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

Thomas Mueller, Benjamin Dieplinger

Department of Laboratory Medicine, Konventhospital Barmherzige Brueder, Linz, Austria

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Corresponding author:
Thomas Mueller
Konventhospital Barmherzige Brueder
Seilerstaette 2-4, A-4020
Linz, Austria
Phone: +43 732 7897 16020
E-mail: thomas.mueller@bs-lab.at

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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].

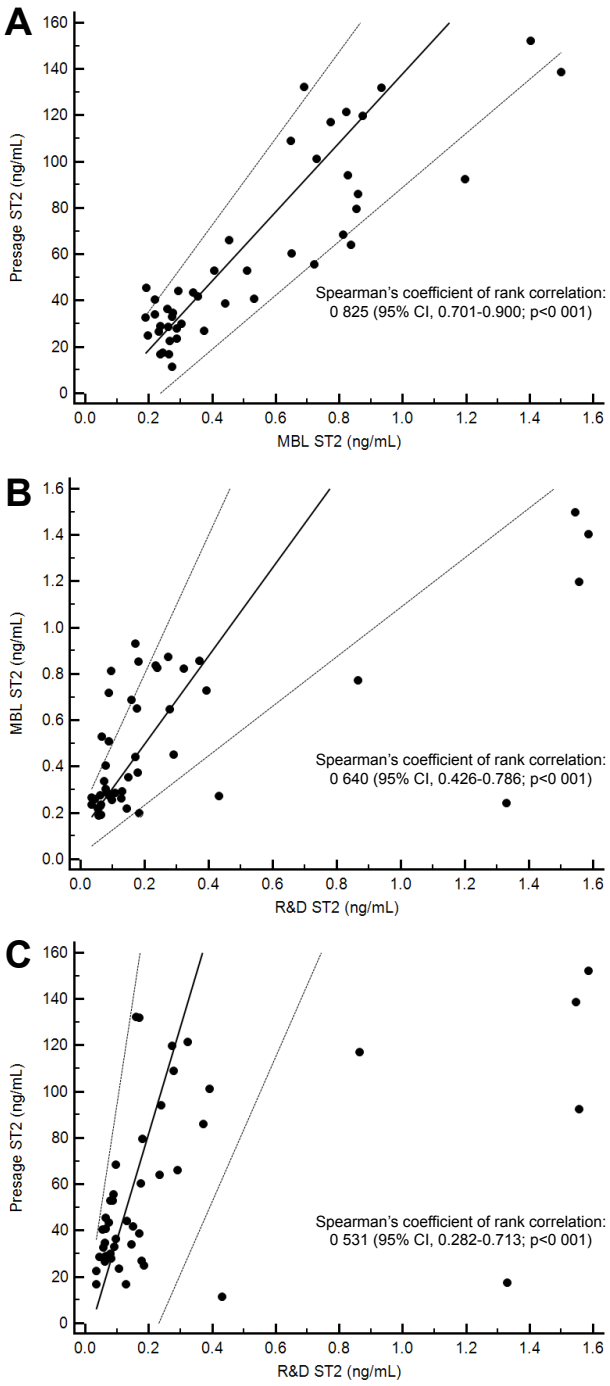
Analyte stability in vitro

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:

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

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

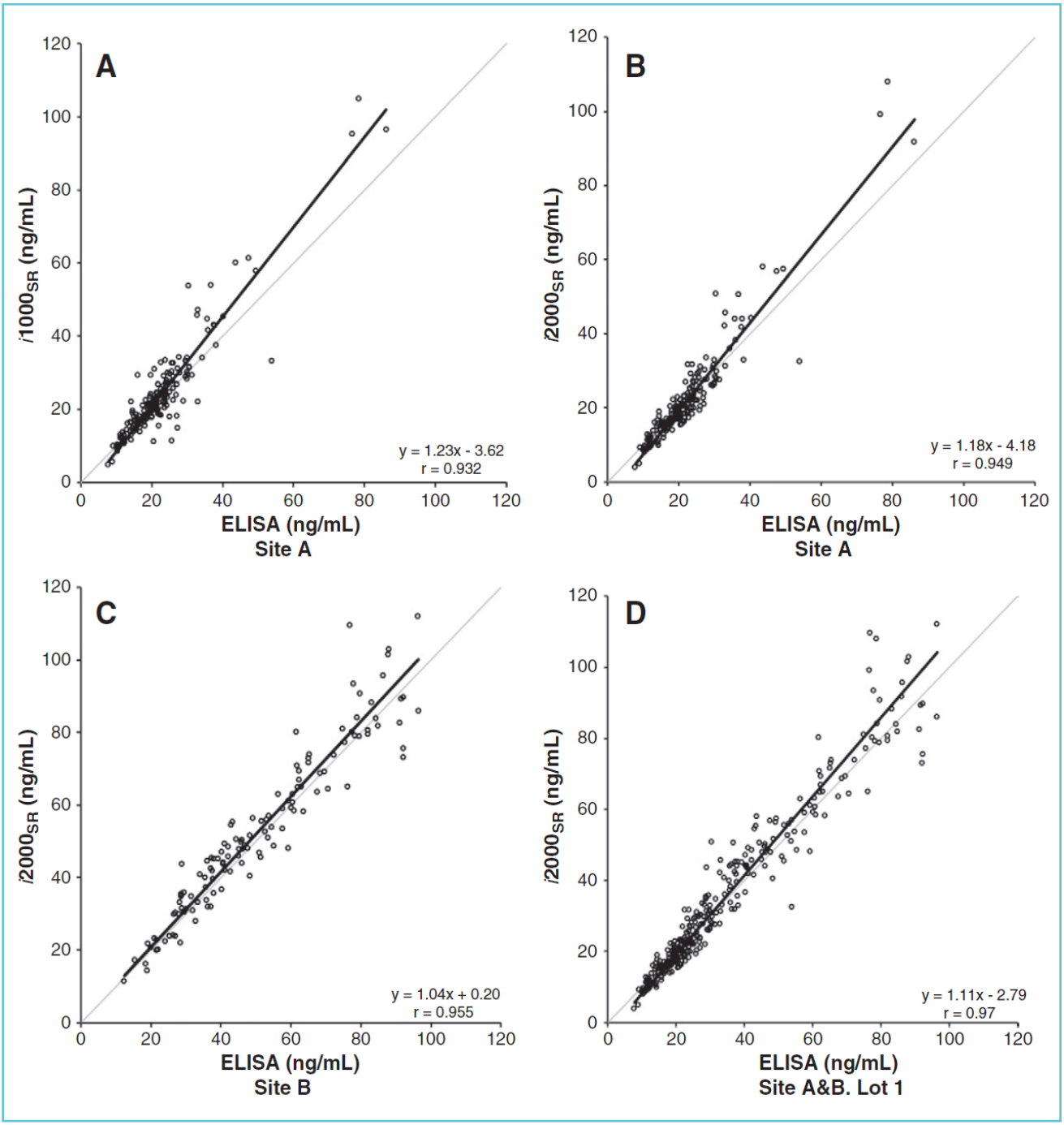
Assay format

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

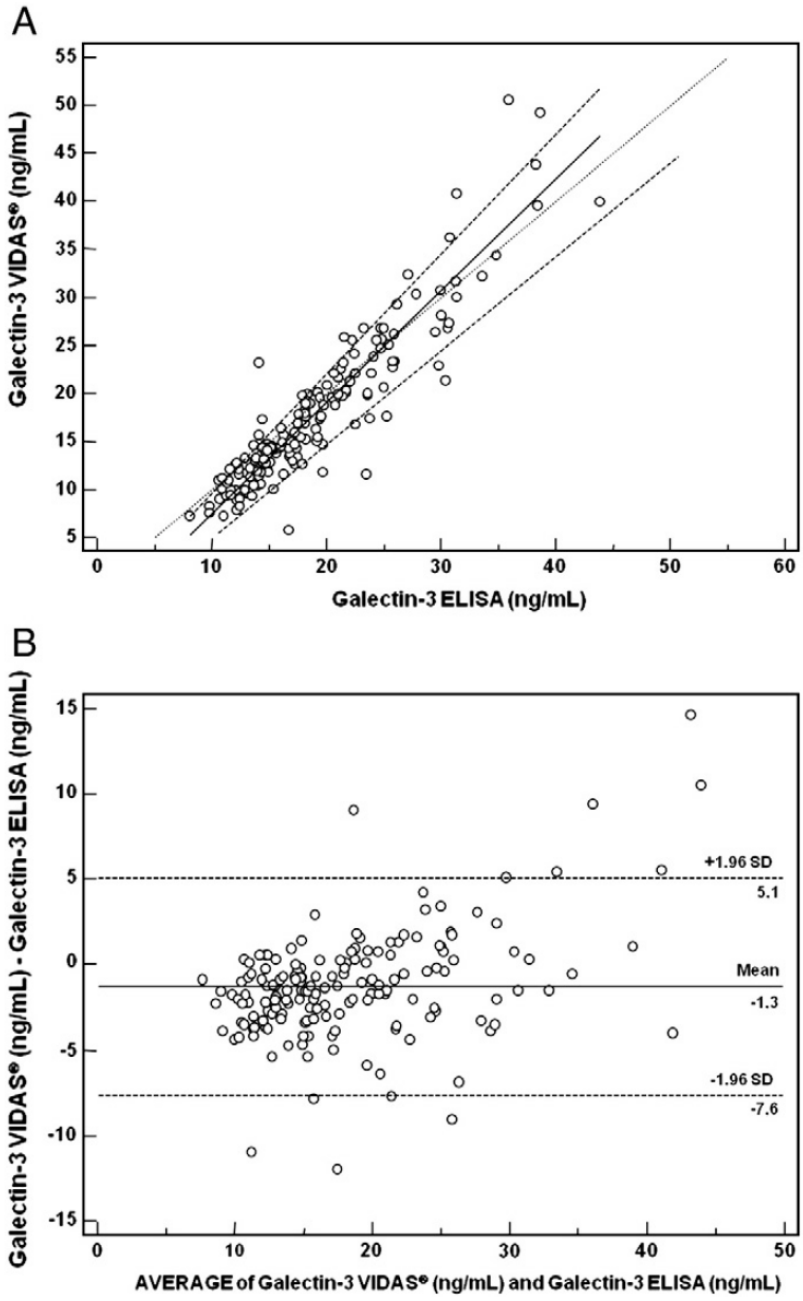
Figure 2 **Scatterplots of galectin-3 plasma concentrations obtained by the ARCHITECT Galectin-3 assay vs. the BGM Galectin-3 assay**



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 *i*1000$_{SR}$ and the ARCHITECT *i*2000$_{SR}$ 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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