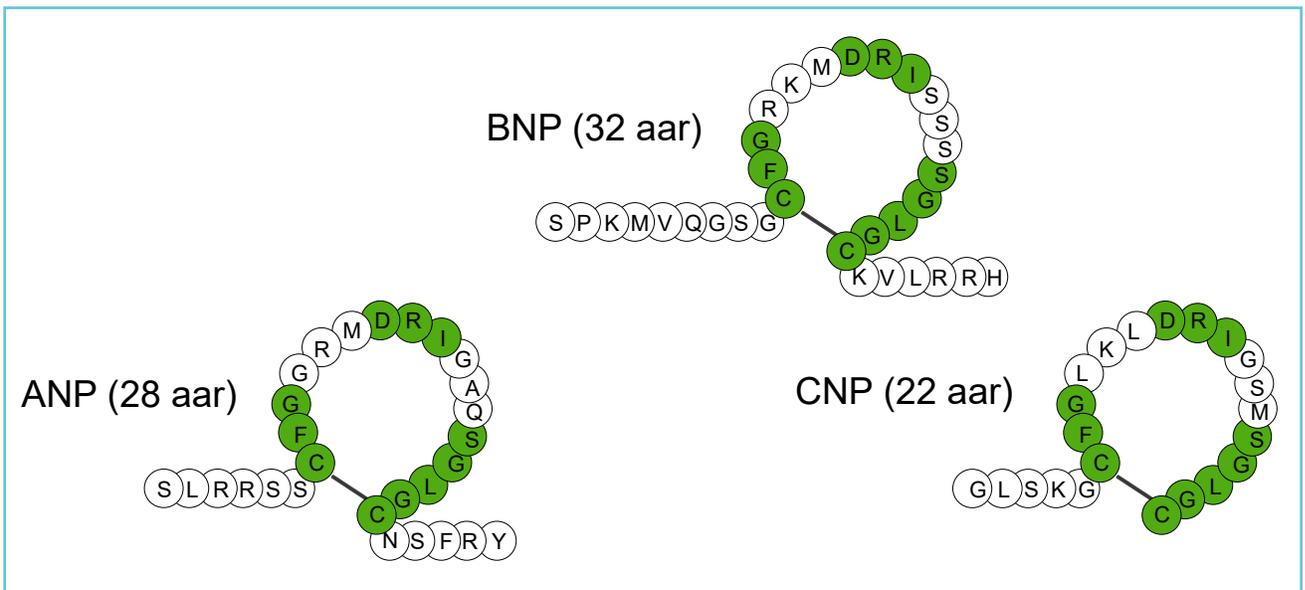
Similar residues are marked by a green background. The ring structure is formed by a disulfide bridge between two cysteine residues.

The expression and secretion of ANP and BNP increase significantly in pathological states accompanied by stretching of the heart chambers, volume overload, and ischemic injury, such as heart failure (HF) and myocardial infarction (MI) (11-13). Because the increase in their production and release in circulation is associated with the cardiac pathologies caused by pressure or volume overload, it was suggested that these peptides may be used as biochemical markers of HF. The diagnostic and prognostic utility of ANP and BNP was later confirmed in a large number of studies (reviewed in (14, 15)).

Considering the beneficial physiological activities of NPs in HF, recombinant forms of ANP and BNP were introduced as therapeutic agents for the treatment of this disease (16-18). Recombinant human ANP (carperitide) was approved in Japan in 1995 for intravenous administration in patients with acute heart failure (AHF) and acute decompensated heart failure (ADHF). Later, in 2001, recombinant BNP (nesiritide) was approved by the Food and Drug Administration (FDA) for acute congestive HF (ACHF). However, questions regarding the efficiency and safety of nesiritide diminished its use. Neseritide is currently considered to be safe, but with little beneficial effect (19).

As mentioned above, ANP and BNP share many common features and may be expected to have similar or even equal value as biomarkers. However, BNP was shown to have greater in vitro stability and superior diagnostic performance compared with ANP, and therefore BNP and its related peptides have emerged as the preferred candidates for the diagnosis of HF, as well as other clinical applications. Recent international guidelines recommend its use for the diagnosis, risk stratification, and follow-up of patients with chronic or acute HF (15, 20). As a consequence, the data regarding BNP-related peptides are currently more comprehensive that the data regarding ANP. In this review, we will focus mostly on the analytical issues related to BNP, as this member of the NP family is more interesting and important from a clinical perspective.

SYNTHESIS AND SECRETION OF ANP

ANP is translated from its gene as a 151-amino acid prepropeptide, preproBNP, that is stored in atrial granules (21). It is secreted and cleaved to a mature peptide, ANP, in response to atrial stretching or stimulation by angiotensin II, endothelin, as well as sympathetic stimulation. After cleavage of the signal peptide (a common element of all secretory proteins, which is responsible for addressing the protein to a secretory pathway) from the 151-amino acid preproANP, the precursor proANP is further processed by atrial convertase corin to produce two circulating peptides, ANP 1-28 and the N-terminal fragment NT-proANP 1-98 (22). The processing of proANP is considered to occur at the time of secretory granule release.

NT-proANP is also cleaved, forming 3 fragments that exhibit physiological activity: proANP 1-30 (long-acting atriuretic peptide), proANP 31-67 (vessel dilator), and proANP 79-98 (kaliuretic peptide, i.e., potassium excretion). All four fragments are present in the circulation (23).

A longer version of ANP called urodilatin, containing four additional N-terminal residues (ANP 1-32), is primarily found in the kidney. It promotes diuresis by increasing renal blood flow (24).

ANP IMMUNOASSAYS

As was discussed above, ANP was less widely accepted as a HF biomarker than BNP and was thus somewhat overshadowed by its sibling, BNP. As a consequence, there is currently only one commercially available immunoassay specific to an ANP-related peptide, which detects the mid-regional zone of pro-ANP (MR-proANP). This assay is manufactured by Thermo Fisher Scientific and it is not yet FDA cleared yet. It utilizes polyclonal sheep antibodies specific to the 50-72 aar of proANP

as coat antibodies along with monoclonal rat antibodies specific to the fragment 73-90 of proANP (25).

SYNTHESIS AND SECRETION OF BNP

The BNP gene encodes a 134-amino acid preproBNP precursor, which is converted to 108-amino acid proBNP by the cleavage of a 26-amino acid signal peptide (26). Interestingly, a fragment of preproBNP signal peptide (17-26 AAR) was shown to be present in the blood of normal individuals and patients with acute MI and was suggested as a circulating biomarker of cardiac ischemia and MI, with some possible advantages over currently used biomarkers such as creatine kinase-MB, myoglobin, and troponins (27). Similarly, a fragment of preproANP signal peptide (16-25 aar) was shown by the same research group to have some potential as an ischemic biomarker (28).

The BNP gene is an early response gene allowing rapid reaction to mechanical, hormonal or sympathetic stimulation: its transcription reaches a maximal level within 1 h after stimulation (29). Synthetized BNP is thought to be stored in limited amount and in acute need is produced de novo. Therefore, the predominant source of circulating BNP appears to be through constitutive secretion from ventricular myocytes. The storage and secretion of ANP are different: ANP is mostly stored in atrial granules and, as a consequence, is available for fast release if needed.

The processing of proBNP gives rise to two fragments: the N-terminal fragment of proBNP (NT-proBNP, 1-76 aar) and the C-terminal region active BNP hormone (77-108 aar) (30). BNP and NT-proBNP appears exclusively as a result of the proteolytic cleavage of proBNP in a stoichiometric ratio of 1:1. For a long time, it was strongly believed and accepted that BNP and NT-proBNP are the principal proBNP-derived molecular forms present in the circulation.

Whether NT-proBNP has any physiological function remains unknown. This fragment is currently considered to be a byproduct formed during maturation of the active BNP hormone. Although NT-proBNP and BNP are produced in an equimolar ratio, the molar plasma concentration of NT-proBNP is several-fold higher than the concentration of BNP. Higher levels of NT-proBNP are thought to be caused by the lower clearance of NT-proBNP from the bloodstream (31). Notably, intact nonprocessed proBNP is also present in the circulation and represents a substantial part of the BNP-immunoreactivity found in the samples of HF patients (discussed below) (32, 33).

POSTTRANSLATIONAL MODIFICATIONS OF proBNP

Both proBNP from the plasma of HF patients and recombinant protein produced in eukaryotic cells were shown to be extensively O-glycosylated at several threonine and serine residues within the N-terminal region (1-76 aar), but not within the BNP-portion of proBNP (77-108 aar) (34-36). In a study by Schellenberger et al., 7 sites of O-glycosylation in recombinant proBNP, expressed in Chinese hamster ovary cells, were identified within the region 1-76 aar of proBNP (i.e., NT-proBNP): Thr36, Ser37, Ser44, Thr48, Ser53, Thr58, and Thr71 (34). Notably, no sites of O-glycosylation were identified within the BNP-part of proBNP molecules.

The finding that proBNP undergoes posttranslational modifications during its maturation had a great impact on the understanding of the biochemistry and complexity of circulating proBNP-derived peptides. Although the exact glycosylation sites of endogenous proBNP and NT-proBNP are still not precisely characterized, indirect data indicate the presence of carbohydrate residues in specific parts of the molecules. According to Seferian et al., the central region (28-56 aar) of NT-proBNP is glycosylated, whereas the C-terminal portion of the molecule (61-76 aar) is mostly free of O-glycans (37). However, endogenous proBNP was shown to be glycosylated both in the central region and in the region located close to the cleavage site, in the region 63-76 for proBNP, which was inaccessible to site-specific antibodies because of glycosylation (38).

The level of endogenous NT-proBNP and proBNP glycosylation in humans seems to be characterized by high interindividual variability, which may arise from the site occupancy, structure, and length of oligosaccharide chains (36, 37). The clinical significance of this variability is currently unknown, and it might be interesting to explore whether it is related to the severity of HF or its etiology.

GLYCOSYLATION OF proBNP AND THE EFFICIENCY OF ITS PROCESSING

The diversity of circulating proBNP-derived peptides found in the circulation can be partially explained by the mechanisms of proBNP processing. The processing of proBNP is considered to occur prior to or in the moment of its secretion into the circulation. However, this understanding is primarily based on indirect observations. As for many other precursor polypeptides, the processing of proBNP is mediated by enzyme(s), namely prohormone convertases. Whether there is a unique convertase or several enzymes are responsible for the processing of proBNP remains an open question. Two proprotein convertases, furin and corin, are considered the most likely proBNP-processing enzymes. In vitro experiments have shown both furin and corin to process proBNP, with the formation of distinct BNP forms: BNP 1-32 (furin) and BNP 4-32 (corin) (39). As corin produced a shorter BNP form (i.e., BNP 4-32), this convertase is relatively unlikely to be the main enzyme responsible

for the processing of proBNP, which highlights the relevance of furin as a proBNP-processing enzyme (reviewed in (40)).

Glycosylation in the region close to the proBNP cleavage site was shown to play a pivotal role in the regulation of the enzyme-mediated processing of proBNP (38). The presence of glycosidic residues in this region of the proBNP molecule was found to suppress the processing of proBNP. In cell-based assays both furin- and corin-mediated processing of proBNP were shown to be suppressed by O-glycans attached to Thr71. It is currently believed that only proBNP molecules that are not glycosylated at Thr71 can be effectively processed into BNP and NT-proBNP. Whether this suppression of proBNP processing by glycosylation at the Thr71 residue is a physiological regulatory process or a pathophysiological mechanism leading to HF progression remains an open question.

The role of glycosidic residues at other sites of proBNP molecule is currently unclear. Because glycosylation is known to be a highly energy-consuming process, it is very unlikely that it has no specific role in the function of BNP. One possibility is that the glycosylation of proBNP within the central region might protect it from undesirable cleavage at other monobasic or dibasic sites in the human proBNP sequence and thus prevent the formation of longer BNP forms. Additionally, in vitro experiments have shown O-glycosylation to increase the stability of proBNP, which may be essential in light of the presence of proBNP and its potential function in the circulation (discussed below) (41).

MOLECULAR FORMS OF BNP IN PLASMA

Initially, it was believed that there were two circulating fragments present in the circulation, BNP 1-32 and NT-proBNP 1-76, formed by endoproteolytic cleavage of proBNP between the Arg76 and Ser77 residues. However, this

concept has recently been greatly modified (42, 43). It was found that only a tiny portion of circulating BNP consists of intact BNP 1-32, which was initially considered a main form of immunoreactive BNP. The absence of BNP 1-32 in plasma samples from patients with advanced HF was reported by Hawkridge et al., challenging the primary simplified concept of BNP 1-32 as a major component of BNP-immunoreactivity in the blood samples of HF patients (44). The work of Niederkofler and coworkers accurately showed that in the plasma of HF patients, BNP 1-32 is present alongside various N- and C-terminal truncated BNP forms, i.e., BNP 3-32, BNP 4-32, BNP 5-32, BNP 5-31, BNP 1-26, and BNP 1-25 (43).

The proteolytic degradation of BNP 1-32 in the circulation is thought to be responsible for the diversity of BNP-derived forms found in the collected samples. Peptidases such as dipeptidyl peptidase IV (DPP IV) and neutral endopeptidase (neprilysin, NEP) were reported to degrade BNP, giving rise to BNP 3-32 and BNP 5-32, respectively (45, 46). Some studies have suggested that insulin-degrading enzyme (IDE) can also degrade BNP to smaller peptides (47, 48). However, BNP was shown to be a poor substrate for neprilysin and IDE, suggesting that another protease is likely responsible for its cleavage. Additionally, the appearance of BNP 4-32 in the circulation may be due to the specific processing activity of corin, as shown by in vitro experiments with exogenous proBNP (39). According to the study of Belenky et al., peptidyl arginine aldehyde protease can degrade BNP at sites in the peptide chain where arginine is present, as specific inhibitors of this enzyme greatly reduce the degradation of the hormone in vitro (49). In mice, plasma protease meprin was shown to cleave BNP (46); however, its ability to degrade BNP in humans is rather questionable (50). The known sites of BNP proteolytic degradation are summarized in Figure 2.

Figure 2   The known main degradation sites of BNP by the action of DPP IV, NEP and IDE
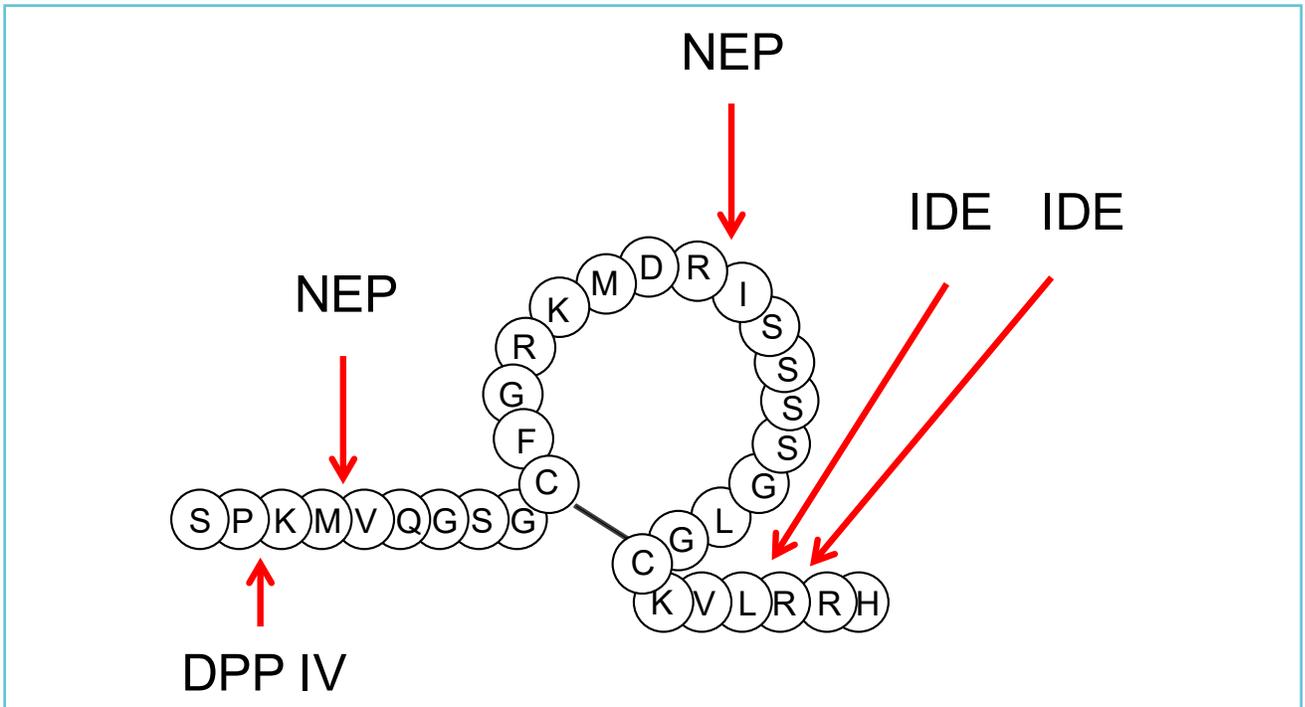
Notably, the instability of BNP in EDTA-plasma samples has been reported even at -80 °C. Thus, to protect BNP from degradation during sample storage, high concentrations of protease inhibitors are required (benzamidine up to 10 mmol/L and AEBSF up to 5 mmol/L) (42).

There is still an open question regarding whether all these BNP forms are equally bioactive. The data on this subject are not consistent. In cell-based assays with cardiac fibroblasts and cardiomyocytes, human BNP 3-32 exhibited similar activity to BNP 1-32 (51). However, in vivo studies in canine models revealed that human BNP 3-32 exhibited reduced natriuresis and diuresis and a lack of vasodilating actions compared to BNP 1-32 (52). Thus, this perspective might suggest that shorter BNP forms do exhibit reduced biological activity compared to the full-length BNP molecule.

ProBNP AS A MAJOR COMPONENT OF BNP-IMMUNOREACTIVITY

A number of studies have convincingly shown that the intact precursor proBNP is the major BNP-immunoreactive form found in collected plasma samples, both in healthy subjects and especially in patients with congestive HF. These findings had a great impact on our understanding of the results of BNP measurements by the routinely used immunoassays, as most of them exhibit cross-reactivity with proBNP due to the presence of BNP-structure within the proBNP sequence. The degree of cross-reactivity was reported to be different for different assays and different forms of proBNP (e.g., glycosylated vs. nonglycosylated) (53).

Because proBNP shares a common 32-amino acid structure with BNP, it is logical to suggest that proBNP might be capable of mediating physiological functions similarly to BNP.

However, in cell-based assays, unprocessed proBNP exhibited markedly reduced physiological activity compared with BNP and is currently considered to be insufficient to promote an adequate physiological natriuretic hormone response in HF patients (54). Additionally, proBNP was shown to have significantly lower affinity for NPR-C than BNP and to be more resistant to proteolytic inactivation by human kidney membranes (55).

The role of proBNP in the circulation and why it is released into the circulation in its unprocessed form remain intriguing questions. Whether it is a normal physiological or rather a pathophysiological process still needs to be clarified to better understand its clinical significance.

IS proBNP A CIRCULATING SOURCE OF BNP HORMONE?

High plasma levels of unprocessed proBNP and the potentially reduced bioactivity of proBNP compared to BNP suggest that circulating proBNP may serve as a reserve BNP-containing form to be processed in the circulation for the release of active BNP hormone. The question of whether proBNP might undergo processing in the circulation was addressed in several studies by testing the in vitro production of BNP from proBNP(56, 57). Although the results of these studies indicate that the cleavage of exogenous nonglycosylated proBNP may occur in serum samples, they should be interpreted with caution, as the relevance of serum as an in vitro model to study proBNP processing in the circulation is rather questionable

Following from the inhibitory effect of O-glycans attached to the Thr_{71} residue of proBNP, the efficiency of proBNP processing depends not only on the activity of convertase(s) but also the glycosylation status of the residue located close to the cleavage site, i.e., Thr_{71}. As we know, proBNP glycosylated at Thr_{71} is not processed by furin

or corin. Thus, it is straightforward to ask which form of proBNP is present in the circulation.

It was found that there are two distinct forms of proBNP in the circulation, which differ in the glycosylation status of Thr_{71} residue: proBNP glycosylated at Thr_{71} and proBNP, which lacks glycan residues at this site. Among these two forms, only proBNP which does not bear any glycans attached to Thr_{71}, was shown to be susceptible to proteolytic cleavage and may give rise to active BNP 1-32 hormone. Interestingly, this observed variability in glycosylation status is apparently attributed only to this site and is not the case for other sites of glycosylation within the proBNP molecule.

Our studies in rats have revealed that the processing of human nonglycosylated proBNP in the circulation, resulting in the formation of BNP 1-32, is possible (58). These data, taken together with the findings that there are two distinct forms of proBNP in the plasma of HF patients, one glycosylated (processing-unsusceptible) and the other non-glycosylated (processing-susceptible) in the region close to the cleavage site, suggest the possibility of peripheral proBNP processing in the circulation (39).

DIVERSITY OF proBNP FORMS IN ACUTE AND CHRONIC HF

Recent studies by Vodovar and colleagues shed some light on the interplay between proBNP glycosylation and its processing in the circulation (59). In this study, the degree of plasma proBNP glycosylation was assessed in three groups of HF patients, i.e., patients with ADHF, non-ADHF (dyspnea but no HF) patients and chronic HF patients, by means of mass spectrometry. Among these three groups, the highest percentage of glycosylated proBNP was present in chronic HF. In contrast, the percentages of glycosylated proBNP in ADHF and non-ADHF patients were lower than in

chronic HF and similar to each other. These data suggest that proBNP processing is altered more in chronic HF than in ADHF or non-ADHF. Strikingly, furin activity but not its concentration was greater in ADHF than in chronic HF, thus providing a differential mechanism of proBNP processing in disease progression in HF. Considering these findings, one might speculate that the significantly increased production and processing of proBNP in ADHF might represent an attempt by the failing heart to increase the level of circulating BNP and to

reduce the overload. In contrast, in chronic HF, with the release of more glycosylated proBNP into the circulation, there will be a defect in the processing of proBNP to mature BNP hormone, as this proBNP form is not susceptible to processing. These findings may reflect the existence of regulatory mechanisms through which plasma BNP rapidly increases in acute conditions by cleavage of the processing-susceptible proBNP form (60).

From a physiological and clinical prospective, there are several important consequences of

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Figure 3   **The scheme of proBNP maturation and processing with the suggested inhibitory effect of O-glycans bound to the Thr_{71} on processing efficiency**
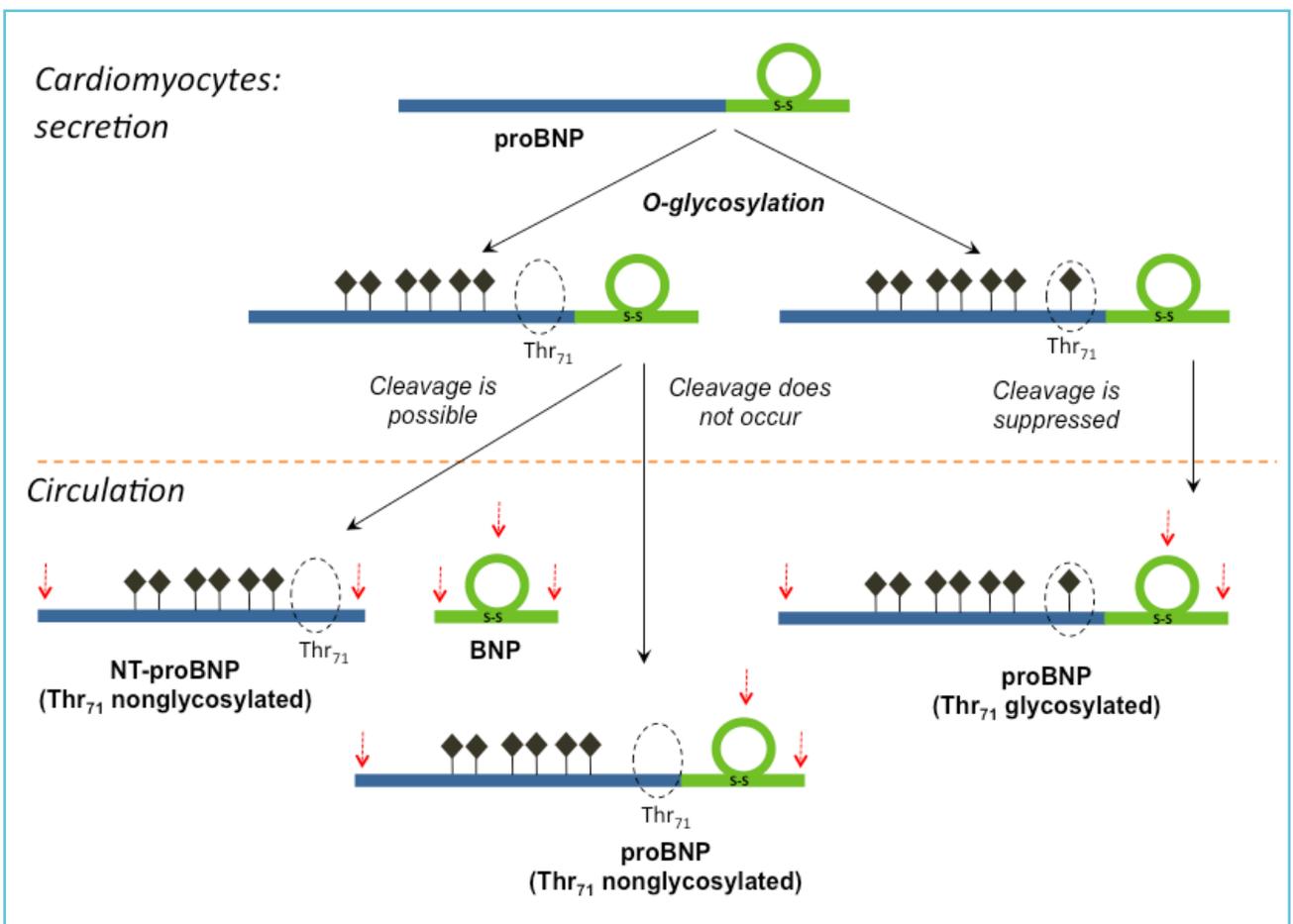
Seven potential sites of O-glycosylation are marked as dark diamonds (34).

The potential N- and C-terminal sites of proteolytic degradation as well as the ones located within the ring structure of BNP (proBNP) are marked by red arrows.

Adapted with modifications from (40).

these new findings. First, it means that the BNP-related peptides are likely different in different HF patients and there is no common BNP status for different forms of HF. Second, there are apparent differences in the processing of NPs between patients with ADHF and patients with chronic HF. From this perspective, one may suggest that immunoassays that can differentiate the glycosylated and nonglycosylated forms of proBNP might have additional value for clinical diagnostics. Such assays are not currently available; however, their development is potentially possible due to the known sensitivity of antibodies to the presence of O-glycans in the recognized epitopes (32).

The recent advances in the understanding of the diversity of circulating BNP forms have considerably changed the initial simplistic scheme of proBNP processing, suggesting that only a few forms are present in the circulation, and have led to a new scheme of proBNP maturation and processing (Fig. 3).

THE DIVERSITY OF IMMUNOASSAYS FOR proBNP-DERIVED PEPTIDES

More than 20 years after the introduction of NPs as cardiac biomarkers, there are a variety of immunoassays, specific for different forms of the peptide, in use by clinicians. We will briefly discuss the most important and recent findings in this field and the impact of the complex biochemistry of proBNP-derived peptides on the interpretation of the test results.

NT-proBNP ASSAYS

The current situation involving NT-proBNP immunoassays is relatively simple. All approved commercially available NT-proBNP immunoassays are based on the same antibodies and calibrator materials distributed by Roche. As a result of a common source of antibodies and calibrator, only small systematic differences between the available NT-proBNP immunoassays have usually been reported, with the total variation across methods within 10%. However, despite the common source of antibodies and standard materials, assay harmonization remains incomplete (61).

The initially proposed cut-off for NT-proBNP assays is below or above 125 ng/L. A value of 300 ng/L works well with the existing assays for the exclusion of acute HF (62).

The first generation of Roche NT-proBNP assays was based on polyclonal antibodies specific for the regions 1-21 and 39-50; the second generation employs monoclonal antibodies (mAbs) specific for the central region of NT-proBNP: 22-28 (27-31) and 42-46. The epitope specificity of the antibodies used in these assays suggests interference from the glycosylation of NT-proBNP molecule, as these regions of NT-proBNP were shown to be glycosylated. Indeed, the negative effect of glycosylation on NT-proBNP recognition by the antibodies specific to the middle fragment of the molecule has been shown in several studies. Commercial NT-proBNP immunoassays were revealed to show substantial cross-reactivity with non-glycosylated proBNP but can barely recognize glycosylated NT-proBNP and proBNP molecules due to the presence of O-glycans in the epitopes recognized by the antibodies. However, although it has been shown that Roche NT-proBNP assays underestimate the concentration of circulating NT-proBNP (up to 10-fold) (37, 63), their diagnostic and prognostic accuracy is quite good, and they are currently widely used in clinical practice.

However, the recent data suggest that underestimating the NT-proBNP concentration due to the influence of glycosylation of NT-proBNP molecules may have some impact on the clinical significance of this biomarker. This subject has been addressed in the work of Helge Røsjø et al., which showed that the deglycosylation

of NT-proBNP by treatment with a mix of specific enzymes (deglycosidases) improved the the diagnostic and prognostic accuracy of the NT-proBNP assay (64). Thus, one may suggest that the epitopes not effected by glycosylation should be preferred for the design of new generations of NT-proBNP assays.

B-TYPE NATRIURETIC PEPTIDE ASSAYS

In contrast to NT-proBNP, the current situation with BNP immunoassay is far more complex. A variety of companies market assays for BNP, which are based on different antibodies and standard materials. Recent studies suggest that there are marked systematic differences among the BNP values obtained using different platforms. The CardioOrmoCheck study reports differences of up to 50% across different BNP immunoassays (65), and such discrepancies occur even for assays using the same antibodies but run on different instruments.

The most common commercial methods for BNP measurement used in the clinical laboratories are sandwich-type immunometric assays. These methods usually employ two antibodies specific for two distantly located epitopes of the BNP peptide chain. One of these antibodies is always specific for the intact cysteine ring, to detect the form, which is considered to be physiologically active, whereas the other is specific either for the C-terminus of the peptide (e.g., in Abbott AxSYM and Architect, Shionogi IRMA) or for the N-terminus (e.g., in Alere Triage and Beckman Access). Obviously, the assays utilizing antibodies specific to the very C-terminus of the BNP molecule should not measure BNP peptides that are degraded at this part of the molecule. Similarly, assays utilizing antibodies specific to the N-terminus of BNP should not measure BNP-related peptides truncated at this part of the molecule.

Among this variety of BNP immunoassays, the "Single Epitope Sandwich" Immunoassay (SES-BNP™) designed by HyTets's specialists and implemented in a platform by ET healthcare differs from conventional sandwich-type BNP assays (66). This assay utilizes one mAb 24C5 specific to the relatively stable ring fragment of the BNP molecule (epitope 11-17), which is within the biologically active cysteine ring, and the second mAb, Ab-BNP2, which recognizes the immune complex of mAb 24C5 with BNP (proBNP) only. Thus, there is no space between epitopes, and consequently, cleavage between the epitopes does not affect it, as only one epitope is needed for BNP measurements in the SES-BNP™ assay. This assay was shown to be able to recognize BNP as well as the recombinant glycosylated and nonglycosylated forms of proBNP with the same efficiency.

The high sensitivity of SES-BNP™ assay (up to 0.5 pg/mL) is most likely achieved by the "locking" effect of the detection antibody – it stabilizes the immune complex of the capture antibody with the antigen and increases the affinity of the capture antibody for its antigen.

The initially proposed cut-off for BNP is 100 ng/L, which excludes acute HF with high negative predictive value. However, it should be stressed that due to the high substantial differences between different BNP immunoassays, this value should be determined for each assay and standard material used in calibration (67).

The diversity of antibodies and standard materials used in commercially available BNP immunoassays is summarized in Figure 4.

The great heterogeneity of proBNP-derived peptides circulating in human blood can partially explain the systematic differences among the results provided by immunoassay methods considered specific for BNP. Another important cause of non-harmonized BNP assays may be the lack of a suitable reference material for the

Figure 4	Antibodies and standard materials used in commercial BNP immunoassays
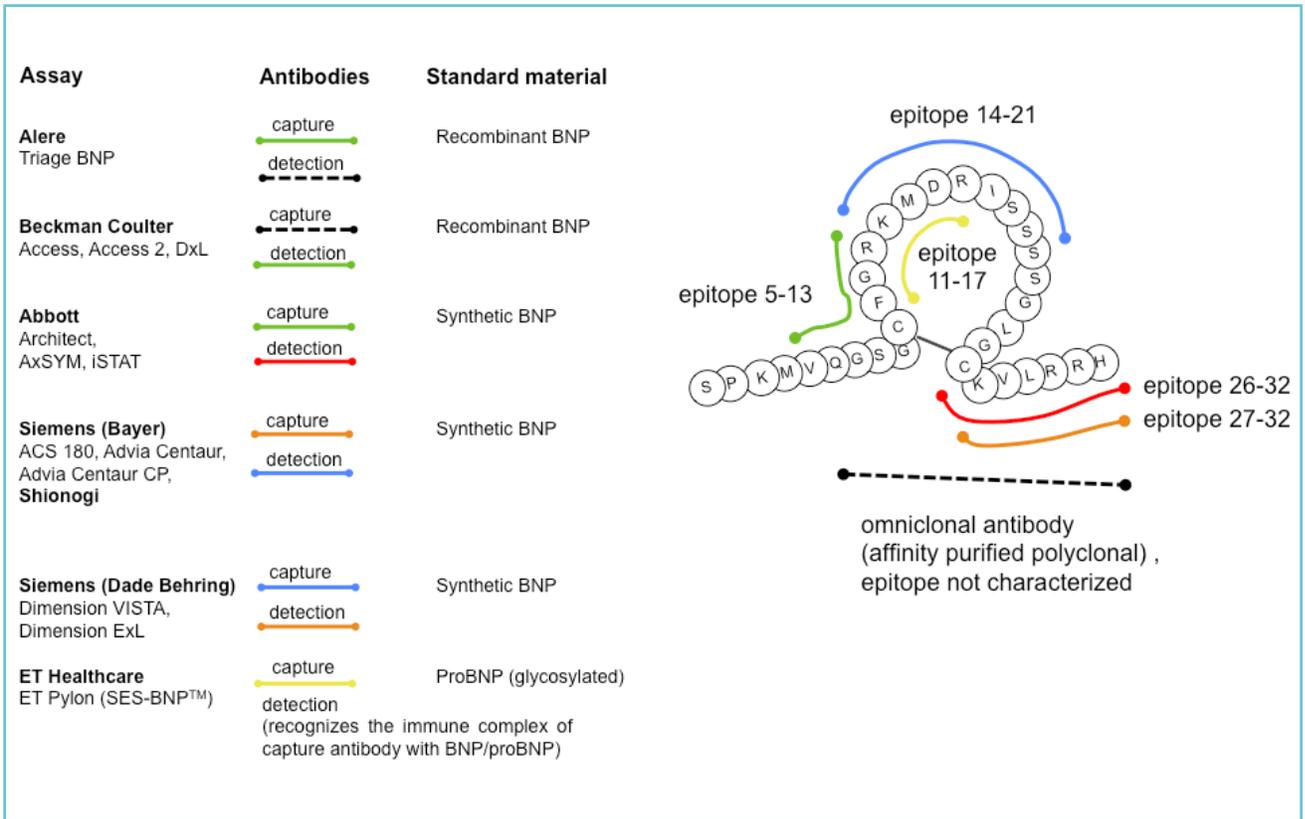
Adapted with modifications from:
http://www.ifcc.org/media/102208/NP%20Assay%20Table%20C%20SMCD%20vJuly_2011.pdf.

calibration of BNP assays by manufacturers. As a consequence of the non-harmonized assays, the obtained results are often unique to a certain method or instrument, so that different results from different assays and platforms are poorly comparable. Thus, a common calibrator used in all these BNP assays might help to reduce the variability of the obtained values. Currently, no such common calibrator has yet been suggested. Considering the prevalence of proBNP as a major BNP-immunoreactive form, one might suggest that proBNP could become a common calibrator and stable standard for BNP immunoassays. The introduction of a common reference material should reduce the systematic differences among current methods and result in better harmonization among results.

Additionally, it was revealed that all BNP immunoassays share some cross-reactivity with proB-NP, as proBNP has the same structure (BNP-part) as the BNP molecule (53). Considering that proBNP is the major BNP-immunoreactive form in the circulation of HF patients, this cross-reactivity is clinically relevant. Some assays may hardly recognize proBNP at all, especially its glycosylated form, due to the steric hindrance of the glycosidic residues.

Therefore, due to the high complexity of BNP-related peptides and the prevalence of proBNP in the circulation, much of the BNP measured by contemporary assays is either nonprocessed proBNP or degradation products of BNP 1-32 rather than intact mature BNP 1-32.

BNP ASSAYS AND THE RECENT ADVANCES IN THE TREATMENT OF HF

The recent data regarding the use of neprilysin inhibitor and angiotensin receptor blocker LCZ696 as a therapeutic agent developed by Novartis in patients exhibiting HF with a reduced ejection fraction have greatly stimulated the interest to the use of BNP and NT-proBNP in HF diagnostics and the monitoring of therapy and also raised some important questions.

The PARADIGM-HF trial (Prospective Comparison of ARNI (angiotensin receptor neprisylin inhibitor) With ACEI (angiotensin-converting enzyme inhibitor) to Determine Impact on Global Mortality and Morbidity in HF) demonstrated a marked improvement in outcomes with LCZ696 compared with enalapril (inhibitor of angiotensin-converting enzyme) alone in patients with predominantly New York Heart Association functional class II HF (68).

Neprilysin is a widely expressed membrane-bound protease, particularly abundant in kidney, that cleaves substrates on the amino side of hydrophobic residues. It has been shown to cleave and inactivate a number of peptides, including glucagon, enkephalins, substance P, neurotensin, oxytocin, bradykinin and amyloid beta (reviewed in (69)). Both ANP and CNP are known to be substrates of neprilysin (70). However, BNP was shown to be a poor substrate for neprilysin, as its specific inhibitors failed to block BNP degradation by human kidney membranes, suggesting that it is unlikely to be a significant regulator of BNP concentration in the kidney (50).

As neprilysin is thought to be responsible for degrading NPs, it is possible that the beneficial effect of this new drug is achieved by inhibiting NP degradation, increasing the level of NPs, ANP and BNP and, as a consequence, improving HF.

However, the suggestion that inhibition of neprilysin should lead to a prompt and prominent increase in BNP level is rather debatable. The effect of neprilysin inhibition should be more prominent for ANP than for BNP, as ANP is known to be a much better substrate for neprilysin than BNP (71). Given the complexity and diversity of proBNP-derived peptides, it is hardly possible that the effect of LCZ696 on the BNP level can be so simple. As the major form of BNP-immunoreactivity is proBNP, we should rather consider its degradation and the effect of the drug on the level of proBNP rather than BNP. Unfortunately, there are currently no data on the degradation of proBNP by the action of neprilysin.

Whether treatment with neprilysin inhibitor will interfere with the use of BNP measurement for HF diagnosis and prognosis or treatment monitoring remains an open question. Considering the complex biochemistry of proBNP-derived peptides, it is definitely not obvious how the BNP and NT-proBNP levels are affected by treatment with LCZ696 in different disease states (72).

On the one hand, if treatment with LCZ696 indeed affects the BNP levels measured by BNP immunoassays, then it seems that BNP measurements may be ambiguous in this case. For this purpose, NT-proBNP measurements seem to be preferred, although it should be considered that the increase in circulating BNP might decrease proBNP production and thus decrease the NT-proBNP level, which would then fail to reflect the improvement of cardiac function. On the other hand, measurements of BNP may be very important to understand at what level of BNP increase the drug therapy works and reflect the action of the drug, whereas NT-proBNP levels may reflect the effects of the drug on the heart.

The complexity of the NP system and the diversity of HF states suggest that the measurements of either BNP or NT-proBNP alone might not be sufficient to fully understand the HF status of patients, but rather both biomarkers (or their ratio) should be used to fully utilize the diagnostic and prognostic value of these biomarkers.

Thus, the use of this new and seemingly promising HF drug generates a number of serious questions for clinical diagnostics that must be answered before this drug becomes routinely used in clinical practice along with NP measurements.

ProBNP IMMUNOASSAYS

By its nature, the proBNP molecule shares a common structure with both BNP (BNP part within proBNP sequence) and NT-proBNP (N-terminal part within proBNP sequence). Thus, a proBNP-specific assay should be based on one antibody specific to the N-terminal part and the second one to the C-terminal part. An assay based on a capture mAb specific for the region 26-32 of the BNP molecule and a detection antibody specific

for the fragment 13-20 of proBNP was designed by HyTest specialists (32). It was shown that this highly sensitive immunoassay is not affected by the glycosylation of proBNP molecules, as the epitopes of the utilized antibodies are free of O-glycans.

Giuliani et al. developed a specific mAb that recognizes the hinge region of the proBNP molecule (75-80 aar) (73). A sandwich immunoassay for the measurement of proBNP was designed by combining this mAb with a polyclonal antibody directed against the BNP part of the proBNP molecule (Fig. 5). An automated version of this method was performed on the BioPlex 2200 Analyzer Multiplex System (Bio-Rad), and its analytical characteristics were evaluated. Notably, the presence of O-glycans at Thr71 may affect the interaction of the hinge-specific antibodies with endogenous proBNP due to the close proximity to the recognized epitope and, as a consequence, underestimate the amount of intact proBNP detected by this assay in plasma samples of HF patients.

| Figure 5 | Schematic representation of proBNP-specific assays designed by HyTest and Bio-Rad. Seven potential O-glycosylation sites within the proBNP sequence are marked with dark diamonds (34) |
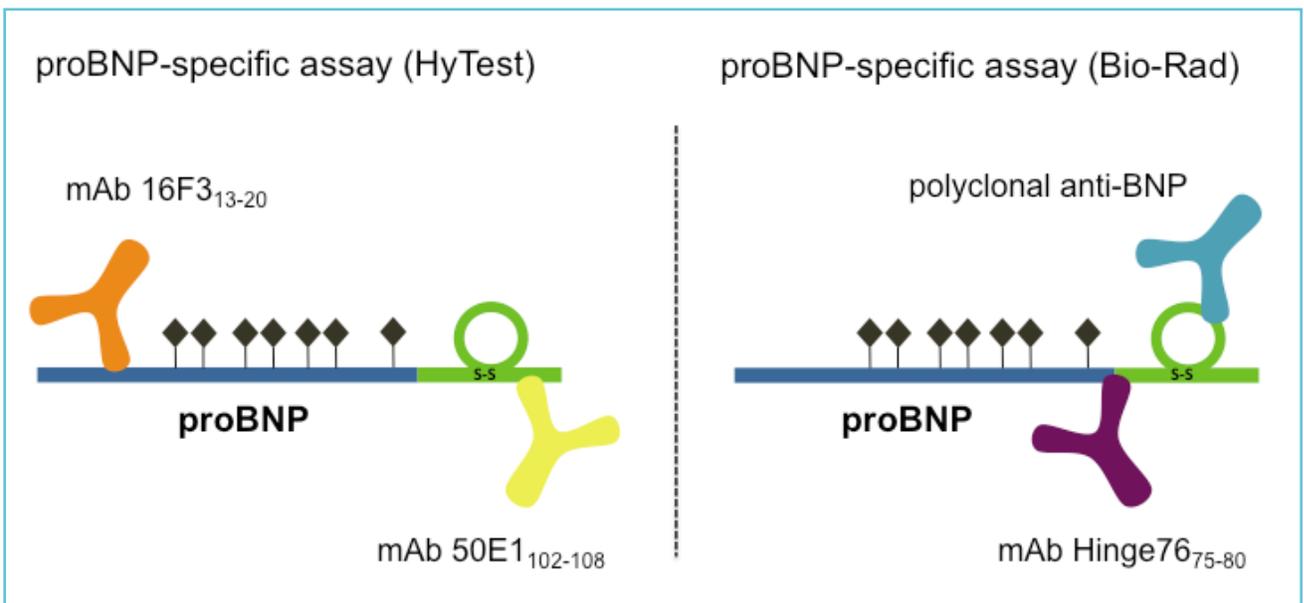
Thus far, proBNP-specific assays have been shown to be equivalent to but no better than BNP or NT-proBNP assays. (74). However, one may speculate that considering the potentially reduced bioactivity of unprocessed proBNP, measurements of the concentration of bioactive BNP or, alternatively, the ratio of BNP to unprocessed proBNP might be clinically relevant.

It should be stressed, however, that there are currently no assays that are specific to BNP with no cross-reactivity to proBNP. The development of such assays is rather challenging due to the presence of the common structure in the proB-NP molecule. Whether such an assay would have additional clinical significance over conventional BNP assays, which detect both BNP and proBNP, remains a question to be answered in future clinical studies.

CONCLUSIONS

NPs are widely accepted to be useful and cost-effective biomarkers for HF. Both BNP and NT-proBNP testing are currently routinely used in clinical practice and have been incorporated into most national and international cardiovascular guidelines. Despite this wide acceptance, the complex biochemistry of NPs requires deep insight into analytical issues for the accurate interpretation of test results in clinical practice. Moreover, the constant implementation of new therapeutic agents (e.g., LCZ696) for HF treatment generates new challenges for their use in diagnostics and the monitoring of therapy and requires comprehensive understanding of how this complex system works. Thus, new immunoassays based on the improved understanding of the complex biochemistry of proBNP-derived peptides should perhaps be considered for future development.

The large systematic differences among methods when comparing the results obtained from different laboratories using different assays represents another important issue to be solved. Novel approaches such as the introduction of a common reference material for BNP immunoassays should be considered to improve this situation.

Additionally, the diversity of circulating forms of BNP-related peptides with different physiological activities suggests that a new generation of immunoassays specific to the distinct forms of BNP, NT-proBNP and proBNP or able to measure the ratio between different BNP forms might offer potential clinical significance over existing assays that do not distinguish different circulating forms of proBNP-derived peptides.

To summarize, we may conclude that recent advances in understanding the complexity of the NP system have both improved the comprehension of the clinical meaning of test results and generated a number of new challenges to be addressed in future studies to improve the diagnostics and treatment of cardiovascular complications.

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Can natriuretic peptides be used to guide therapy?

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ABSTRACT

Over the last 15 years, the hypothesis that intensified treatment directed at reducing natriuretic peptide (NP) concentrations may improve the outcomes of patients with heart failure (HF) has been scrutinized in several prospective clinical trials, with conflicting results. Collectively, however, the data suggest that NP concentrations may be useful in guiding HF management and improving HF-related morbidity and mortality. In this review, we summarize the existing data investigating the use of NPs as targets for outpatient HF therapy. We focus on the information gathered in randomized clinical trials and comprehensive meta-analyses, and also on the recommendations of international guidelines (primarily guidelines from the European Society of Cardiology and the American College of Cardiology/American Heart Association). Although the results for this approach are promising overall, additional well-designed prospective randomized controlled trials (e.g., the GUIDE-IT trial) are necessary to confirm or refute the utility of NP-guided outpatient HF management.

CAN NATRIURETIC PEPTIDES VALUES BE USED TO GUIDE HEART FAILURE THERAPY?

Clinicians have been asking this question for 15 years now, and the answer is still unclear. Richard Troughton and Mark Richards published a seminal paper in *The Lancet* in 2000, in which they launched the hypothesis of guiding heart failure (HF) treatment with objective measurement of natriuretic peptides (NPs). This prospective pilot study was conducted in Christchurch, New Zealand and included 69 patients with a history of decompensated HF and systolic dysfunction. The participants were randomized to management by a standardized clinical algorithm or to clinical management with NP-guided drug uptitration (1). The goal in the NP-guided arm was to drive plasma concentrations of NTproBNP to <200 pmol/L (approximately 1700 pg/mL). During 9 months of follow-up, patients who received NP-guided treatment had significantly fewer deaths or hospitalizations for HF. This study recruited relatively young patients with reduced left ventricular ejection fraction (LVEF); however, due to patient enrollment in the late 1990s, very few of the patients were on beta blockers or mineralocorticoid receptor antagonists (MRAs). This initial study has provided the nucleus for a multitude of prospective studies launched in the forthcoming years. Nevertheless, as of 2015, the results from the clinical trials published to date are in most cases conflicting, in part due to disparities in their design. Thus, the multiple meta-analyses that have been performed to clarify the situation have been less definitive.

WHAT DO THE CLINICAL TRIALS SAY?

Because NPs are reflective of hemodynamic state and disease severity in HF, their role in therapeutic guidance has been investigated in several clinical trials. Three potential strategies for using cardiac peptides in the management of HF patients may be considered.

The first approach consists of targeting pharmacologic therapy to prespecified NP concentrations to optimize the effects of drugs. This approach has received much interest and has been tested in several prospective randomized trials that yielded conflicting results. Some studies demonstrated mortality or morbidity benefits from NP-guided therapy: Troughton, STARS-BNP, Berger, PROTECT (1,3-5); others reported benefits only in younger patients: TIME-CHF, BATTLESCARRED (6,7); or only in responder patients: UPSTEP (8); and other studies showed no advantages for NP-guided compared to clinically guided therapy: Beck-da-Silva, SIGNAL-HF, PRIMA, Anguita, STARBRITE (9-13).

The second strategy, reported in the recent NorthStar trial (2), assessed whether high-risk but stable chronic HF patients, identified as those with NTproBNP levels >1000 pg/mL, would benefit from prolonged specialized HF clinical assistance (pre-PARADIGM clinical treatment) compared to referral back to general practitioners. The results demonstrated no differences in the composite score for mortality and hospitalization for cardiac causes, suggesting that baseline NP had limited value in the selection of out-of-hospital management strategy in HF patients.

The problems with these trials are multiple but in many ways understandable as clinicians struggle to find the right metrics to use to guide therapy. Although some trials have had targets for titration of the natriuretic peptides, in many instances, these goals have not been achieved in a majority of the patients. If only 30% of the cohort reaches the goal suggested, one might ask "has the hypothesis really been tested?" In addition, should the goals of therapy be a fixed level of natriuretic peptide regardless of the starting point or should it be some percentage change in the baseline value or is there a need

for both types of criteria. This is of particular importance because of the marked biological variation of natriuretic peptides (14). In some studies, very large changes are necessary to be sure that the changes observed are due to treatment and not conjoint biological and clinical variability (15). Finally, the types of patients included may make a huge difference. Those with heart failure with preserved ejection fractions (HFPEF) tend to have different natriuretic peptide levels than those with heart failure with reduced ejection fractions (HFREF). Those with valvular heart disease may or may not be similar to either of those groups.

A third strategy may emerge with availability of LCZ696 in the market (post-PARADIGM clinical treatment). Although the mechanisms involved are complex, it appears that BNP levels are increased by LCZ696. On the other hand, NTproBNP values are reduced although we do not know if they are reduced commensurate with the levels that would be necessary to make outcomes with agents that do not include Neprilysin inhibition. Thus, it could be that NTproBNP will become the preferred peptide biomarker for therapy guidance (16).

In addition, given its markedly improved efficacy, it may be that natriuretic peptides elevations will help to identify those who may benefit from Neprilysin inhibition. An analogy between acute coronary syndrome (ACS) and chronic HF relative to the use of biomarkers and their impact on therapy is clear. In ACS, the presence of chest pain, ST segment ups and downs in the ECG and cardiac troponin rise and fall is indicative of a high-risk patient that requires urgent-preferred catheterization to open the culprit artery in order to relief symptoms and improve prognosis. In chronic HF, the presence of dyspnea, a reduced ejection fraction in the echocardiogram and a very high level of circulating natriuretic peptides may identify a high-risk patient who is a

candidate to switch to LCZ696 in order to improve symptoms, reduce mortality (both sudden and pump failure death), and reduce HF-hospitalizations (Figure 1). This is not strictly NP guided therapy, as it was firstly hypothesized by Troughton and Richards, but rather using NPs to prescribe a new treatment option which has shown a dramatic beneficial effect compared with conventional treatment. This new strategy is supported by the data from the PARADIGM Trial, the first trial in incorporating an objective measure of severity using NPs into the inclusion criteria (17).

WHAT DO THE META-ANALYSES SAY?

To overcome the uncertainty produced by the conflicting results of single studies of the first strategy described above, three meta-analyses investigated the utility of NP-guided therapy in patients with chronic HF (18-20). These meta-analyses comprised data from six, eight, or 12 randomized clinical trials.

In the meta-analysis by Felker et al. (18), only six studies were collected, which reported on 1,627 patients. Although a significant benefit for all-cause mortality in patients assigned to NP-guided therapy was reported, the analysis was limited by the inclusion of three still unpublished studies, which prevented detailed collection of patient population characteristics.

The meta-analysis by Porapakkham et al. (19) included 1,726 patients in eight studies. In this analysis, the favorable effect on all-cause mortality in patients assigned to NP-guided therapy was mostly driven by the TIME-CHF trial (6) in the sensitivity analysis section of the meta-analysis. The statistical significance of the effect was lost when the TIME-CHF trial, but not any other trial included in the meta-analysis, was removed from the analysis. Notably, no difference was observed for all-cause or HF-related hospitalization.

Figure 1	Scheme of the analogy between ACS and chronic HF (with the advent of LCZ696), and the value of biomarkers to guide decisions*
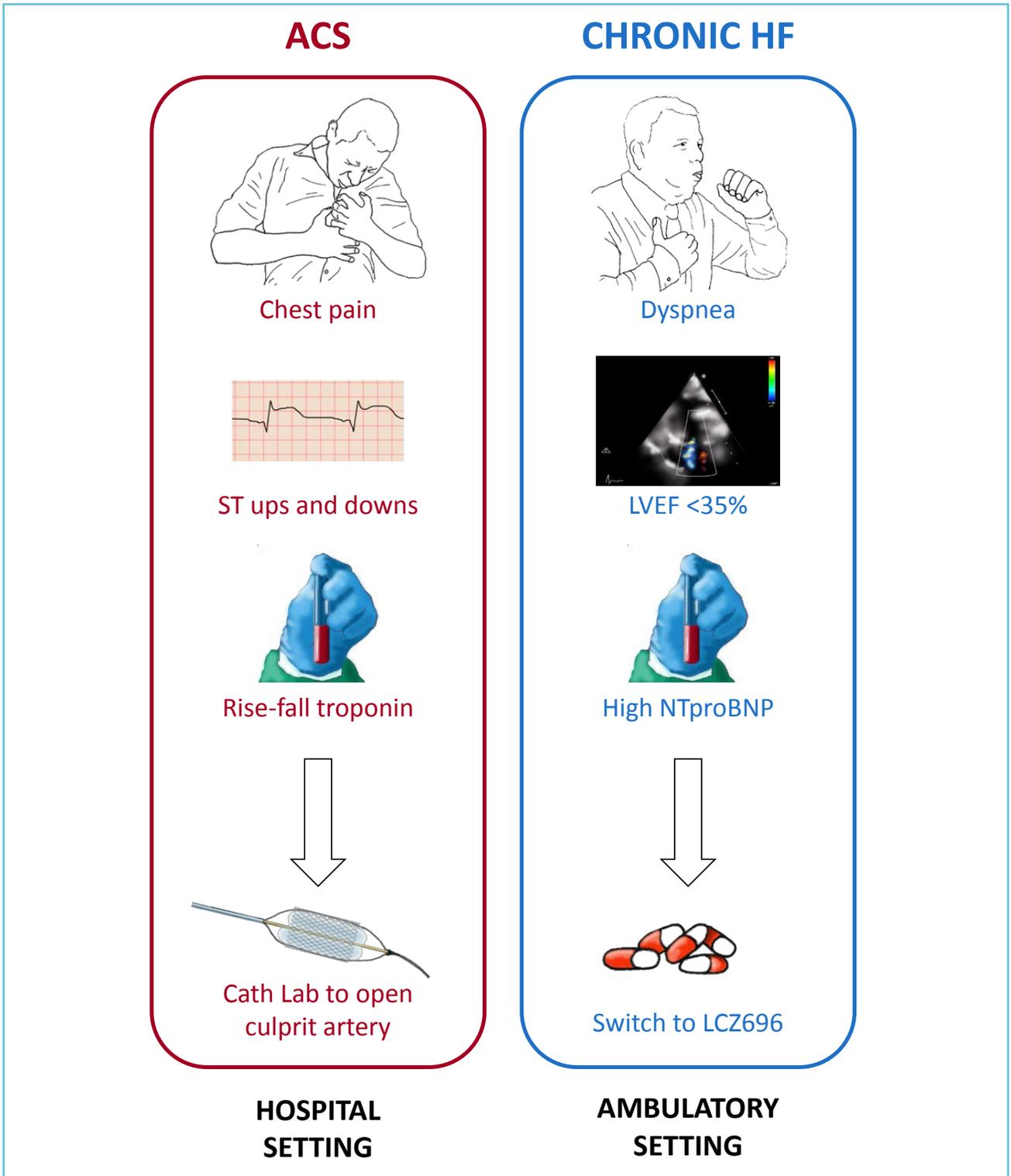


* Created by Carolina Gálvez-Montón

The most recent and largest meta-analysis by Savarese et al. (20) included 2,686 patients included in 12 studies (Figures 2,3). This meta-analysis for the first time reports a benefit for HF-related hospitalization; moreover, the mortality benefit observed was more consistent and not influenced in the sensitivity analysis by any single study or by any potential confounders. This meta-analysis was the only one to investigate separately the effects of BNP- and NTproBNP-guided therapy, suggesting that NTproBNP- but not BNP-guided therapy was significantly associated with improved survival as well reduced hospitalization. A word of caution is necessary here, since no single trial has been designed specifically to compare head-to-head BNP- vs. NTproBNP-guided therapy.

Meta-analysis data did not find a significant benefit for elderly patients when, elderly subgroups from three trials were analyzed: TIME-CHF, BATTLESCARRED, and UPSTEP Trials (6-8). It is conceivable that the more frequent presence of comorbidities may prevent or even promote potentially harmful up titration of HF drugs in elderly patients; however, this speculation requires further confirmation.

Figure 2	Odds ratios of all-cause mortality*
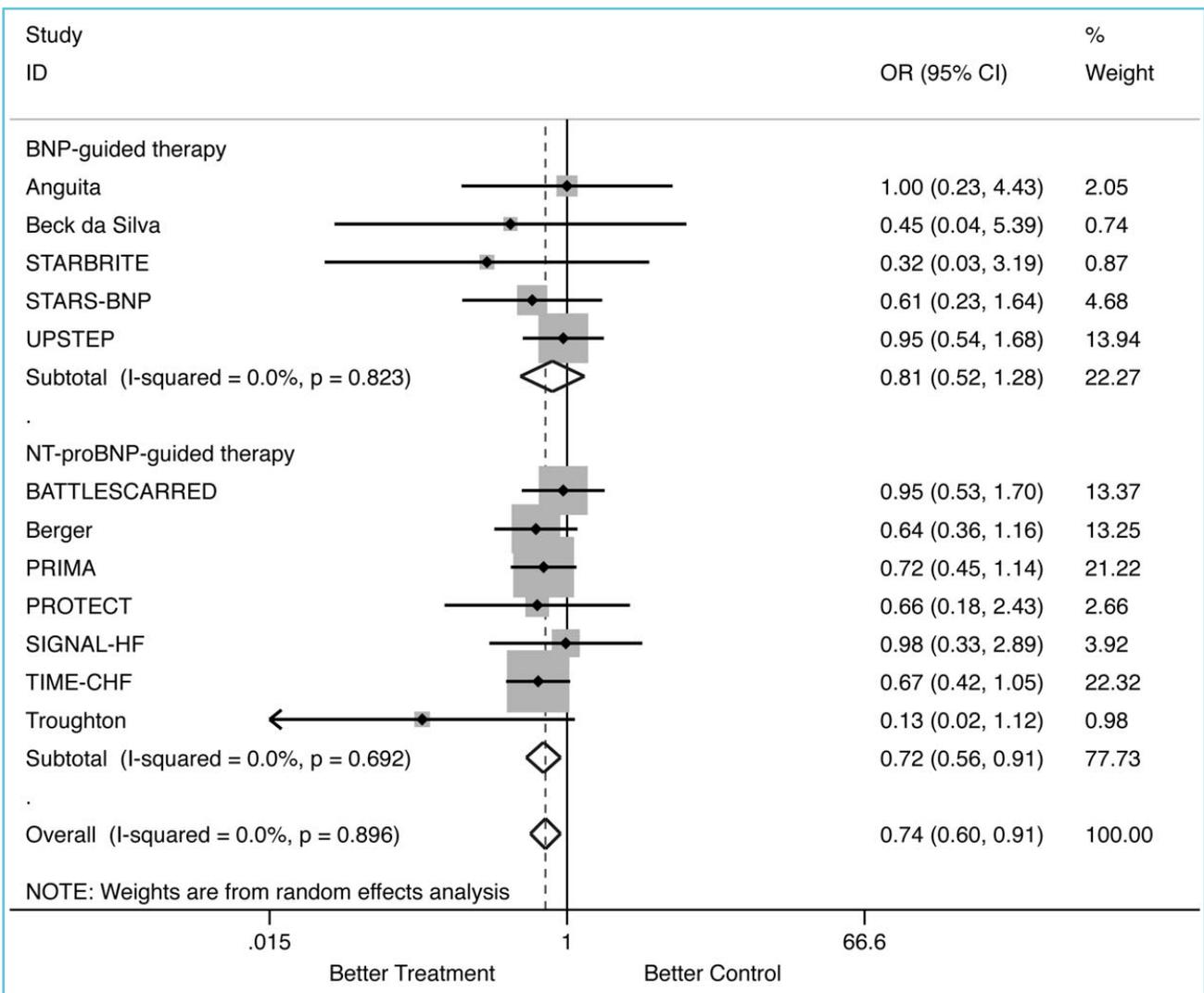
* Taken from (20).

Figure 3	Odds ratios of heart failure–related hospitalization*
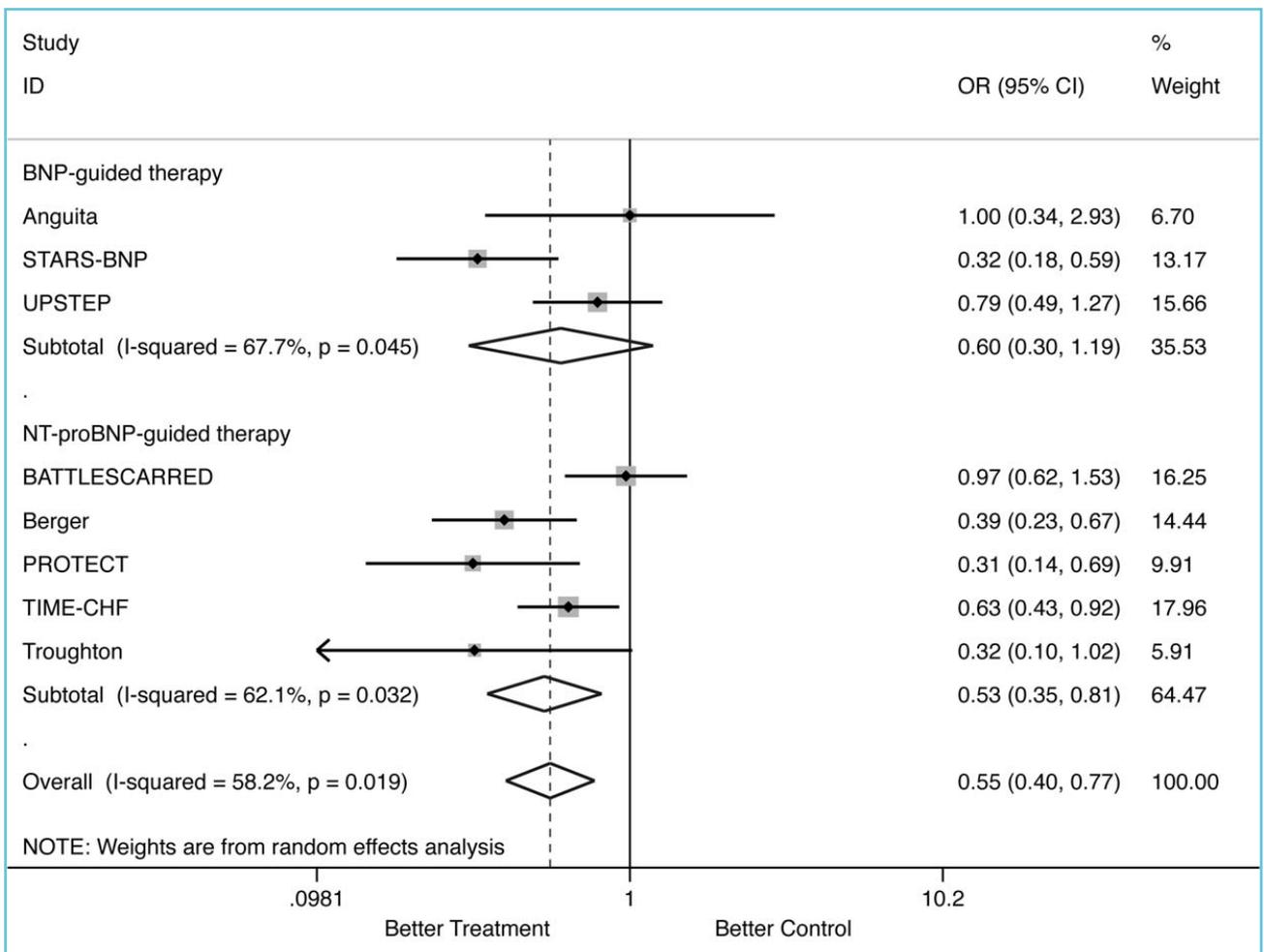
* Taken from (20).

WHAT DO THE GUIDELINES SAY?

The dense and comprehensive guidelines on HF from both the European Society of Cardiology (ESC) (65 pages) (21) and the American College of Cardiology/American Heart Association (ACC/AHA) (92 pages)(22) devote just a few lines to the issue of NP-guided therapy.

The ESC guidelines for the diagnosis and treatment of HF published in 2012 state that "High NP concentrations are associated with a poor prognosis, and a fall in peptide levels correlates with a better prognosis. However, several randomized clinical trials that evaluated NP-guided treatment (intensifying treatment in order to lower peptide levels) have given conflicting results. It is uncertain whether outcome is better using this approach than by simply optimizing treatment (combinations and doses of drugs, devices) according to guidelines" (21). No indications on Class of Recommendation or Level of Evidence are provided in the ESC guidelines.

The 2013 ACC/AHA guidelines for the management of HF state that NP-guided HF therapy can be useful in achieving optimal dosing of guideline-directed medical treatment in select clinically euvolemic patients who are followed

in a well-structured HF disease management program with a Class of Recommendation IIa and a Level of Evidence B (22). This statement is followed by the explanatory text: "NP levels improve with treatment of chronic HF, with lowering of levels over time in general, correlating with improved clinical outcomes. Thus, NP "guided" therapy has been studied against standard care without NP measurement to determine whether guided therapy renders superior achievement of guideline-directed medical treatment in patients with HF. However, randomized clinical trials have yielded inconsistent results. The positive and negative NP-guided therapy trials differ primarily in their study populations, with successful trials enrolling younger patients and only those with HFrEF. In addition, a lower NP goal and/or a substantial reduction in NPs during treatment are consistently present in the positive "guided" therapy trials. Although most trials examining the strategy of biomarker "guided" HF management were small and underpowered, two comprehensive meta-analyses concluded that NP-guided therapy reduces all-cause mortality in patients with chronic HF compared with usual clinical care, especially in patients <75 years of age. This survival benefit may be attributed to increased achievement of guideline-directed medical treatment. In some cases, NP levels may not be easily modifiable. If the NP value does not fall after aggressive HF care, risk for death or hospitalization for HF is significant" (22). In sum, both guidelines solicit additional information.

WHAT IS THE FUTURE?

Where to next for the biomarker-guided management of HF? There is no doubt that further trials are required to provide conclusive evidence. Such a confirmation study is currently under way: the GUIDE-IT (Guiding Evidence Based Therapy Using Biomarker Intensified Treatment

in Heart Failure) study is designed to definitively assess the effects of an NP-guided strategy in high-risk patients with systolic HF on clinically relevant endpoints of mortality, hospitalization, quality of life, and medical resource use. GUIDE-IT is a prospective, randomized, controlled, unblinded, multicenter clinical trial designed to randomize approximately 1,100 high-risk subjects with systolic HF (LVEF ≤ 40%) to either usual care (optimized guideline-recommended therapy) or a strategy of adjusting therapy with the goal of achieving and maintaining a target NT-proBNP level of <1,000 pg/ml (23). The estimated study completion date is December 2017.

In addition to revisiting the strategy in the event of new effective drugs, such as the groundbreaking LCZ696 (17), which has been approved for use in the United States, further studies should examine the potential utility of other markers, such as ST2, either alone or in combination with NPs (24). ST2 manifests much less variability than do natriuretic peptides which may be ideal for following changes with treatment (25). However, the targets that need to be achieved are still unclear.

Despite the uncertainties, the consistently strong and independent relationship of NPs with prognosis should encourage physicians to measure NPs early after diagnosis and periodically thereafter for risk stratification. This will allow appropriate surveillance and fully informed counselling of both patients and their families.

CONCLUSIONS

In spite of the fact that the trials conducted to date have had different designs and pursued different NP targets in varied populations of patients with HF, the use of NPs to guide pharmacologic therapy in patients with chronic HF seems to be associated with a reductions in mortality and HF-related hospitalization, especially in younger patients (<75 years) with reduced

LVEF. There remains a need for definitive trials with sufficient power to confirm the efficacy of this strategy (e.g., GUIDE-IT), yet the existing evidence suggests that serial NP measurement as an audit and/or adjunct to decision making for dose titration in HF is rational and likely to improve outcomes.

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High sensitivity cardiac troponin assays – how to implement them successfully

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ABSTRACT

High sensitivity troponin (hsTn) assays provide an unprecedented opportunity to improve the detection and treatment of cardiac injury from coronary and non-coronary causes. They may also play a role in guiding the primary and secondary prevention of cardiovascular disease. However, to derive maximal benefit from their use, careful planning for the implementation of these new assays is required. In this manuscript, we will discuss actions that can be taken during hsTn pre-implementation, implementation and post-implementation phases. Key concepts for consideration in the pre-implementation phase include: the establishment of a multi-disciplinary implementation team; development of quality control procedures; education of clinical staff; modification of existing clinical workflow and provision of computerized decision support. Strategies for ensuring successful implementation and post-implementation phases will also be discussed.
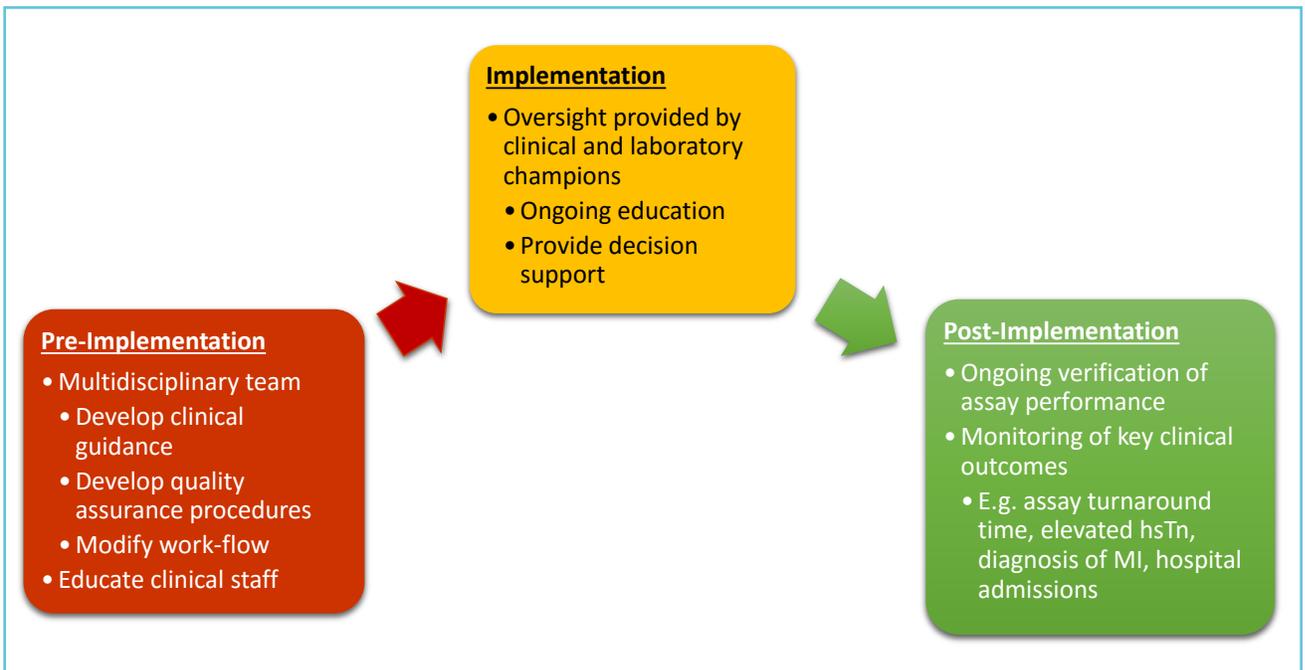
INTRODUCTION

The introduction of high sensitivity cardiac troponin (hsTn) assays provide an unprecedented opportunity for earlier and more accurate diagnosis of myocardial infarction; improved diagnosis of myocardial injury from non-coronary etiologies and guidance for primary and secondary prevention of cardiovascular disease.[1, 2] hsTn assays are able to accurately measure 10-fold lower concentrations of cardiac troponin than contemporary assays and therefore can measure troponin values in healthy persons. By definition, a hsTn assay measures troponin values in at least 50% of healthy individuals.[3] Additionally, hsTn assays measure troponin precisely with little variation between repeat measurements (co-efficient of variation (CV) $\leq$ 10% at the 99th% of a reference population).[4] hsTn assays have been approved for clinical use in most parts of the world, with the exception of the United States (it is anticipated that approval in the United States will occur within the coming 1-2 years).

Clinical use of hsTn has been associated with an increase in the diagnosis of myocardial infarction (especially in women[5]) and a reduction in morbidity and mortality of patients evaluated for acute coronary syndrome (ACS).[6] It also results in a reduction in the emergency department length of stay for patients evaluated for suspected ACS.[7] However, a number of questions remain unanswered regarding the potential negative consequences of using hsTn clinically. First, there is a concern that hsTn will lead to an increase in the number of patients with elevated troponin values and consequently, an increase in hospital admissions and downstream testing. Although some studies have reported increases in the frequency of troponin values >99th percentile with the use of hsTn,[8, 9] others have not.[10] The change in frequency of troponin elevations with implementation of hsTn is related to analytic sensitivity of the contemporary assay used and whether or not the 99th percentile of the contemporary assay was used for decision making. Additionally, an increase in the frequency of elevated troponin values may not necessarily result in an increase in the diagnosis of myocardial infarction (MI).[11] Second, there is no consensus on the appropriate thresholds for clinical decision making.[12] The threshold for ruling out MI, diagnosing MI, performing additional diagnostic evaluation for MI,[13, 14] and for risk-stratification of different cardiovascular diseases may be different.[15] Furthermore, gender-specific cut-offs may provide improved diagnostic value over gender-neutral cut-offs.[5] Third, optimal timing of serial troponin measurements and the ideal change values (relative versus absolute) for distinguishing between acute and chronic troponin elevations remain under investigation.[16, 17] Fourth, pre-analytical and analytical factors remain important in ensuring the accuracy of hsTn measures.[18]

Successful implementation of a novel test occurs in three phases: the pre-implementation phase, implementation phase and post-implementation phase (Figure 1). During the pre-implementation phase, a multi-disciplinary team tasked with developing clinical guidance and quality assurance procedures and educating clinical staff should be established. The implementation phase should be guided by clinical and laboratory medicine champions. Each discipline will need one or two champions who will provide ongoing education and decision support and be available to troubleshoot problems as they arise. During the post-implementation phase, ongoing assay performance verification should be performed. Additionally, key clinical outcomes such as: assay turnaround time, prevalence of elevated hsTn, and the number of hospital admissions for evaluation of ACS should be monitored. The following paragraphs discuss these concepts further.

Figure 1   Overview of strategies for successful implementation of hsTn

Implementation
- Oversight provided by clinical and laboratory champions
- Ongoing education
- Provide decision support

Pre-Implementation
- Multidisciplinary team
- Develop clinical guidance
- Develop quality assurance procedures
- Modify work-flow
- Educate clinical staff

Post-Implementation
- Ongoing verification of assay performance
- Monitoring of key clinical outcomes
- E.g. assay turnaround time, elevated hsTn, diagnosis of MI, hospital admissions

This figure depicts the strategies necessary for successful implementation of hsTn. It highlights important steps that ought to be taken in the pre-implementation, implementation and post-implementation phases.

ESTABLISHING A MULTI-DISCIPLINARY IMPLEMENTATION TEAM

Clinical implementation of hsTn requires a multi-disciplinary approach with the viewpoints of all stakeholders represented. At minimum, the implementation team should consist of representatives from cardiology, emergency medicine, internal medicine/hospitalists and laboratory medicine. This team will oversee the entire implementation process and will be responsible for making recommendations regarding protocols for interpreting hsTn values, threshold values for clinical decision making, education programs for clinical staff, and monitoring on-going quality improvement measures. This team will also be responsible for creating or endorsing a new diagnostic algorithm for ruling-out MI. The unique perspectives of each team member are important. For example, from the perspective of cardiologists who have the benefit of being able to observe the clinical course of hospitalized patients, a

test that facilitates diagnosing MI with high specificity (i.e. low-likelihood of false positives) is desirable to avoid subjecting patients unnecessarily to procedures and treatments that can have adverse consequences. However, from the perspective of the ED physician challenged with determining the disposition of patients with symptoms suspicious of MI, a test that facilitates diagnosing MI with high sensitivity (i.e. low-likelihood of false negatives) is desirable to avoid missing the diagnosis of MI and inadvertently discharging MI patients to their homes. Despite having these conflicting perspectives, clinicians of different specialties trust clinical laboratory values implicitly[19] and don't always appreciate potential analytical confounds that often influence laboratory values. Therefore a multi-disciplinary approach will ensure that the viewpoint of all key stakeholders are represented, with the primary objective of doing what's best for patients. A number of key questions worth considering by the multi-disciplinary implementation team are presented in Table 1.

Table 1	Key questions for multi-disciplinary implementation team
1	What is the appropriate threshold for the 99th percentile (gender-specific versus gender-neutral versus a combination of the two)?
2	How much change in hsTn constitutes sufficient change for an acute process? Should absolute changes, relative changes or a combination of the two be used?
3	What is the recommended practice for managing patients with acute or chronic myocardial injury but no evidence of myocardial infarction?
4	What quality control checks are need to ensure the accuracy of troponin values?
5	How should results be reported? (preferably with whole numbers)
6	What decision support should be provided to clinicians?
7	How should clinicians be educated about hsTn?

DEVELOPING QUALITY CONTROL PROCEDURES

As part of the pre-implementation phase, quality control procedures should be developed. These should include assay verification procedures such as validating the limit of blank (LoB), evaluating precision at the reported limit of detection (LoD), and assessing the linearity range of the assay. Monitoring the accuracy of assay values below the 99th percentile will be of critical importance since rapid rule-out MI protocols rely on the accuracy of low hsTn values for risk-stratification. For example, a rule-out ACS strategy based on the 2015 European Society of Cardiology (ESC) guidelines will recommend discharging patients with an initial hsTn<99th percentile whose symptoms started more than 6 hours prior to blood draw, are pain free, have a Global Registry of Acute Coronary Event (GRACE) score <140 and in whom other life-threatening conditions have been excluded.[20] Therefore if the 99th percentile of an assay is 26 ng/L and the clinical chemistry laboratory reports an erroneous value of 25 ng/L instead of an actual value of 27 ng/L (7.4% difference), an MI patient may be inadvertently discharged home. The likelihood of inadvertently discharging MI patients will be even more significant if the rule-out ACS strategy is based on studies that deem it safe to rule-out MI in patients with initial hsTn<LoD.[21-23] Processes must be established to allow clinicians report cases in which hsTn values do not match the clinical scenario. Consequently, clinical chemistry laboratories should also have established protocols for addressing these inconsistencies.

Although hsTn assays produce more robust results and fewer outlier values than contemporary troponin (cTn) assays,[24] analytical confounds that currently affect cTn assays will continue to influence hsTn values. For example, hemolysis may result in decreases in hsTnT values and increases in hsTnI values.[25, 26] Additionally, the imprecision of hsTnI values is influenced by the extent of centrifugation performed.[27] Therefore quality control procedures should include actions that reinforce careful sample acquisition and preparation.

EDUCATING CLINICAL STAFF

In preparation for the implementation phase, physicians, nurse practitioners (NPs), physician assistants (PAs), nurses and ancillary support staff who perform blood draws should receive

education tailored to their role. Nurses and support staff who perform blood draws should be reminded of the importance using appropriate sample acquisition techniques. Nurses should receive additional education that highlights the differences between hsTn and cTn, and explains the new diagnostic algorithm formulated by the multidisciplinary team for ruling out MI. Physicians, NPs and PAs who will be utilizing hsTn tests need comprehensive education on hsTn. For many of them, learning to use hsTn will represent a paradigm shift in how they interpret troponin values. More than ever before, they will have to remember that troponin elevation is not synonymous with myocardial infarction. Understanding the differences between acute myocardial injury, chronic myocardial injury and myocardial infarction will continue to be critically important. Additionally, with the increase in the detection of type 2 MIs in the hsTn era, clinicians will have to learn to distinguish between type 1 and type 2 MIs,[28] and avoid treating type 2 MI patients the same way they treat type 1 MI patients. Improved understanding of the prognostic value of minor troponin elevations will be important in the hsTn era. On one hand, the often causal dismissal of minor troponin elevations as "troponemia" needs to be moderated by the realization that patients with any elevated troponin values have higher risk of adverse cardiovascular events than those without troponin elevations.[9, 29] On the other hand, the conservative approach to admit any patient with any troponin elevation also needs to be avoided. The success or failure of hsTn implementation will largely depend on the success or failure of clinician education.

MODIFY EXISTING CLINICAL WORKFLOW

Implementation of hsTn can result in a decrease in the length of stay of ED patients evaluated for ACS.[7] However, indiscriminate use of hsTn may also lead to an increase in the number of patients with elevated troponin values, and a potential increase in hospital admissions and downstream testing. Therefore, to derive maximal benefit from hsTn implementation, modification of the existing clinical workflow during the pre-implementation phase will be necessary. In United States EDs, nursing triage orders are often placed to facilitate patient evaluations and decrease time-to-treatment.[30] Troponin is often included in the list laboratory tests that triage nurses are allowed to order. In the era of hsTn, it will be important to provide clear guidance regarding the criteria for ordering hsTn by triage nurses. Lack of such guidance may lead to hsTn testing in patients with a very low pre-test probability of having ACS.

Decreasing the time it takes to rule-out MI using new protocols that incorporate hsTn testing also requires modifications to existing clinical workflow. For example, a number of studies have reported that 1-hour algorithms perform well in ruling-out MI.[31-33] The turnaround time for hsTn assays may be approximately 60 minutes,[34] thus to derive benefit from the 1-hour protocol, modifications to the work-flow, including obtaining the second troponin sample prior to receiving the results of the first troponin sample deserves consideration.

CONSIDER PROVIDING COMPUTERIZED DECISION SUPPORT

Interpreting hsTn values is not always simple. It often involves remembering complex decision making algorithms. The introduction of electronic medical records (EMR) provides a unique opportunity to provide computerized decision support that can guide clinical decision making. Diagnostic algorithms can be easily embedded into existing EMR to provide recommendations regarding the next steps of a patient's work-up. Absolute and relative changes in hsTn can also be calculated automatically and integrated in

decision support algorithms, fostering a systematic approach to patient evaluation and treatment using hsTn.

IMPLEMENTATION PHASE AND POST-IMPLEMENTATION PHASE

The implementation of hsTn should be led by champions from clinical chemistry, cardiology, emergency medicine and internal medicine. These champions should be available to provide ongoing education and decision support especially during the first month of implementation. They will also help troubleshoot problems as they arise. By modeling behaviors that exemplify best practices, clinical champions can be powerful agents of change during the implementation of hsTn.

The post-implementation phase is critical to ensuring a successful hsTn. During this period, ongoing verification of the accuracy of assay performance should be continued as is done for most clinical assays. To keep track of the effect of hsTn on clinical care, key clinical outcomes such as laboratory turnaround time, the prevalence of elevated hsTn, hospital admissions for suspected ACS should be monitored.

CONCLUSIONS

HsTn holds promise for transforming the diagnostic evaluations for cardiovascular disease. Careful planning for the implementation of these assays will allow patients to derive maximal benefit from their promise.

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Soluble ST2 and galectin-3: what we know and don't know analytically

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ABSTRACT

The proteins soluble ST2 (sST2) and galectin-3 are currently gaining mounting interest as candidate biomarkers in cardiac disease. Both, sST2 and galectin-3 have been included in the 2013 ACCF/AHA guideline for additive risk stratification of patients with acute and chronic heart failure. The aim of this review is to provide information on analytical considerations of measuring circulating sST2 and galectin-3 including knowledge on in vitro stability, biological variation and reference ranges of both analytes.

INTRODUCTION

The proteins soluble ST2 (sST2) and galectin-3 are currently gaining growing interest as candidate biomarkers in cardiac disease [1-3]. There is increasing evidence that plasma concentrations of these two analytes provide prognostic information in patients with cardiac disease independently of and additive to other established markers such as cardiac troponins or natriuretic peptides [1-4]. Both, sST2 and galectin-3 have been included in the 2013 ACCF/AHA guideline for additive risk stratification of patients with acute and chronic heart failure [4].

The protein sST2 (also termed Interleukin-1 receptor-like 1, isoform B) is 328 amino acids in length, has a molecular weight of 36,993 Da [http://www.uniprot.org/uniprot/Q01638; accessed September 14, 2015], and is glycosylated at several positions. The protein galectin-3 (also termed Mac-2 antigen or Carbohydrate-binding protein 35) is 250 amino acids in length, has a molecular weight of 26,152 Da [http://www.uniprot.org/uniprot/P17931; accessed September 14, 2015], and can form dimers and higher order oligomers.

The aim of this review is to provide information on analytical considerations of measuring circulating sST2 and galectin-3 including knowledge on *in vitro* stability, biological variation and reference ranges of both analytes.

PATHOPHYSIOLOGY OF sST2 AND GALECTIN-3

ST2 is an interleukin-1 receptor family member with transmembrane (ST2L) and soluble isoforms (sST2) [5-8]. ST2L is a membrane bound receptor, and interleukin-33 (IL-33) is the functional ligand for ST2L [5-8]. In principle, IL-33 functions as a danger signal or an alarmin by signaling the presence of tissue damage to local immune cells after exposure to pathogens, injury-induced stress, or death by necrosis [6-8].

IL-33/ST2L signaling leads to inflammatory gene transcription and ultimately to the production of inflammatory cytokines/chemokines and induction of immune response [7,8]. sST2, a soluble truncated form of ST2, is secreted into the circulation and is believed to function as a "decoy" receptor for IL-33, inhibiting the effects of IL-33/ST2L signaling [5-8]. Thus, increased concentrations of sST2 in the circulation attenuate the systemic biologic effects of IL-33. Blood concentrations of sST2 are significantly increased, e.g., in inflammatory/infectious diseases, in cancer and in cardiac disease but not in chronic kidney disease [1-3,7-9]. The major source of circulating sST2 in healthy individuals and in patients with distinct diseases (especially in human cardiac disease) is, however, currently not established [7,8].

Galectin-3 is a unique member of chimera type galectins and is involved in a large number of disease processes [10,11]. Galectin-3 contains a carbohydrate-recognition-binding domain that enables the specific binding of β-galactosides [10,11]. Galectin-3 exhibits both intracellular and extracellular functions and it has a concentration dependent ability to be monomeric or form oligomers [10,11]. Galectin-3 is involved in cell adhesion, activation, proliferation, apoptosis as well as cell migration [10-12]. It plays an important role not only in cancer [13] but also in inflammation [10,11,13]. In this context, galectin-3 can be viewed as regulatory protein acting at several stages along the continuum from acute inflammation to chronic inflammation and tissue fibrinogenesis [10]. Indeed, the involvement of galectin-3 in various "inflammatory/fibrotic" conditions such as arthritis, asthma, pneumonia, atherosclerosis, and kidney disease has been described [9-11,13]. Even in the pathophysiology of heart failure, galectin-3 plays a biological role through inflammation and fibrosis [1-3,9,13].

ASSAYS FOR MEASURING CIRCULATING sST2 AND GALECTIN-3

Table 1 provides information on selected commercially available assays for measurement of sST2 and galectin-3 in human serum/plasma.

Among the ST2 assays specified in Table 1, the Presage ST2 assay (Critical Diagnostics) is the only method that has been cleared by the U.S. Food and Drug Administration (FDA) and has received Conformitè Europèenne (CE) mark; this is an enzyme-linked immunosorbant assay (ELISA) [14,15]. Furthermore, the manufacturer of the Presage ST2 assay recently started to market the ASPECT-PLUS ST2 Test (quantitative sandwich monoclonal lateral flow immunoassay), a point-of-care assay for quantitatively measuring sST2. In the future, assays for measurement of sST2 on automated platforms will probably also be made available. In contrast to the FDA cleared Presage assay, the MBL ST2 ELISA, the RayBiotech ST2 ELISA and the R&D ST2 ELISA are research assays [7,16].

Table 1	Information on selected commercially available assays for measurement of sST2 and galectin-3 in human serum/plasma			
Manufacturer	**Assay/kit**	**Limit of detection[†]**	**Measurement range[†]**	**Inter-assay CV or total CV[†]**
Critical Diagnostics (www.criticaldiagnostics.com)	ASPECT-PLUS ST2 test	12.5 ng/mL	up to 250 ng/mL	<23%
Critical Diagnostics (www.criticaldiagnostics.com)	Presage ST2 kit	1.3 ng/mL	up to 200 ng/mL	<9%
MBL International (www.mblintl.com)	Human ST2 ELISA kit	0.032 ng/mL	up to 20 ng/mL	<6%
RayBiotech (www.raybiotech.com)	Human IL-1 R4/ST2 ELISA kit	0.002 ng/mL	up to 1.2 ng/mL	<12%
R&D Systems (www.rndsystems.com)	ST2/IL-1 R4 DuoSet ELISA or Quantikine ELISA	0.005 ng/mL	up to 2.0 ng/mL	<8%
Abbott Diagnostics (www.abbottdiagnostics.com)	ARCHITECT Galectin-3 test	1.0 ng/mL	up to 114 ng/mL	<9%
BG Medicine (www.bg-medicine.com)	BGM Galectin-3 test	1.1 ng/mL	up to 95 ng/mL	<12%
bioMérieux (www.biomerieux-diagnostics.com)	VIDAS Galectin-3 assay	2.4 ng/mL	up to 100 ng/mL	<6%
R&D Systems (www.rndsystems.com)	Human Galectin-3 Quantikine ELISA	0.085 ng/mL	up to 10 ng/mL	<7%

[†] *Information derived from the package inserts (effective September 14, 2015).*

The first assay for measurement of galectin-3 that has been cleared by the FDA and has received CE mark was the BGM Galectin-3 ELISA (BG Medicine) [17,18]. Afterwards, Abbott and bioMérieux have entered agreements with BG Medicine to commercialize the assay for use on their own automated platforms. In the meanwhile, the ARCHITECT Galectin-3 assay (Abbott Diagnostics) has also received FDA approval and CE mark. The ARCHITECT Galectin-3 assay is a chemiluminescent microparticle immunoassay [18,19], and the VIDAS Galectin-3 assay uses the enzyme-linked fluorescent assay technology [18,20]. In contrast, the R&D galectin-3 assay is a research assay in an ELISA format.

In this review, we used the approach to describe the analytical properties of the Presage ST2 assay and the BGM Galectin-3 assays first and afterwards discuss their features in comparison with other methods for sST2 and galectin-3 measurement, respectively.

THE PRESAGE ST2 ASSAY

Assay format

The Presage ST2 assay is an *in vitro* diagnostic device that quantitatively measures sST2 in serum or plasma by ELISA [14,15]. The Presage ST2 assay kit is provided in microplate configuration. The assay uses mouse monoclonal anti-human sST2 capture and detection antibodies. Real time testing has revealed a shelf life of 12 month for the Presage ST2 assay kit when stored at 2-8°C. Serum, lithium heparin plasma and EDTA plasma have been validated as possible sample types for the Presage ST2 assay.

Precision, linearity, limit of detection, limit of quantification

The range of standards is 3.1-200.0 ng/mL when used with specimens diluted 1:50. The manufacturer claims a Limit of Blank (LoB) of 0.5 ng/mL, a Limit of Detection (LoD) of 1.3 ng/mL, and

a Limit of Quantification (LoQ) of 2.4 ng/mL. In two published studies, a Limit of Detection (LoD) of <2.0 ng/mL was found [14,21]. The Presage ST2 assay had a within-run coefficient of variation (CV) of <2.5% and a total CV of <4.0% in one of those studies [14], and in the other study a within-day CV of <7.6% and a total CV of <14% [21]. Results from linearity analyses indicate that the method is linear within the dynamic range of the assay calibration curve [14,21]. There are minimal effects induced by hemolysis, lipemia, icterus or rheumatoid factor [7].

Analyte stability in vitro

The results of studies on the *in vitro* stability of sST2 indicate that the analyte is stable for 48 hours at room temperature, for at least 7 days at 4°C, and for at least 1.5 years at −20°C and at −80°C [14,21,22]. Thus, the analyte as measured with the Presage ST2 assay is well suitable for routine use in laboratory settings, also facilitating unproblematic conditions for sample shipment and storage. Three freeze and thaw cycles do not seem to affect sST2 analyte concentrations [7].

Biological variation of sST2

The components of biological variation of sST2 in healthy individuals with a median sST2 plasma concentration of 10 ng/mL (range, 5–34 ng/mL) were studied at one week intervals for six weeks. An intra-individual biological CV of 11%, an inter-individual biological CV of 46%, and a reference change value of 30% was found [14]. The reference change value indicates the difference required for 2 serial measurements of sST2 to be significantly different at p <0.05. In a similar study also using the Presage ST2 assay, the authors revealed exactly the same results on the components of biological variation when blood was taken every two weeks for eight weeks from individuals

Figure 1 **Scatterplots of sST2 plasma concentrations obtained by three different methods**
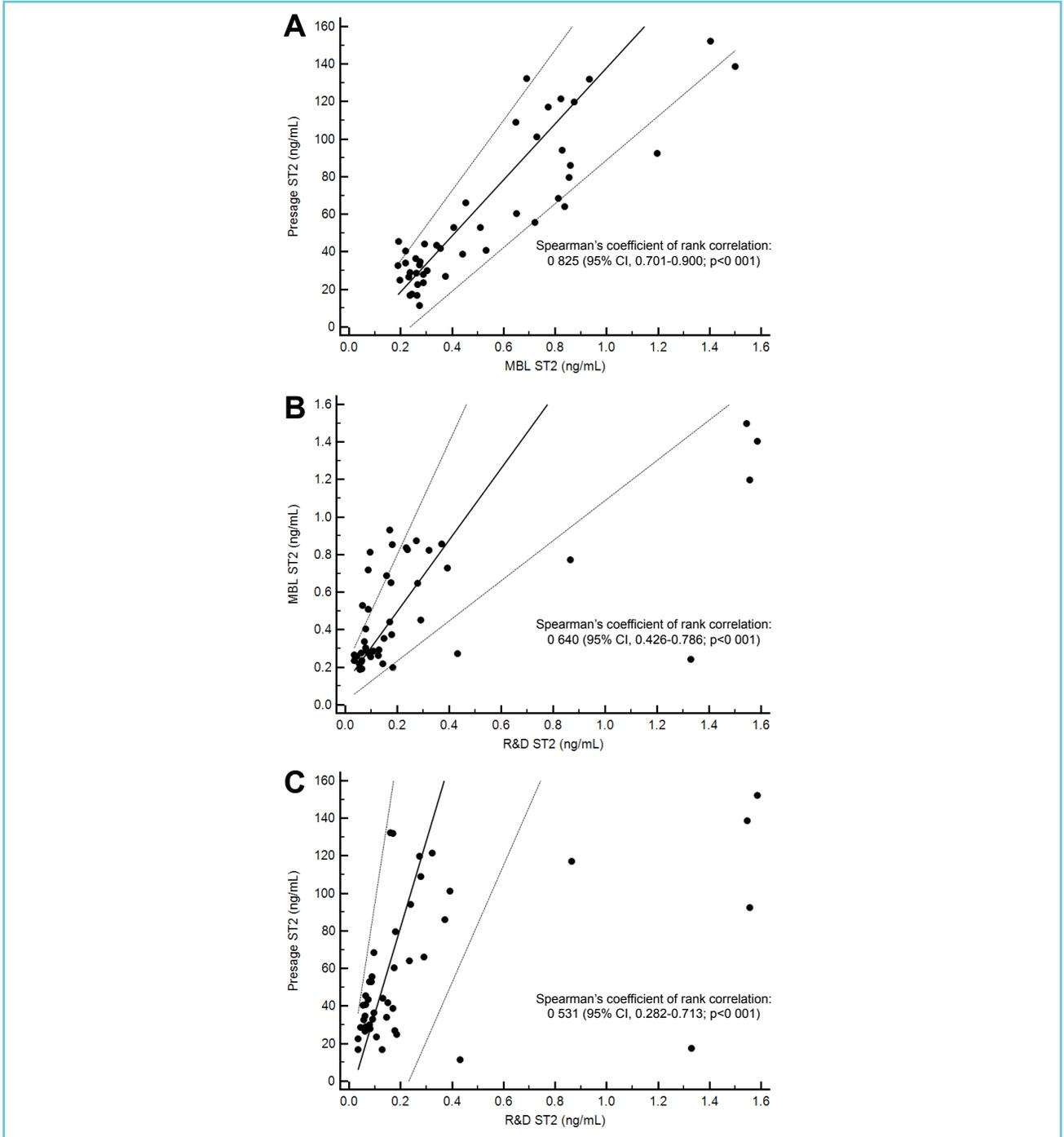
(A) MBL assay vs. Presage assay; (B) R&D assay vs. MBL assay; and (C) R&D assay vs. Presage assay. The method comparison graphs display the scatter diagrams with the regression line (solid line) and the 95% confidence intervals for the regression line (dashed lines) according to Passing & Bablok as derived from Table 2. In addition, the results of Spearman rank correlation are given for each graph. Samples from 45 male patients with a variety of diseases were analyzed.

Adopted from [16].

with a median sST2 plasma concentration of 29 ng/mL (range, 12–75 ng/mL) [23]. The reference change value of 30% might be the basis for further studies attempting to demonstrate that sST2 can be used to monitor the results of treatments over time.

sST2 concentrations in reference value studies

From a reference value study of adult healthy blood donors from Europe, it became obvious that sex-specific reference values might be necessary for sST2 measured with the Presage ST2 assay. There was a significant difference of plasma concentrations between genders; in the male sample, the reference interval for sST2 was 4-31 ng/mL, and in the female sample it was 2-21 ng/mL [14]. Another evaluation on a US population revealed slightly higher reference intervals for male and female adults, but still a considerable difference between both genders; in the male sample, the reference interval for sST2 was 9-50 ng/mL, and for the female sample it was 7-33 ng/mL [21]. More recently, reference values for circulating sST2 were also derived from a subset of the Framingham study again revealing a considerable difference between male and female individuals; in the male sample, the reference interval for sST2 was 11-45 ng/mL, and for the female sample it was 9-35 ng/mL in this study [24].

In pediatric patients without heart failure and renal disease, sST2 plasma concentrations were not associated with age, gender or body mass index; the reference interval was 8-64 ng/mL including four outliers, and 9-50 ng/mL excluding outliers [25].

COMPARISON OF THE PRESAGE ST2 ASSAY WITH OTHER COMMERCIALLY AVAILABLE ASSAYS FOR sST2 MEASUREMENT

Mainly three different assays have been used to determine circulating sST2 concentrations in published clinical studies: the Presage ST2

assay, the MBL ST2 assay, and the R&D ST2 assay. The original development of the MBL ST2 assay was by the research group of Tominaga and co-workers in Japan [26].

In a previously published study, sST2 plasma concentrations as measured by these three commercially available assays were compared [16]. In the study participants, the median sST2 plasma concentrations were 43.5 ng/mL as measured by the Presage ST2 assay, 0.375 ng/mL by the MBL ST2 assay, and 0.144 ng/mL by the R&D ST2 assay. Regression analyses revealed that there were major differences between the three methods. The results of this study are summarized in Table 2 and in the scatterplots shown in Figure 1. Concentrations of sST2 obtained with the Presage ST2 assay, the MBL ST2 assay, and the R&D ST2 assay are not equivalent. The reasons for the lack of agreement between the three methods are most probably different standards, antibodies, reagents and buffers [7,16]. Therefore, it is important to be aware that the results reported in published studies obtained with the three methods are not directly comparable.

Currently, no studies have been published comparing sST2 plasma concentrations as measured with the ASPECT-PLUS ST2 Test vs. the Presage ST2 test. We were able to find respective information in the package insert of the ASPECT-PLUS ST2 test only, where a concordance analysis of EDTA plasma from 60 individuals is described.

The respective Passing-Bablok regression analysis revealed the following equitation with the Presage assay as the reference method:

$$y \ [ng/mL] = 1.01 \ x +5.8 \ [ng/mL].$$

The Cusum test did not show a significant deviation from linearity.

Table 2	Data on an analytical assay comparison of the Presage ST2 assay, the MBL ST2 assay, and a R&D ST2 assay*

sST2 plasma concentrations as measured by the tree assays					
Assay	Lowest value	25th percentile value	Median value	75th percentile value	Highest value
Presage ST2 assay	11.5 ng/mL	28.9 ng/mL	43.5 ng/mL	87.8 ng/mL	152 ng/mL
MBL ST2 assay	0.189 ng/mL	0.263 ng/mL	0.375 ng/mL	0.784 ng/mL	1.500 ng/mL
R&D ST2 assay	0.034 ng/mL	0.077 ng/mL	0.144 ng/mL	0.274 ng/mL	1.586 ng/mL

Passing and Bablok regression equitations			
Assays compared	Regression equitation	Intercept (95% confidence interval)	Slope (95% confidence interval)
MBL (variable x) vs. Presage (variable y)	y [ng/mL] = −11 ng/mL + 149 x [ng/mL]	−11 ng/mL (−28 to −2)	149 (117 to 187)
R&D (variable x) vs. MBL (variable y)	y [ng/mL] = 0.118 ng/mL + 1.902 x [ng/mL]	0.118 ng/mL (0.021 to 0.200)	1.902 (1.069 to 3.000)
R&D (variable x) vs. Presage (variable y)	y [ng/mL] = −9 ng/mL + 459 x [ng/mL]	−9 ng/mL (−72 to 6)	459 (312 to 891)

* Plasma samples of 45 patients with a variety of diseases were measured with all three commercially available assay kits.
Adopted from [16].

### THE BGM GALECTIN-3 ASSAY

Assay format

The BGM Galectin-3 assay is an *in vitro* diagnostic device that quantitatively measures galectin-3 by ELISA [17,18]. A rat monoclonal anti-mouse galectin-3 antibody serves as the capture antibody. The overall homology between mouse and human galectin-3 is 85%, and in the N-terminal proportion of the protein, where the epitope for the assay is located, there is 100% homology between human and murine galectin-3 [17]. A mouse monoclonal anti-human galectin-3 antibody functions as the detection antibody [17]. The shelf life is 27 month for the BGM Galectin-3 assay kit when stored at 2-8°C. Serum and EDTA plasma have been validated as possible sample types for the BGM Galectin-3 assay.

Precision, linearity, limit of detection, limit of quantification

The measurement range based on the standards is 1.4-94.8 ng/mL when used with specimens diluted 1:10. The manufacturer claims a Limit of Blank (LoB) of 0.86 ng/mL, a Limit of Detection (LoD) of 1.13 ng/mL, and a Limit of Quantification (LoQ) of 1.32 ng/mL. These data were derived from a multi-center evaluation study [17]. In the same study, the BGM Galectin-3 assay had a within-run

Figure 2   **Scatterplots of galectin-3 plasma concentrations obtained by the ARCHITECT Galectin-3 assay vs. the BGM Galectin-3 assay**
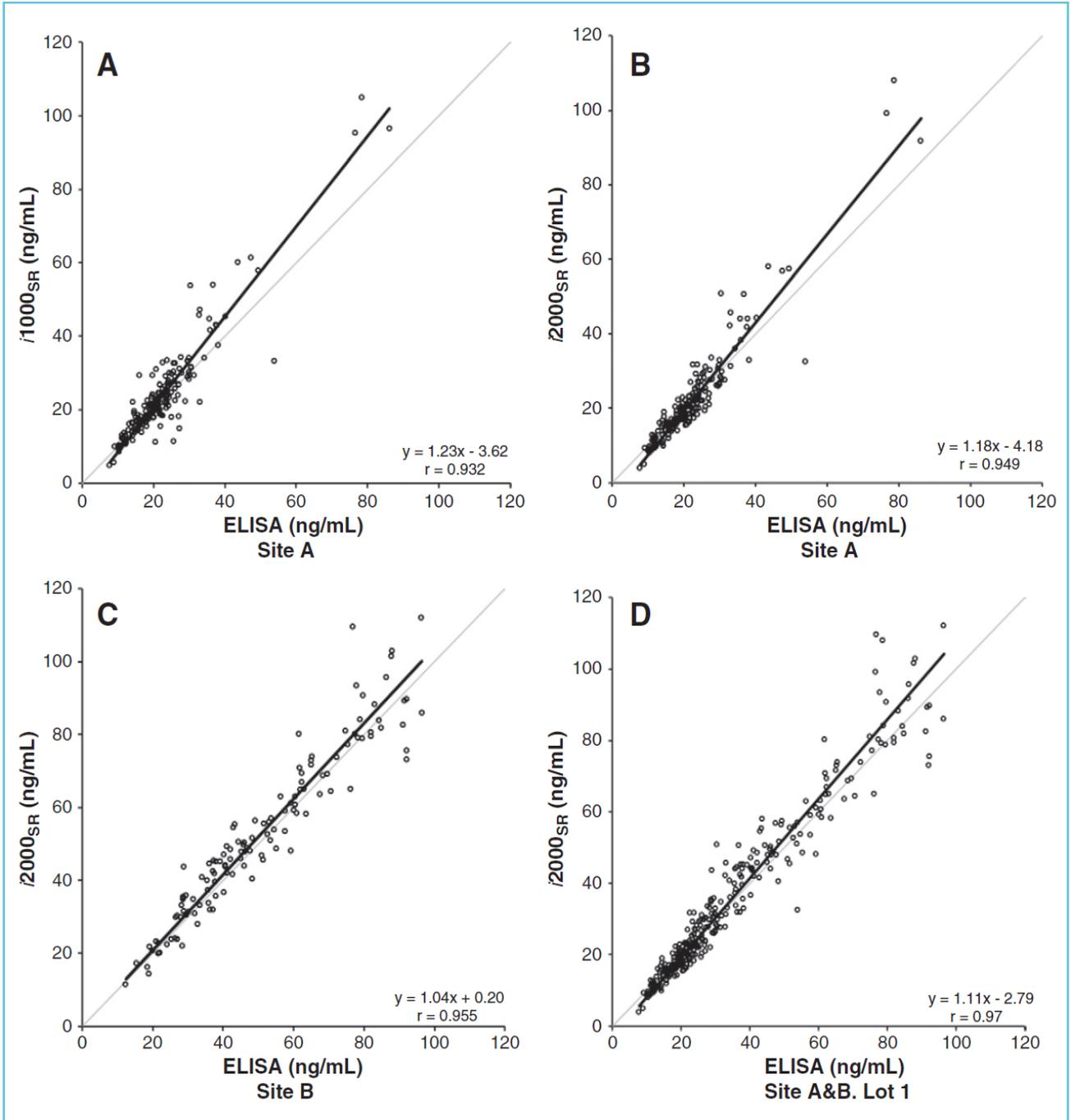
Panel A represents the i1000$_{SR}$ compared to the ELISA at site A; Panel B represents the i2000$_{SR}$ compared to the ELISA at site A; Panel C represents the i2000$_{SR}$ compared to the ELISA at site B; Panel D represents the combined i2000$_{SR}$ data from site A and B compared to the ELISA. A total of 190 samples at site A and 129 samples at site B were analyzed. The grey line represents x=y and the solid black line indicates the Passing-Bablok regression line. Equitations of the regression line and correlation coefficients are shown.

Adopted from [19].

Figure 3	Scatterplots of galectin-3 plasma concentrations obtained by the VIDAS Galectin-3 assay vs. the BGM Galectin-3 assay
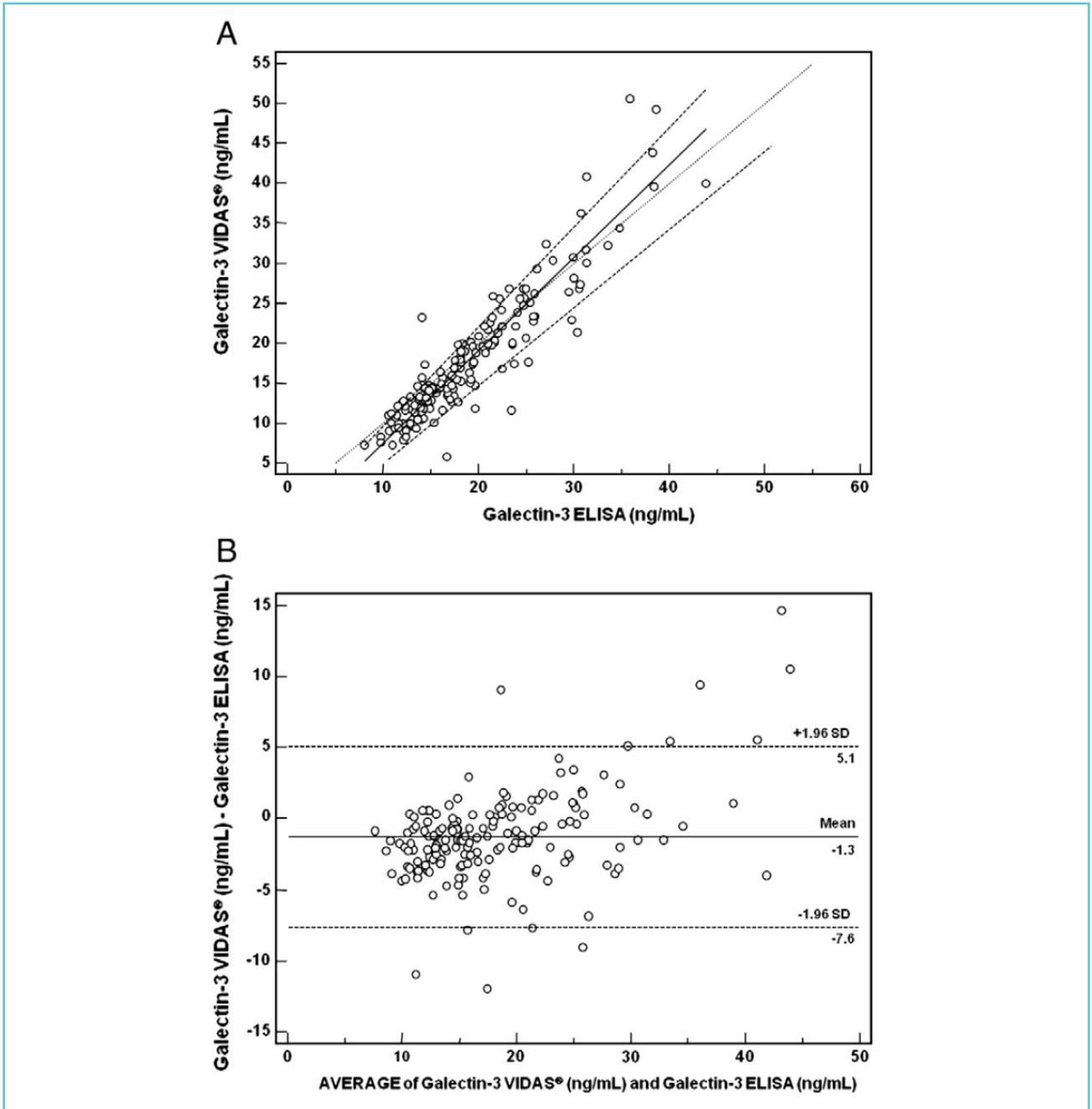
Galectin-3 plasma concentrations measured with both methods were obtained in 137 heart failure patients with reduced ejection fraction. Passing and Bablok regression analysis (A) and Bland and Altman plot (B) are shown.

Adopted from [20].

coefficient of variation (CV) of <7.4% and a total CV of <17.0%. Linearity of BGM Galectin-3 was demonstrated within the dynamic range of the assay calibration curve [17]. No cross-reactivity and no interference from common medications, lipemia or icterus were found.

Analyte stability 'in vitro'

The authors of a published assay evaluation study claim that the analyte is stable for 15 days at room temperature, for 15 days at 4°C, and for at least 6 months at –20°C and at –70°C [17]. Thus, the analyte as measured with the BGM Galectin-3 assay is considered well suitable for routine use in laboratory settings, also facilitating unproblematic conditions for sample shipment and storage. Six freeze and thaw cycles do not seem to affect galectin-3 analyte concentrations.

Biological variation of galectin-3

The components of biological variation of galectin-3 in healthy individuals with a median galectin-3 plasma concentration of 12 ng/mL (range, 7–20 ng/mL) were studied at two week intervals for eight weeks [23]. An intra-individual biological CV of 20%, an inter-individual biological CV of 23%, and a reference change value of 61% was found [23]. The reference change value indicates the difference required for 2 serial measurements of galectin-3 to be significantly different at $p < 0.05$. In the same study, the authors revealed the following results on the components of biological variation when blood was taken hourly for four hours from individuals with a median galectin-3 plasma concentration of 12 ng/mL (range, 6–28 ng/mL): intra-individual biological CV of 16%, an inter-individual biological CV of 16%, and a reference change value of 39% [23]. Both reference change values might be the basis for further studies attempting to demonstrate that galectin-3 can be used to monitor the results of treatments over time.

Galectin-3 concentrations in reference value studies

The upper reference value determined in adult individuals without known cardiac disease from the BioImage study was 22 ng/mL [17]. All individuals had detectable galectin-3 levels within the measuring range of the BGM Galectin-3

assay [17]. No distinction was made with respect to the individuals' age, gender or renal function.

In pediatric patients without heart failure and renal disease, galectin-3 plasma concentrations were not associated with age, gender or body mass index; the reference interval was 7-44 ng/mL including two outliers, and 7-33 ng/mL excluding outliers [25].

COMPARISON OF THE BGM GALECTIN-3 ASSAY WITH OTHER COMMERCIALLY AVAILABLE ASSAYS FOR GALECTIN-3 MEASUREMENT

In the vast majority of published clinical studies, the BGM Galectin-3 test (and the former version of this assay) has been used; until now relatively few studies have been performed with the ARCHITECT Galectin-3, the VIDAS Galectin-3 or the R&D galectin-3 assays.

The ARCHITECT Galectin-3 test uses the same monoclonal antibodies and conjugate used in the BGM Galectin-3 test [18]. Thus, besides the fact that the ARCHITECT Galectin-3 test demonstrates acceptable analytical performance on both the ARCHITECT $i1000_{SR}$ and the ARCHITECT $i2000_{SR}$ platforms, a method comparison between the ARCHITECT Galectin-3 test as the comparative method and the BGM Galectin-3 test as the reference method revealed slopes of 1.0 to 1.2, intercept of <-3.6 ng/mL and correlation coefficients of >0.90 [19]. The results of this multi-center assay comparison are shown in Figure 2.

According to our information, the VIDAS Galectin-3 assay is standardized against the BGM Galectin-3 test. However, due to proprietary reasons, bioMérieux does not report the details of the VIDAS antibodies. A comparison study with the BGM Galectin-3 test, however, showed an acceptable correlation (correlation coefficient of 0.90) and agreement between

both methods, with a rather small bias (i.e., a slope of 1.13 and an intercept of -3.83 ng/mL) [18,20] as depicted in Figure 3.

To our knowledge, no studies have been published comparing galectin-3 plasma concentrations as measured with the R&D galectin-3 assay vs. the BGM Galectin-3 assay. However, if we interpret the different limits of detection and measurement ranges given in Table 1 correctly, we assume a substantial bias (proportional and/or constant bias) between these two methods.

WHAT WE DON'T KNOW ANALYTICALLY

Greater clarity regarding the similarities and differences between the ST2 assays and the galectin-3 assays, respectively, would be welcome in order to minimize confusion when interpreting data in the published literature. At present, little recognition is given to the likely considerable differences between the sST2 and galectin-3 methods listed in Table 1. Emphasizing that results for one method do not necessary indicate results from another is important. This is applicable especially for the sST2 assays where a large bias between the methods can be observed, but to a lesser extent even for the galectin-3 assays where the concentrations obtained may be also dependent on the method used.

The currently commercially available methods for measurement of sST2 are not standardized. It is unclear that any of the methods has a calibrator which quantifies the analyte correctly. To resolve this issue it would be necessary to quantify the standards of the sST2 assays by a golden standard method. Similar considerations hold true for the galectin-3 methods as well, although the BGM Galectin-3 test, the ARCHITECT Galectin-3 test and the VIDAS Galectin-3 assay are "harmonized" to each other but obviously not to the R&D galectin-3 assay.

In addition, it is not published in the literature, which exact epitopes are detected by the antibodies against sST2 and galectin-3 used for the methods listed in Table 1. Therefore, it should be clarified whether the specific antibodies used in the assays recognize primary, secondary or tertiary structures of the sST2 and galectin-3 protein, respectively. If antibodies do not recognize the primary structure epitopes of the analyte, the ratio of available epitopes to the mass of protein will be dependent on retention of the structure of the epitope during the purification process of the standards. Consequently, this ratio might vary with each purification of the standard during production processes for different lots of the assays.

As detailed earlier in this review, increased concentrations of sST2 in the circulation can attenuate the systemic biologic effects of IL-33 by functioning as a "decoy" receptor for IL-33. Thus, sST2 and IL-33 could be measurable in different forms in the circulation. Theoretically, three analytes namely "free sST2", "free IL-33" and "complexed sST2" (i.e., sST2 bound to IL-33) should be present in the circulation [7]. Assuming non-competitive assays for the detection of sST2 and IL-33 by using capture and detection antibodies, different combination options are present. It is unclear, but it appears likely in our opinion that we measure the sum of "free sST2" and "complexed sST2" with the assays described in the literature. Therefore, a better understanding of what is detected by using different sST2 assays is needed. In order to clarify the situation for the sST2 assays available, elucidation of the protein crystal structure combined with epitope mapping would be necessary for the analyte sST2 and the assay antibodies. Considering the consequences of the IL-33/ST2L signaling pathway it might be illuminating to measure circulating concentrations of "free sST2", "complexed sST2", and "free IL-33" with different assays in the same patients. This is of course speculative, but measurement of these three analytes and calculating ratios

might provide insight into the pathophysiology of diseases with increased sST2 and/or IL-33 serum/plasma concentrations. One would like to suggest that with such assays even the prognostic information for patients with, e.g., inflammatory disease or heart disease could be increased.

As pointed out, galectin-3 exhibits both intracellular and extracellular functions and it has a concentration dependent ability to be monomeric or form oligomers. It is not well described in the literature, whether galectin-3 monomers, dimers and higher order oligomers are present in the circulation of humans [10,27,28]. If yes, this could have implications on what is analytically detected by different galectin-3 assays, especially if assay antibodies are directed against the N-terminal non-carbohydrate recognition domains, which are involved in higher order oligomerization. In addition, as studies suggest that various biological activities of galectin-3 are dependent on its ability to form higher order oligomers, it would be interesting to measure to which extent monomers and different oligomers are present in the circulation of healthy and diseased individuals. Similarly to the above considerations on sST2, measurement of the different isoforms of galectin-3 in the same patients and calculating ratios might provide insight into the pathophysiology of diseases with increased galectin-3 concentrations. Again, we would like to suggest that with such assays even the prognostic information for patients with, e.g., inflammatory disease or heart disease could be increased.

An important issue is the capability of any given assay to accurately measure low circulating concentrations of analyte which is method dependent. With respect to sST2, it is documented that it is not feasible with the MBL ST2 assay to accurately measure sST2 concentrations in healthy individuals [29]. In contrast, available data substantiate that it is possible to determine

sST2 concentrations in the vast majority of healthy individuals with the Presage ST2 assay [14,30]. As a consequence, the Presage ST2 assay is considered a high-sensitivity assay for measurement of soluble ST2 [14,15]. Similarly, it is documented in the literature, that it is possible to determine galectin-3 concentrations in the vast majority of healthy individuals with the BGM Galectin-3 test, the ARCHITECT Galectin-3 test and the VIDAS Galectin-3 assay [17-20]. No published study has evaluated the analytical sensitivity of the R&D assays for measurement of sST2 and galectin-3. Therefore, no statement can be made whether these assays facilitates reliable measurement of analyte concentrations in healthy individuals at low serum/plasma concentrations. Understanding distinctions between the sST2 and galectin-3 assays is critical, as they obviously vary quite substantially with respect to their low-end sensitivity and precision.

Reference values of sST2 are higher in males than in females, but are independent of age, body-mass index and renal function [14,21,24,25]. Thus, decision regarding the need for sex-based cut offs values for sST2 measurement requires more in-depth study. The physiological reasons and clinical relevance of these gender-specific differences among healthy adult individuals remain to be determined. One possibility is that sST2-synthesis or secretion might be (at least in part) under androgen control. Although a study was not able to demonstrate an independent association between sST2 and various sex hormones in healthy individuals [31], that does not necessarily imply that sST2-synthesis or secretion is not under androgen control. Specific *in vitro* experiments need to be designed addressing this issue. In the Framingham study, it was found that women taking estrogen had the lowest values when the authors stratified analyses by estrogen replacement status [24]. No relevant relationship was found between

circulating galectin-3 concentrations and age or gender in "healthy" individuals [17-20,25]. However, because it is evident from the literature that circulation galectin-3 concentrations are increased in renal disease and inflammatory disease, reference value studies on galectin-3 should consider both conditions. To our opinion, it is mandatory that reference value studies rely on pediatric or adult individuals without any impairment of kidney function and without any indication of inflammatory disease.

Lastly, further disease specific studies are necessary in order to elucidate the relationship between the progression of cardiac disease among pediatric and adult patients and sST2 and galectin-3, respectively. Additionally, increased ST2 and galectin-3 concentrations have been reported in cardiac disease but also in association with inflammatory disease (e.g., pneumonia and chronic obstructive pulmonary disease) [9]. This emphasizes the importance of considering non-cardiac co-morbidities and underlying inflammation in planning disease specific studies.

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ST2 and galectin-3: ready for prime time?

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ABSTRACT

ST2 and galectin-3 are emerging biomarkers in the field of heart failure and have been extensively studied, and that whether they provide additional prognostic value on top of the clinical models and the gold standard in HF, (NT-pro)BNP. Our aim was to provide a comprehensive review of these emerging HF-related biomarkers in chronic, acute and incident heart failure. Regardless of the type of heart failure, both biomarkers seem to have an additional effect on top of the clinical model including natriuretic peptides. Strategies that combine multiple biomarkers may ultimately prove to be beneficial in the guidance of HF therapy in the future. However, additional prognostic value appears to be limited, and what we need is to prospectively test the consistent observations, which then might lead to the implementation of ST2 and galectin-3 in heart failure algorithms.

1. INTRODUCTION

Heart failure (HF) is a major public health problem and affects more than 25 million patients worldwide. The lifetime risk for development of heart failure (HF) is more than 20% for people at the age of 40 and it is a major cause of morbidity and mortality in the western world (1). Although considerable improvements have been made in HF therapy, 5-year mortality rates remain unacceptably high, exceeding 50% (2). We can expect that, due to an aging population, the prevalence of HF will rise, at an alarming rate (3).

Therefore, better insight in the pathophysiological mechanisms that cause HF is needed. Biomarkers that reflect such mechanisms may assist in risk stratification and may help to create treatment strategies for the individual patient. Biomarkers may aid in the diagnosis of heart failure, or may be used to risk stratify patients, or to guide treatment. As such, numerous biomarkers have entered the heart failure arena. The vast number of biomarker articles has been referred to as a "biomarker tsunami" (4), but most biomarkers are still under investigation as therapeutic consequence and their role in disease management remains unclear at this stage.

The biomarker that is considered the gold standard, and is mentioned as such in HF guidelines, is B-type natriuretic peptide (BNP, or its stable precursor, NT-proBNP); this biomarker has established itself to be useful in diagnosis, prognosis, and disease management (5). In acutely decompensated patients with high volume load, the cardiac wall endures stress resulting in highly elevated BNP levels, which is loading dependent, and therefore will drop after unloading (6). However, BNP has its drawbacks, and is influenced by the "loading status" of the patient during presentation, but also by renal function, and obesity (7-9).

As mentioned by the 2013 American College of Cardiology/American Heart Association guideline for the management of heart failure, both galectin-3 and ST2 are emerging biomarkers that are not only predictive for hospitalization and death in patients with HF, but also add additional prognostic value over natriuretic peptides (10).

### ST2 and galectin-3: basic biology and functions

Suppression of tumorigenicity 2 (ST2), also known as IL1-RL1, is a member of the Toll-like/IL-1 receptor superfamily. As member of this family, ST2 consists of a common intracellular domain, the Toll/Interleukin-1 receptor (TIR). The gene for ST2 is located on chromosome 2q12 and is conserved across species. Four isoforms of ST2 exist namely, sST2, ST2L, ST2V and ST2LV. The soluble (sST2) and the transmembrane (ST2L) are mostly studied in HF research. sST2 lacks the transmembrane and cytoplasmic domains and includes a nine amino-acid C-terminal sequence. ST2 is upregulated by cardiomyocytes and cardiac fibroblasts when mechanical stress is imposed, for instance stretch. The ligand for ST2 is IL-33, another of member of the IL-1 interleukin family. When bound to IL33, ST2L confers an inhibitory effect on the Th2-dependent inflammatory response. Soluble ST2 can bind IL33, and it is hypothesized that sST2 works as a decoy receptor to IL-33 (11) (Figure 1A). Nowadays, it is thought that IL-33 signalling through ST2L provides a cardioprotective phenotype to protect the heart from excess stress, and that sST2 may neutralize this protective effect (12). In this article, we will use ST2 invariably, regardless if we refer to sST2 or ST2L.

Galectin-3 is encoded by a single gene, LGALS3, which is located on chromosome 14. It consists of two domains, namely an atypical N-terminal domain and a C-terminal carbohydrate-recognition domain (CRD). During differentiation of monocytes into macrophages galectin-3 is released and is involved in many processes during the acute inflammatory response such as

Figure 1A,B **(Patho) physiological processes of sST2 and galectin-3 in Heart Failure**
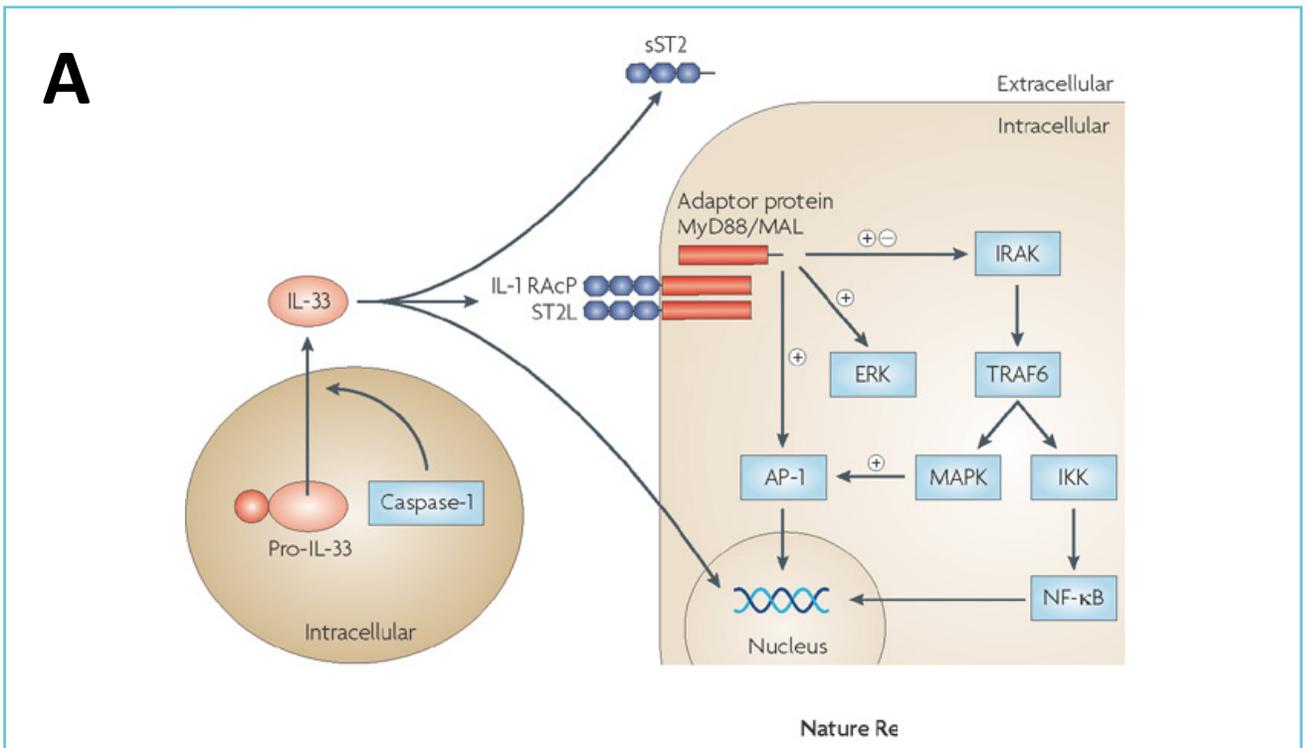
A. sST2 in the extracellular environment might bind free IL-33, thereby effectively decreasing the concentration of IL-33 that is available for ST2L binding and reducing the biological effect of IL-33 (11).
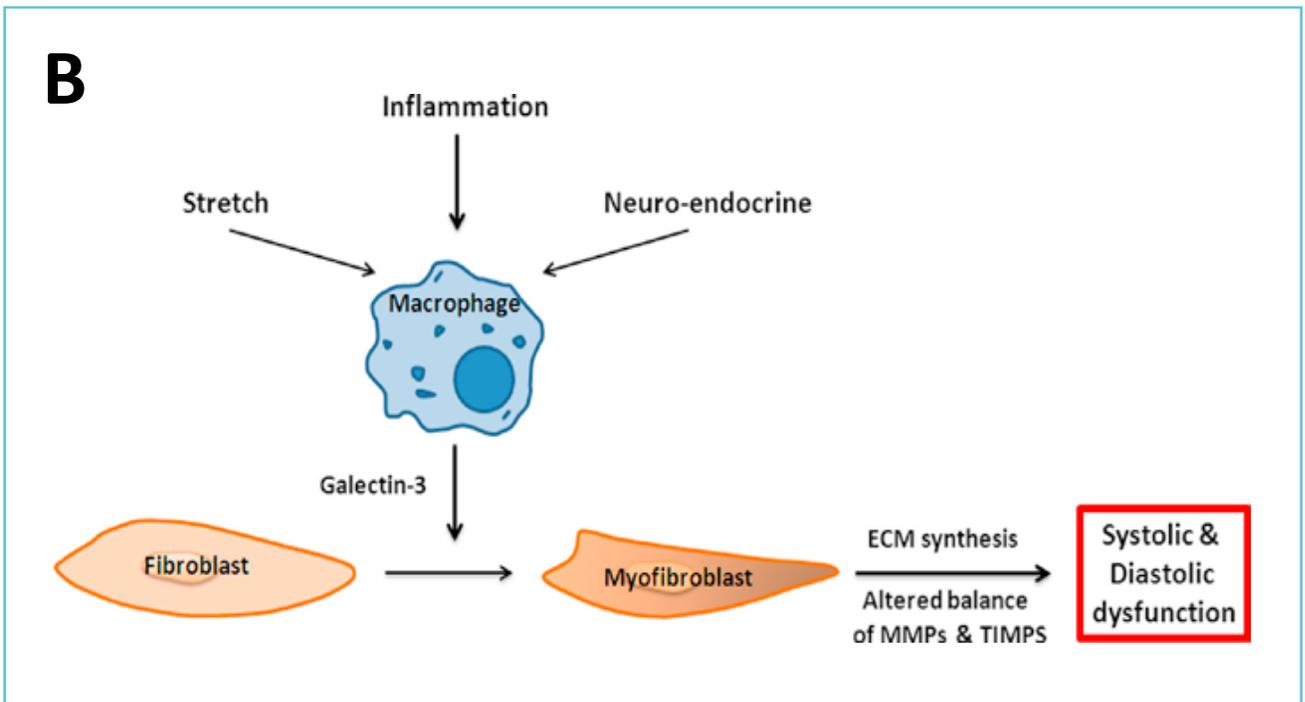
B. The transition of fibroblast to myofibroblast and the involvement of galectin-3 leading to systolic and diastolic dysfunction (35).

neutrophil activation and adhesion, chemoattraction of monocytes, opsonization of apoptotic neutrophils and activation of mast cells. Galectin-3 has been identified as a causal factor in the development of fibrosis of the heart (and other organs). The potential roles of galectin-3 in HF are displayed in Figure 1B (13,14).

Established risk factors, such as New York Heart Association (NYHA) functional class, medication use, routine laboratory values, and left ventricular ejection fraction (LVEF), do not fully explain the mortality risk of patients with chronic HF and do not estimate the prognosis of individuals (15,16). Both ST2 and galectin-3 reflect tissue damage, independent of cardiac loading conditions. As such, they may supplement the currently used biomarkers. We herewith discuss articles describing these emerging HF-related biomarkers in chronic, acute and incident heart failure.

2. CHRONIC HEART FAILURE

2.1. ST2

ST2 can reliably be measured with three different assays. The MBL assay, the R&D assay and the Presage assay. The latter assay is FDA-cleared and CE marked, while the other two methods are research assays. These three methods are not directly comparable and it is important to be informed which method was used when interpreting the results (17). Dieplinger et al. reported that only the Presage ST2 assay meets the needs of quality specification of laboratory medicine (18).

We can only discuss a subset of the published studies, and there are many more. We refer to recent excellent review articles that summarize all the available evidence for ST2 (19-21). Currently, we lack a meta-analysis that would help to compile the aggregate evidence. We discuss a few of the most interesting articles.

One of the first published reports on ST2 and chronic HF was published by Pascual-Figal et al. (22). They demonstrated that ST2 could predict sudden cardiac death in ambulatory patients (N=99) with mild to moderate HF and systolic dysfunction. >70% of sudden cardiac death occurred in patients with both elevated ST2 and NT-proBNP levels compared to 4% when both markers were low (13).

Daniels et al. (23) reported in a larger cohort of HF patients which were referred for echocardiogram (N=588) that heart rate, current diuretic use, estimated creatinine clearance, the presence/absence of right ventricular hypokinesia, and mitral valve E wave velocity were independently associated with ST2 levels. In addition to association analyses, they also observed that multivariate adjusted ST2 levels were significantly predictive for all-cause mortality after one year.

In a much larger study in 2011, Ky et al. (24) reported of 1141 chronic HF patients. The Penn Heart Failure Study (PHFS) investigators concluded that ST2 is strongly associated with HF severity. Patients with elevated levels of circulating ST2 had a markedly increased risk of death or heart transplantation. In the assessment of individual patient risk, ST2 performed equally well compared to the established biomarker NT-proBNP.

The relationship of ST2 and renal function was studied in the Barcelona study (25). This study included 891 patients, and demonstrated that the prognostic value of ST2 was not influenced by renal function. This finding suggests that ST2 may be advocated as a preferable biomarker in patients with renal insufficiency, a co-morbidity that is very common in HF and is among the best predictors of adverse outcomes.

Functional capacity and long-term clinical outcomes in ambulatory patients was studied by Felker et al. (26) in a Controlled Trial Investigating

Outcomes of Exercise Training (HF-ACTION) study. ST2 was measured in a sub-set of 910 patients and was associated with cardiovascular death and HF hospitalization even after comprehensive covariate adjustment. Combining ST2 with NT-proBNP rendered the strongest predictive value (Figure 2A). However, the addition of ST2 to the model did not result in significant reclassification in this study.

Another large trial, the Controlled Rosuvastatin Multinational Trial in Heart Failure (CORONA) study, comprising of 1449 patients, demonstrated the association between ST2 and cause-specific outcomes (27). Next to the primary endpoint (a composite of cardiovascular (CV) death, non-fatal myocardial infarction, and stroke), the authors also studied the association of ST2 with worsening HF. ST2 indeed was associated with the primary endpoint, but was no longer associated after full adjustment including NT-proBNP and CRP, however ST2 remained associated with death due to worsening HF, hospitalization due to worsening HF, and hospitalization due to any CV cause, also after full adjustments. The latter could suggest that the ST2 pathway, in addition to its value in risk stratification of death is also relevant in determining disease progression in HF.

A recent study with a long follow-up of nearly 4 years by Gruson et al. showed in 137 HF patients with reduced LVEF that ST2 predicted long-term CV death, even stronger then NT-proBNP (28).

2.2. Galectin-3

Several commercially available assays can measure galectin-3 plasma or serum levels (29). Manually, galectin-3 can be measured with the BG Medicine assay (30) and the R&D assay (31). But several automated assays are available to measure galectin-3, including the ARCHITECT assay (32), the Vidas assay (33), and an Alere assay. The possibility to measure galectin-3 values on an automated platform allows for a quick, easy and reliable measurement of galectin-3 levels. By far, most published data have been measured with the BGM assay, however the ARCHITECT and VIDAS assays use the same antibodies and are calibrated against the BGM assay, making reported values comparable throughout the literature.

We can only discuss a subset of the published studies, and there are many more. We refer to recent excellent review articles that summarize all the available evidence for galectin-3 (19,34,35). Also, there is a recent meta-analysis that helps to compile the aggregate evidence (36). We discuss a few of the most interesting articles.

Lin et al. observed a correlation of galectin-3 with cardiac extracellular matrix (ECM) turnover markers in 106 CHF patients. These correlations were still abundant after adjusting for age, sex, smoking status and NYHA class (37).

The prognostic importance of galectin-3 was analyzed for the first time in 232 chronic HF patients with systolic dysfunction and severe HF (38). These patients, by Lok et al., were also studied in a follow up study, with 9-years follow-up. After adjustment for several established risk factors, galectin-3 remained an independent prognostic marker for long-term all-cause mortality (Figure 2B) (23,39). Next to this, they observed an independent relationship between galectin-3 and left ventricular remodeling determined by serial echocardiography (24). The latter finding strengthened the hypothesis that galectin-3 is involved in cardiac remodeling. The HF-ACTION investigators did not only measure ST2 as described above, but also measured galectin-3 in a sub-cohort of 895 patients (40). Galectin-3 levels at baseline were related to the primary outcome of the study, all-cause mortality or rehospitalization. Patients with both elevated levels of galectin-3 and NT-proBNP had a two times higher risk for all-cause death or rehospitalization. Galectin-3

Figure 2A,B   sST2 and Galectin-3 combined with NT-proBNP in Chronic Heart Failure
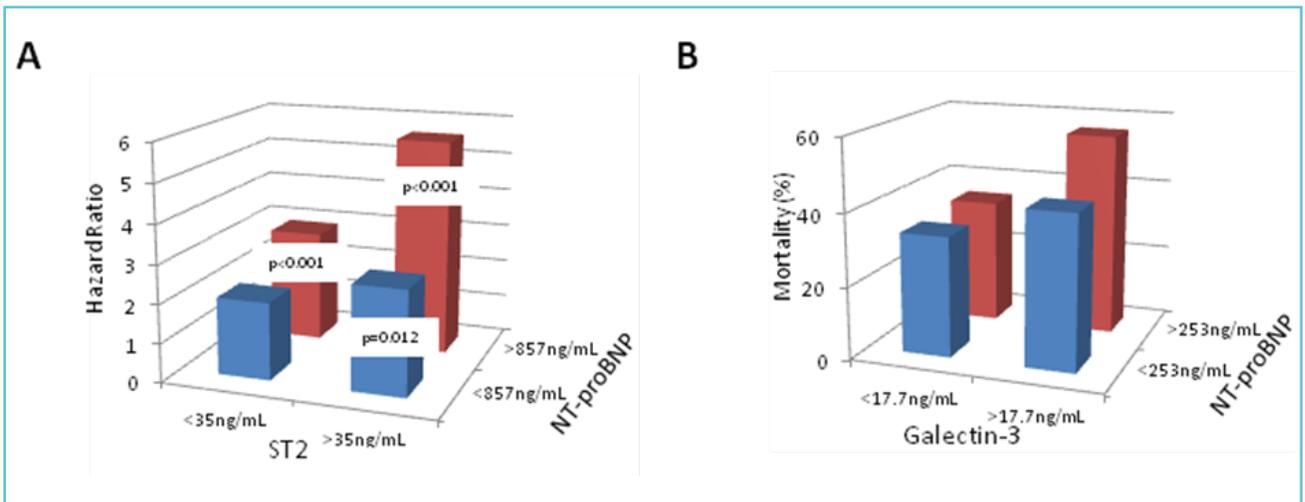
A. Hazard ratio for all-cause mortality based on groups defined by NT-proBNP and ST2, compared with a reference group with low NT-proBNP and low ST2. P values are for change relative to reference group; Figure adapted from Felker et al (26).

B. Mortality rate by galectin-3/NTproBNP subgroups; Figure adapted from Lok et al. (38).

alone lost its predictive value after adjustment for NT-proBNP for the composite endpoint, cardiovascular death or cardiovascular hospitalization. In a more recent analysis of the same study, mortality was divided in death due to heart failure and sudden cardiac death (SCD), and it was shown that galectin-3 had no incremental value on top of clinical factors and NT-proBNP to predict death due to heart failure, while it did add incremental value for the prediction of SCD (41). The low baseline levels of galectin-3 in this cohort could possibly explain this.

In the CORONA trial (42), after a median follow-up of 33 months, galectin-3 univariably predicted the composite endpoint of cardiovascular death, nonfatal myocardial infarction and stroke, but after adjustment for NT-proBNP this was no longer statistically significant. In an additional analysis it was shown that, seemingly paradoxically, patients with low galectin-3 levels may benefit from statin therapy (43). However, in the Val-HeFT trial, originally designed to evaluate the efficacy of valsartan in chronic systolic HF patients, galectin-3 remained significantly associated with mortality and HF

hospitalization, also after addition of both eGFR and NT-proBNP to the model (44). The same results were reported in a smaller trial comprising of 133 CHF patients (45). Galectin-3 seems to be more influenced by kidney function (46,47) than ST2, and it has been shown that elevated galectin-3 levels precede and predict renal insufficiency (48).

In a head-to-head comparison of ST2 and galectin-3 in 876 ambulatory patients, both ST2 and galectin-3 were associated with an increased risk for all-cause mortality but ST2 was only associated with cardiovascular mortality. Galectin-3 did not significantly improve the performance for prediction of mortality when added to a (extensive) base model. The authors discussed that the absence of prognostic value of galectin-3 in this study may be explained by the observation that galectin-3 might confer stronger prognostic information in early disease as compared to progressed disease (49).

Finally, a recent meta-analysis by Chen et al. (36) investigated the relationship between galectin-3 and all-cause mortality in 8,419 participants enrolled in 9 studies, with a follow up

period ranging from 1 to 8.7 years. After correction for well-established risk factors, including in all studies at least age, creatinine (or eGFR) and BNP (or NTproBNP), galectin-3 was shown to have an independent predictive value for mortality in chronic HF.

3. ACUTE HEART FAILURE

Acute HF (AHF) is a main cause of hospitalizations for people over the age of 65 in the western world (50) and a leading cause of mortality. Dyspnea is the most common symptom of AHF patients who are presented at the emergency department (ED) (51). Usually a first assessment of AHF patients occurs at the ED, which commonly consists of clinical parameters that are easy to obtain, such as medical history, use of drugs, signs and symptoms of heart failure, ECG, chest X-ray and laboratory assessment. Currently, biomarker assessment including BNP or NT-proBNP and hs-Troponin is commonly part of this work up. However, despite all these parameters, it has been reported that nearly half of the HF hospitalizations were unnecessary in retrospect (52). This highlights that there is room for improvement. Hospitalization for AHF is a crucial moment in a patients course with the diagnosis, it is estimated that risk for death by 1 year is as high as 30% following discharge, with rehospitalization rates exceeding 50% (53).

3.1. ST2

The first study of ST2 measurement in patients with suspected or proven AHF was in the Pro-BNP Investigation of Dyspnea in the Emergency Department (PRIDE) study (54). In 593 patients admitted to the emergency department with acute dyspnea ST2 was measured. ST2 levels were significantly higher in patients with AHF than patients with non-HF dyspnea and the likelihood for HF diagnosis was greater in patients in the highest deciles of ST2. The sensitivity to

correctly diagnose AHF was significantly better for NT-proBNP than for ST2. ST2 concentrations predicted 1-year mortality in both patients with and without HF, where ST2 showed additive prognostic value on top of NT-proBNP (Figure 3A). In a sub-study of PRIDE in 139 subjects conducted by Shah et al. (55) ST2 concentrations were associated with higher LV end-systolic dimensions and volumes, lower LV ejection fraction, lower right ventricular fractional area change, larger RV systolic pressure and hypokinesia. They also demonstrated that ST2 predicted death at 4 years, independent from other traditional clinical, biochemical and echocardiographic risk markers.

Friões et al. (56) divided AHF patients into HFpEF and HFrEF patients, and reported that NT-proBNP predicted all-cause mortality or readmission at 6 month for both types of patients. Interestingly, ST2 was reported to be a significant marker for prognosis in HFrEF patients, but not so in HFpEF patients. However, in a pooled analysis of three cohorts from Boston, Massachusetts, Linz, Austria and Murcia, Spain, data were available for 447 patients with AHF. They found equally strong prognostic value of ST2 irrespective of LVEF (57). The data suggest that different pathways for the establishment and progression of HF are present, and biomarkers might help to characterize between these phenotypes.

3.2. Galectin-3

As for ST2, galectin-3 was initially evaluated in the PRIDE study published by van Kimmenade et al. (58). They demonstrated that galectin-3 was a better prognostic predictor for 60-day mortality compared to NT-proBNP and that the combination of both predicted mortality even better (Figure 3B). This finding was strengthened by the comparable observations in the Coordinating study evaluating Outcomes of Advising and Counseling in Heart Failure

Figure 3A,B   sST2 and Galectin-3 combined with NT-proBNP in Acute Heart Failure
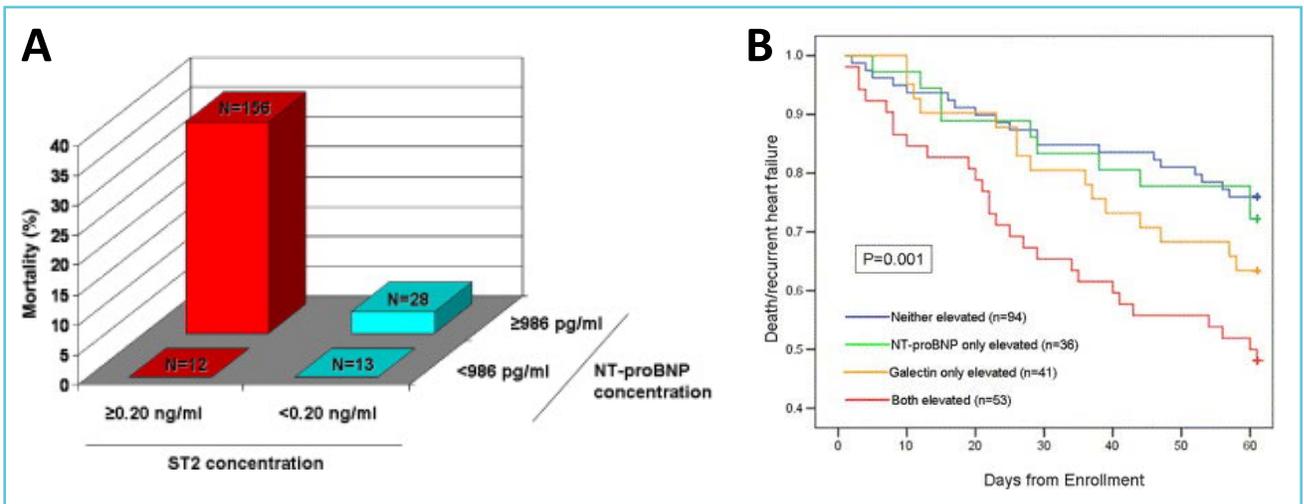
A. Mortality rates at 1 year as a function of ST2 and NT-proBNP concentrations among patients with acute HF (n = 208) (54).

B. Elevated galectin-3 and NT-proBNP levels were in acute heart failure associated with higher rates of mortality/recurrent heart failure (60).

(COACH) study that enrolled 592 AHF patients at discharge (59). In the COACH study galectin-3 seemed to have particularly strong predictive value in patients with HFpEF, compared to patients with HFrEF (59). This finding was validated in a PRIDE sub-study which included echocardiographic data showing significant relations between galectin-3 and diastolic echo parameters. Galectin-3 was significantly correlated with parameters of diastolic function such as E/E' (60). Another large HFpEF cohort comprising 419 patients admitted with AHF, demonstrated the same predictive value of galectin-3 in patients with HFpEF. Galectin-3 emerged as an independent predictor of unfavorable outcome (mortality and HF hospitalizations), and addition of galectin-3 to base models increased c-statistics and yielded significant reclassification indices. The particularly strong value in HFpEF is promising, as currently, the diagnosis and therapy of HFpEF are difficult and further insight in possible mechanism are needed (59).

Further, Meijers et al. showed that the predictive value of galectin-3 is particularly useful for short-term outcomes. Elevated galectin-3 was strongly associated with near-term rehospitalization at 30, 60, 90 and 120 days in a pooled analysis comprising three AHF studies (PRIDE, COACH and the Maryland-study (UMD-H23258), totaling 902 AHF patients (61). In this study, galectin-3 showed numerically very high and statistically very strong reclassification indices.

Another large study focusing on short-term outcome of 603 patients presenting with acute dyspnea in the emergency department showed that galectin-3 was a strong predictor of 90-day outcome. However, in a multivariable model it lost its predictive value (62).

Finally, while most studies report on identification of high-risk patients, Meijers et al. recently assessed whether biomarkers could be used for low-risk prediction in AHF patients. Out of a large panel of biomarkers galectin-3 was the only biomarker able to predict low-risk for all cause mortality and/or HF rehospitalization adjusted for multiple variables including NT-proBNP (63).

4. INCIDENT HEART FAILURE

Despite significant progress in the treatment of heart failure (HF), the incidence and prevalence of this diagnosis are rising. This trend is expected to continue and is attributed primarily to the increasing proportion of elderly in the population, improved care of acute heart diseases resulting in improved patient survival, and increasing prevalence of cardiovascular risk factors such as obesity and diabetes. Biomarkers might identify subjects at risk for incident HF.

4.1. ST2

Four community-based cohorts have examined ST2 concentrations at baseline namely the FINRISK97 population cohort (64), Framingham Heart Study (65), the Dallas Heart Study (66), and the Cardiovascular Health Study (67).

Hughes et al. investigated the predictive value of ST2 in a large Finnish general population study (N=8444). In this cohort of healthy individuals, ST2 adds little predictive information beyond standard risk markers for cardiovascular endpoints, such as MACE, CVD and stroke, and is of marginal benefit to all-cause mortality prediction in a general population of healthy participants. ST2 also failed to improve prediction for heart failure either as an individual marker following adjustment for Framingham risk factors or in addition to the established cardiac marker NT-proBNP and/or eGFR.

In contrast, in 3,428 Framingham participants followed for approximately 11 years, sST2 was associated with both incident heart failure and all-cause mortality. Subjects in the highest quartile of sST2 had a 2.5-fold increased risk of incident heart failure compared with those in the lowest quartile.

ST2 was also measured (using a less sensitive research-use-only method) in 3,294 participants of the Dallas Heart Study, who were followed for a median of 8 years. In this analysis, most subjects did not have measurable sST2. In contrast to data from other cohorts, ST2 concentrations were not associated with most traditional cardiovascular risk factors. Since the assay used in the Dallas Heart Study differs so strongly from the ones used in other studies, it is difficult to compare the studies.

Most recently, ST2 concentrations were measured in 3,915 participants of the Cardiovascular Health Study free of heart failure. During a median follow-up of 15 years, ST2 concentrations at baseline predicted incident cardiovascular events in multivariable-adjusted analyses. Specifically, participants in the upper quintile of sST2 had a greater risk of heart failure and cardiovascular death compared with participants in the lowest quintile.

4.2. Galectin-3

Six community-based cohorts have examined galectin-3 concentrations at baseline, namely the Framingham Heart Study (68), the Physician Health study (case control) (69), the PREVEND study (70), FINRISK97 population cohort (71), the Rancho Bernado study (72), and the Cardiovascular Health Study (67).

In the Framingham (offspring) study, galectin-3 levels were available in 3,353 participants, who were observed during a mean follow-up of 11 years. Galectin-3 was a significant predictor of HF risk, also after multivariable adjustment. This observation was validated in the Physicians' Health Study, which used a case-control design, describing 462 cases and 462 controls. After adjusting for clinical variables there was a significant relation between galectin-3 and new onset HF.

The Physicians' Health Study validated the Framingham findings in a nested case control design, including approximately 900 subjects. After adjusting for BMI, diabetes, AF, hypertension, CRP, alcohol and smoking there was a significant

relation between galectin-3 and new onset HF, with or and without prior CHD.

In the PREVEND (PREvention of Vascular and End stage ReNal Disease) study, a total of 8,569 subjects were followed during a median follow-up of 13 years. Galectin-3 emerged as a predictor of new onset HF in subjects with high CV risk compared to subjects with low CV risk.

The same cohort as described earlier, the general population-based FINRISK 1997 cohort evaluated the usefulness of galectin-3. They observed that galectin-3 levels were predictive for future cardiovascular events, also after adjustment for gender. However, the reclassification indices were modestly improved.

In an elderly cohort (mean age 70 years), the Rancho Bernardo Study, baseline galectin-3 levels were independently associated with all-cause and CVD mortality. These elderly subjects had no known CVD prior to the study participation. Addition of galectin-3 to the model resulted in significant reclassification indices.

Both ST2 and galectin-3 was measured in the Cardiovascular Health Study. Comparable results for both biomarkers were observed and subjects in the upper quintile of galectin-3 level had a greater risk of heart failure and cardiovascular death compared to participants in the lowest quintile.

5. THERAPEUTIC GUIDANCE

Serial ST2 sampling over time during aggressive HF therapy seems to be a strong prognostic indicator for future outcomes (73). Especially adjustment of β-blocker dose might be associated with changes in ST2 levels (74). In a post hoc analysis from the PROTECT trial (75), it was observed that β-blocker therapy exerted beneficial effects across the complete study population. Patients with high ST2 levels and a low dose of β-blocker were at the highest risk for cardiovascular events. However, up-titration of beta-blocker therapy nowadays is part of the standard clinical care so it is questionable if we would need a biomarker-guided treatment.

Next to an association with β-blockers an association for both biomarkers with mineralocorticoid receptor antagonists (MRAs) is present. Recent experimental data showed that MRA treatment after MI strongly reduced both galectin-3 and ST2 expression in the myocardium and improved LVEF (76). Aldosterone might play a role as mediator of the pro-fibrotic effects of galectin-3. However in clinical studies (77,78) no evidence of the interaction between galectin-3 and MRA treatment were observed. Therefore, it remains unclear whether we should use biomarker-guided therapy in the future.

6. TARGETED THERAPY

Unraveling the pathophysiological mechanism of both biomarkers could be of significant importance. As already described and proven in experimental studies, galectin-3 inhibitors, such as complex carbohydrates, attenuate the cardiac remodeling processes and reduce fibrosis formation after different cardiac stressors (79,80). These inhibitors also resulted in improved function parameters as fractional shortening. Galectin-3 has the potential as a new modifiable risk factor in HF patients and it would be very interesting to monitor galectin-3 levels pre and post anti-galectin-3 treatment (81). No such data exist for ST2.

7. CONCLUSION –
READY FOR PRIME TIME?

We have provided an overview of the potential value of ST2 and galectin-3 in chronic heart failure, acute heart failure and incident heart failure. Both ST2 and galectin-3 show prognostic value in mostly all patients with chronic and acute HF, on top of natriuretic peptides.

As clearly shown in all figures, both sST2 and galectin-3 predict outcome more accurately when combined with NT-proBNP. These consistent findings may pave the road for the implementation of sST2 and galectin-3 in chronic heart failure algorithms. Strategies that combine multiple biomarkers may ultimately prove beneficial in guiding HF therapy in the future.

This is also true for AHF patients, but in this setting even serial measurements of both galectin-3 and sST2 would provide further insights in patient management strategies, for example at discharge and 3-6 months later during the "chronic" state of the disease.

When considering risk assessment, there is no urgent need for additional prognostic tests. Current models already very accurately predict prognosis in our HF patients, and addition of more factors show modest improvement of the models. We do not need biomarkers to tell us to take medication that is part of evidence-based therapy, which we should start and up titrate in all HF patients.

So, the most challenging task will be to design, fund and launch prospective studies that incorporate biomarker based treatment algorithms. Such studies will provide definite data as to whether it is useful and economical to order (expensive) biomarker tests on the long run. Such studies should test biomarker specific or targeted therapies, not the state of the art medication that is part of daily care. Only in this manner, HF management will move forward towards more personalized approaches, which might lead to the use of multiple biomarkers. Learning the most efficient ways to exploit them to assess and risk stratify patients should be the goal of the upcoming years. Testing algorithms including ST2 and or galectin-3 could be a first step towards more personalized medicine.

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Emerging and disruptive technologies

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ABSTRACT

Several emerging or disruptive technologies can be identified that might, at some point in the future, displace established laboratory medicine technologies and practices. These include increased automation in the form of robots, 3-D printing, technology convergence (e.g., plug-in glucose meters for smart phones), new point-of-care technologies (e.g., contact lenses with sensors, digital and wireless enabled pregnancy tests) and testing locations (e.g., Retail Health Clinics, new at-home testing formats), new types of specimens (e.g., cell free DNA), big biology/data (e.g., million genome projects), and new regulations (e.g., for laboratory developed tests).

In addition, there are many emerging technologies (e.g., planar arrays, mass spectrometry) that might find even broader application in the future and therefore also disrupt current practice. One interesting source of disruptive technology may prove to be the Qualcomm Tricorder XPrize, currently in its final stages.

INTRODUCTION

We live in an age where many technologies have disrupted or transformed both our everyday lives and commerce (e.g., the worldwide web, tablets, cell phones, wifi, the Cloud, Skype, Amazon, Expedia, Google) (1). Likewise, in laboratory medicine, automation, computers, immunoassay, monoclonal antibodies, and PCR are examples of technologies that were considered disruptive and now are routine in everyday practice. Currently, there are several emerging and potentially disruptive technologies that could unexpectedly displace an established technology and modify the practice of our profession. This article briefly explores a number of technologies that have made a major impact on the practice of laboratory medicine in recent years or are poised to have an impact in the future.

CANDIDATE TECHNOLOGIES

In this article, I have chosen to discuss the following disruptors: robots, 3-D printing, technology and technology convergence, point-of-care technologies, new types of specimens, big biology/data, and the regulations.

Robots

The manual and labor intensive laboratories of the early 1900s have evolved to the highly automated laboratories of today in which robotics plays a major role (e.g., automated analyzers, total automation systems). Mobile general-purpose dual arm robots represent a new phase of automation (http://www.motoman.com). This type of robot is employed in some laboratories to perform repetitive tasks, such as ELISAs (enzyme-linked immunosorbent assay), assays that were previously performed by technologists. This trend inevitably will lead to a depopulation of the laboratory (humans outsourced to machine labor). Based on these advances, it is not difficult to envisage deployment of autonomous artificial intelligence-enabled humanoid robots in the future, which would lead to a further depopulation of the laboratory (2).

3-D printing

Replicators, based on 3-D printing technology, are finding use in research and manufacturing, especially for prototyping (http://3dprinting.com/what-is-3d-printing/). In the routine clinical laboratory, a possible future role for this technology could include on-demand manufacture of spare parts, including key parts and components for laboratory equipment. The use of replicators therefore would result in a greater degree of autonomy for the laboratory (3).

Technology and technology convergence

There are many technologies and facets of technology that may influence the future development of laboratory medicine. Massively-parallel planar microarray-based analytical methods already dominate nucleic acid sequencing. In one version of this type method, an array of micro-sized spots (a microarray) of oligonucleotides on the surface of a small chip are used to simultaneously test a sample for the presence of sequences complementary to the immobilized oligonucleotides. The simultaneous nature of the testing dramatically improves throughput compared to previous methods. It is expected that this type of testing may become more widespread and heavily utilized in the future for both genomic (https://www.genome.gov/10000533) and proteomic applications (4).

At the same time, the test menu for mass spectrometry, a technology that competes with microarrays for some applications, is expanding. The current applications for mass spectrometry span proteomics, genomics, neonatal testing, steroid profiles, drug testing, vitamin D testing, and microbial identification. Mass spectrometry-based microbial identification is an example

of an application that has been disruptive in microbiology. The broad scope of potential applications for mass spectrometry, from metals to macromolecules, positions this technology for even greater disruptive impact in the future.

Finally, the Qualcomm Tricorder XPrize provides a view of several emerging technologies that may ultimately turn out to be disruptive (http://tricorder.xprize.org/). The vision of this $10 million prize is to "Imagine a portable, wireless device in the palm of your hand that monitors and diagnoses your health conditions. That's the technology envisioned by this competition, and it will allow unprecedented access to personal health metrics." The competition currently is in its final stage. DNA Med Institute (DMI), a company that has developed a hand-held analyzer for testing whole blood (http://www.dna-medinstitute.com/), is among the finalists. The DMI technology is based on "nano dipsticks"; small pads on the dipsticks react with blood components, and the resulting fluorescent signals from the pads are scanned in a flow cell. NASA has already utilized this analyzer in zero gravity conditions (5).

Technology convergence is the integration of two or more different technologies within a single device or system. This has been a trend for smart phones that now commonly integrate a telephone with other capabilities and features, including a camera, GPS, PDA, MP3 player, and wireless access (e.g., internet and email). Medical testing now can be added to this list of features. Initially, this was via medical apps, e.g., for heart rate (6), but now there are glucose testing devices that plug into a smart phone port (e.g., iHealth Align) (https://ihealthlabs.com/glucometer/ihealth-align/) or a computer (e.g., CONTOUR® NEXT USB) (https://www.contournext.com/our-products/contour-next-products/contour-next-usb-meter/). Clearly, this technological advance ultimately may disrupt conventional glucose meters.

Point-of-care technologies

The use of disposable electronics is one interesting example of a disruptive trend in point-of-care testing. These devices simplify operator use of lateral flow tests and provide more detailed diagnostic information and an improved user-interface (so-called digital pregnancy tests). The Clearblue Advanced Pregnancy Test with Weeks Estimator is the most sophisticated of these devices. It contains two tests strips: one detects hCG and the other measures the level of hCG to estimate time since ovulation (1-2 weeks, 2-3 weeks, 3+ weeks) (http://www.clearblueeasy.com/pregnancy-test.php).

The device controls the timing of the assay, and the disposable electronics and optical reader within the test cartridge measure the strip results to provide a simple, digital readout in words and numbers on the LCD display.

One of the latest developments in digital pregnancy tests is the First Response™ Pregnancy Pro, a wireless technology-enabled pregnancy test stick that connects via Bluetooth® to a smart phone. An app on the smart phone provides test instructions, indicates the time remaining to the result (and provides distractions to reduce stress while waiting for the test result), displays the result of the test, and suggests next steps (http://www.firstresponse.com/en/Products/Pregnancy/Pregnancy-PRO).

Smart contact lenses are another example of a novel point-of-care technology. One such product, under joint development by Google and Novartis, includes a glucose-sensing electrode and telemetry, so that glucose levels in the fluid bathing the eye can be monitored and the data transmitted to a remote device (7). In addition, the recently FDA-cleared Triggerfish® smart contact lens detects changes in ocular dimension for monitoring the patterns of intraocular pressure fluctuations in order to improve the management of glaucoma (8).

There are also on-going and major changes in access to tests and testing. The consumer now can choose from numerous locations that provide direct testing (e.g., pharmacies, Retail Health Clinics) (9) and also has many available routes to obtain diagnostic medical tests. These include Direct to Consumer Testing via the internet, collection kits for drugs of abuse and infectious diseases (e.g., Home Access® Express HIV-1 Test System) (http://www.homeaccess.com/ExpressHIV_Test.asp), and pharmacogenomic tests available from the pharmacy (e.g., Harmonyx test for attention deficit hyperactivity disorder)(10). One new company's focus is on at-home heart health testing, using a small analyzer and disposable cartridges. Test information is sent to the cloud, and the test results are sent back to the user in ~ 5 minutes, together with tips on how to improve their results (http://techcrunch.com/2016/03/23/former-apple-exec-launches-at-home-blood-test-startup/). Direct to consumer testing is controversial and arguments center around the ethical, legal, medical aspects and the societal impact of such testing (http://www.ascp.org/content/docs/default-source/pdf/2a03e13e-a766-4c4f-aa82-c82f953d2988.pdf).

New types of specimens

Traditionally, laboratory medicine has focused on the analysis of nucleic acid isolated from cellular specimens (e.g., white blood cells, buccal cells, FFPE (formalin-fixed, paraffin-embedded) tissues). The discovery that blood also contains cell-free DNA has led to intense interest in the analytical possibilities of this specimen type. Cell-free DNA refers to relatively small pieces of circulating DNA that originate from a person, a fetus, or a cancer (so-called liquid biopsy) (11). Already, there are several cell-free DNA-based methods for trisomy testing (12), and plans have been announced for large scale tumor-free DNA testing to enable the early detection of cancer in

asymptomatic individuals (http://www.grailbio.com/). The emerging success of cell-free DNA as a specimen is not without controversy, especially in its performance as a screening test for fetal aneuploidy compared to standard methods (13), but in the future, it has the potential to displace other sources of nucleic acid for a range of testing purposes.

Big biology/data

Large-scale mega-sequencing projects involving thousands to millions of subjects are a recent phenomenon (http://blog.oup.com/2015/02/millions-genomes-project/). At the current time, there are several studies (either in planning stages or in progress) involving one million subjects, including studies organized by the Beijing Genomics Institute (BGI) (14), National Institutes of Health (NIH) (https://www.nih.gov/precision-medicine-initiative-cohort-program/scale-scope), Veterans Affairs (VA) (http://www.research.va.gov/mvp/), and others planned for 100,000 (UK, https://www.genomicsengland.co.uk/the-100000-genomes-project) and 200,000 subjects (http://www.grailbio.com/).

The general goal of these studies is to determine how best to use genomics in healthcare. Some studies, such as the UK 100,000 Genomes Project, have focused on specific types of patients, e.g., patients with rare diseases and their families, and patients with cancer. The hope is that these studies will provide new insights into genetics and health that will lead to the development of more effective, and potentially disruptive, testing. In fact, the first patients diagnosed through the UK 100,000 Genomes Project were reported in 2015 (http://www.genomicsengland.co.uk/first-patients-diagnosed-through-the-100000-genomes-project/).

Regulations

The regulation of clinical laboratories (e.g., CLIA'88) and in vitro diagnostic devices (e.g.,

Table 1	Classification of primary problem of problematic LDTs (16)
A	Tests that yield many positive results when the disease or condition is not actually present (false-positives)
B	Tests that yield many negative results when the disease or condition is actually present (false-negatives)
C	Tests with the potential to yield both many false-positive and false-negative results
D	The factor detected has no clear relevance to the disease
E	Tests linked to treatments based on disproven scientific concepts
F	Tests that undermined drug approval or drug treatment selection
G	Other unvalidated tests

FDA clearance, CE Marking) has been beneficial for the quality of clinical laboratories and clinical testing. In the United States, the FDA has announced its plans for regulating laboratory developed tests (LDTs) (15), and a recent publication has outlined the public health evidence for FDA oversight of LDTs (16). This publication provides 20 case studies of problematic LDTs that are classified into seven different primary problems (Table 1) (16). The issues identified relate to test performance (false positive, false negative), lack of clinical validation or validity, and adverse impact on therapy.

The impending regulation of LDTs would be a major disruption for clinical laboratories in terms of the cost and resources that potentially are required to comply with new LDT regulations. At this early stage in the process, it is difficult to predict the effect of such new regulations, but it is possible that the number of laboratories offering LDTs would decrease. Most likely, the tests would be consolidated and sent to laboratories that are willing to make the necessary investment required to comply with the final regulations (e.g., reference laboratories).

CONCLUSIONS

Plotting the future course of laboratory medicine is difficult, and history has shown that attempts to predict future developments are often unsuccessful (17). This article presents selected developments that have the potential to change the way in which laboratory medicine is practiced in central laboratories or at the point of care. Many of these are based on emerging technologies that are still in the early stages of development and thus their potential for disruption has yet to be determined.

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