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Research paper

Critical analytical evaluation of promising markers for sarcopenia

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ABSTRACT

We tested and validated irisin (IRI), myostatin (MYO), PIIINP, osteoglycin (OGN), TMEM119 (TMEM) and activin A (AA) and established the analytical performance, reference range and stability (considered unstable if more than 20% increase/decrease in the levels was observed in more than 10% of the samples). We were unable to obtain a valuable calibration curve with the Cusabio kits (TMEM and OGN). Coefficient of variation (CV) was too high for IRI (CV 17–30%), but were $\leq 10\%$ for the 3 other analytes. AA and MYO were stable up to 3 months at $-20\text{ }^{\circ}\text{C}$ and $-80\text{ }^{\circ}\text{C}$ in serum or EDTA plasma and up to 6 months at $-80\text{ }^{\circ}\text{C}$. PIIINP was stable only 1 month in EDTA plasma (but not in serum) at $-20\text{ }^{\circ}\text{C}$ or $-80\text{ }^{\circ}\text{C}$. After 3 months of storage, PIIINP was not stable anymore, in serum or EDTA plasma, at $-20\text{ }^{\circ}\text{C}$ or $-80\text{ }^{\circ}\text{C}$. Surprisingly, after 6 months at $-80\text{ }^{\circ}\text{C}$, results returned in the $\pm 20\%$ for both serum and EDTA plasma. PIIINP levels did not differ between men and women and the RR was (median, 90% CI) 1.2 (0.8–1.6)–6.0 (5.6–6.4) $\mu\text{g/L}$. The RR for MYO was 845 (437–1312)–6067 (5524–6552) pg/mL for men and 600 (268–1027)–4438 (4026–4837) pg/mL for women and the RR for AA was 177 (132–210)–622 (580–661) pg/mL for men and 98 (49–147)–480 (430–525) pg/mL for women. PIIINP and AA but not MYO accumulated in CKD as values observed in 10 hemodialyzed patients were higher than in normal individuals.

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1. Introduction

Sarcopenia is a disease characterized by a loss of muscle mass and muscle function and has become a major health condition associated with ageing, which contributes to many components of public health at both the patient and the societal levels. In 2010, the European Working Group on Sarcopenia in Older People (EWGSOP) has published recommendations for a clinical definition and consensual diagnosis criteria of sarcopenia [1]. According to the EWGSOP, sarcopenia is defined by the presence of low muscle mass or low muscle performance that can lead to adverse outcomes like physical disability, poor quality of life and death. Prevalence of this disease is difficult to establish and can vary according to the cut-offs points that are taken into consideration [2]. Different tools exist to assess the diagnostic of sarcopenia (recently reviewed in [3]) but the estimation of the prevalence remains linked to the diagnostic tool that has been used [4]. Contrary to many other diseases, literature is scarce on serum biomarkers that could potentially help in the diagnostic of the

disease, or in the follow-up of treated or untreated patients. Hence, a biomarker could be of interest for many reasons. Among them, a biomarker is often easily obtained via a simple blood sampling, its determination is generally reproducible, can be achieved by different labs throughout the world, the levels obtained do not leave any room for subjectivity and, compared to more sophisticated techniques, it is often cheaper. However, biomarker determination is not necessarily so “simple” and several pitfalls can occur and flaw the results of a study. Among them, the precision of the assay is of course a major issue. It is indeed difficult to rely on results that have been obtained with a method presenting a coefficient of variation (CV) higher than 15%. Next to precision, two major points are often eluded in clinical studies, namely the reference ranges and the stability of the marker. Indeed, next to classical, well-established biomarkers, many emerging biomarkers used in clinical studies are generally obtained with kits for “research use only”. In other words, it means that no robust reference ranges are proposed by the manufacturer and that no short or long term stability of the analyte in serum or EDTA plasma has been studied, which is of course of importance when samples are collected prospectively in clinical studies, many months before the determination of biomarkers. Finally, little is generally known on the clearance of the biomarker once in the circulation, and on its possible increase when kidney

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function declines. As mentioned, these important factors can totally fool the investigators of a study if they have not been established prior to sample collection. The clinical laboratory of our institution has an extensive experience in the validation and the handling of new biomarkers and, in conjunction with the clinicians that follow sarcopenic patients, we have decided to evaluate from an analytical point of view six emerging biomarkers that could potentially play a role in the management and follow-up of the patients.

2. Material and methods

2.1. Biomarkers

Six biomarkers have been selected for this validation study, namely activin A (AA) and myostatin (MYO) (R&D Systems, Abingdon, UK), procollagen III N-terminal peptide (PIIINP) (Orion Diagnostica, Espoo, Finland), osteoglycin (OGN) and human transmembrane protein 119 (TMEM119) (Cusabio, Wuhan, PR of China) and irisin (IRI) (Phoenix Pharmaceuticals, Karlsruhe, Germany). All of these assays were ELISA methods, except PIIINP, which was a radio-immunoassay (RIA). The lots numbers used in this evaluation were 322439 for AA, 324636 and 322411 for MYO, 1621019 and 1635398 for PIIINP, A06076961 for OGN, Z23076960 for TMEM119 and 604944 for IRI.

These markers have been chosen because they play a role in the linkage of muscle to bone [5–7], are associated with lean mass [8] or are regulators of muscle mass [9–11].

2.2. Performance study

The precision (CV) was evaluated in accordance with a modified protocol based on CLSI EP-5A2 by running five serum samples in triplicate on five consecutive days. To obtain values spanning the

dosing range, we screened for that purpose different clinical samples issued from diabetic, hemodialyzed, healthy and obese individuals.

The reference ranges were established in 120 healthy individuals (60 men and 60 non-menopause women). We evaluated the renal clearance of the markers by comparing the results obtained in the reference population and in 10 hemodialyzed patients. Finally, we studied the short-term (24 hours) and the long-term (1, 3 and 6 months) stability of the biomarkers in serum and EDTA plasma. For that purpose, we drew 5 SST tubes with gel separator and 5 tubes containing EDTA in 10 healthy volunteers. SST tubes were allowed to clot at room temperature for 30 minutes, spun 10 minutes at +4 °C and aliquoted. One fresh serum and EDTA plasma was immediately run to give the “T0” value. One aliquote of serum and EDTA plasma was determined after 24 hours of storage at +4 °C, and the others after 1, 3 and 6 months of storage at –20 °C and –80 °C. We considered that, to be reliable, the CV of an assay should be < 15%. A matrix (serum or EDTA plasma) was considered as unstable if more than 20% of the samples increased or decreased by more than 20%, compared to T0.

All the analyses have been performed in duplicates.

The characteristics of the kits, as provided by the manufacturers are presented in Table 1.

All the study was performed with the agreement of the Ethics Committee of the CHU de Liege and participants gave their informed consent.

3. Results

3.1. Analytical precision

3.1.1. Osteoglycin

The quantifiable point of the curve presented a value of 0.156 ng/mL. Unfortunately, all the human samples that we tested

Table 1
Characteristics of the different assays presented in this study, as presented by the manufacturers.

	Detection range	Sensitivity	Precision (inter-assay)	Research use only?	Reference range	Stability	Specificity	Internal QC?
AA	15.6–1000 pg/mL	From 0.75 to 7.85 pg/mL	From 4.7 to 7.9%	Yes	Human serum (n=35): 142–753 pg/mL	Not provided	Natural and recombinant Activin A. No significant cross-reaction with different peptides tested	Yes; bought separately
MYO	31.3–2000 pg/mL	From 0.9 to 5.3 pg/mL	From 3.1 to 6%	Yes	Human serum (n=35): 1264–8588 pg/mL	Not provided	Natural and recombinant mature myostatin. No cross-reaction with myostatin propeptide, follistatin. Recombinant human GASP-1 interferes at levels > 10 ng/mL	Yes; bought separately
PIIINP	1–50 µg/L	0.3 µg/L	From 6.5 to 7.2%	No	232 healthy adults (19–65 yo): 2.3–6.4 µg/L	5 days between 2 and 8 °C. For longer periods, store at < –20 °C	Not sensible to smaller degradation products found in blood. It measures the propeptide and its higher molecular weight form. Does not cross-react with PINP	Yes
OGN	0.156–10 ng/mL	0.039 ng/mL	< 10%	Yes	Not provided	Not provided	Osteoglycin	No
TMEM119	62.5–4000 pg/mL	< 15.6 pg/mL	< 10%	Yes	Not provided	Not provided	TMEM119	No
IRI	0.1–1000 ng/mL	Not provided	Not provided	Not provided	Not mentioned	Not provided	Not provided	Yes

with this assay had a value that was below the absorbance of the standard 0. It seems that this kit does not have the sensitivity enough to detect circulating concentrations of OGN in human blood. We thus had to stop the evaluation of the OGN assay provided by Cusabio.

3.1.2. Other biomarkers

The inter-assay coefficient of variation ranged from 5.5 to 8.4% for AA (studied range: 57.1–724 pg/mL), from 7.2 to 8.9% for MYO (57–1158 pg/mL), 5.2 to 10.1% for PIIINP (2.85–20.5 $\mu\text{g/L}$), 14.1 to 38.7% for TMEM119 (468–1426 pg/mL), and 19.7 to 46.9% for IRI (3.9–5.4 ng/mL). According to the high CV that we obtained for TMEM119 and IRI, we did not explore these parameters any further.

3.2. Reference range

The reference population was constituted of 120 healthy subjects (60 men and 60 non-menopause women). The mean age was 36.2 ± 9.7 years old for men and 30.1 ± 5.5 years old for women.

For AA, we observed a significant difference between the values observed in both sex with a median of AA [95% interval confidence] of 398 pg/mL [344–446] observed in men vs. 281 pg/mL [254–321] observed in women, $P < 0.0001$. The reference range (90% IC) calculated with the robust method requested by CLSI 28-A3 guideline was 177 (132–210)–622 (580–661) pg/mL for men and 98 (49–147)–480 (430–525) pg/mL for women.

In the same healthy population, we also observed a difference between men and women for MYO. The median [95% IC] was 3330 pg/mL [2943–4003] for men vs. 2540 pg/mL [2374–2879] for women, $P < 0.0001$. The derived reference range was 845 (437–1312)–6067 (5524–6552) pg/mL for men and 600 (268–1027)–4438 (4026–4837) pg/mL for women.

Finally, we did not observe a significant difference between men and women regarding PIIINP. The median (95% IC) obtained in the 120 individuals was 3.6 $\mu\text{g/L}$ [3.4–3.9] and the reference range was 1.2 (0.8–1.6)–6.0 (5.6–6.4) $\mu\text{g/L}$.

3.3. Renal clearance

MYO does not seem to accumulate in CKD as the median observed in 10 hemodialyzed (HD) patients males (3376 ± 1723 pg/mL) was not different from the one observed in the healthy subjects. On the contrary, we observed significantly ($P < 0.0001$) higher values in HD patients for AA (854 ± 278 pg/mL) and PIIINP (11.24 ± 4.12 $\mu\text{g/L}$) than the ones observed in the healthy subjects.

3.4. Correlation between the markers

In the healthy population, AA and MYO were significantly ($P = 0.0001$) correlated. The coefficient of correlation (95% CI) observed was $r = 0.34$ (0.18–0.50). The degree of correlation observed between MYO and PIIINP remained significant ($P < 0.05$) but the coefficient of correlation was lower [$r = 0.20$ (0.02–0.36)]. There was no significant correlation between AA and PIIINP.

3.5. Stability

The stability of AA, MYO and PIIINP are presented in Fig. 1. AA and MYO were shown to be stable up to 3 months at -20°C and -80°C in serum or EDTA plasma and up to 6 months at -80°C whereas PIIINP was stable only 1 month in EDTA plasma (but not in serum) at -20°C or -80°C . After 3 months of storage, PIIINP

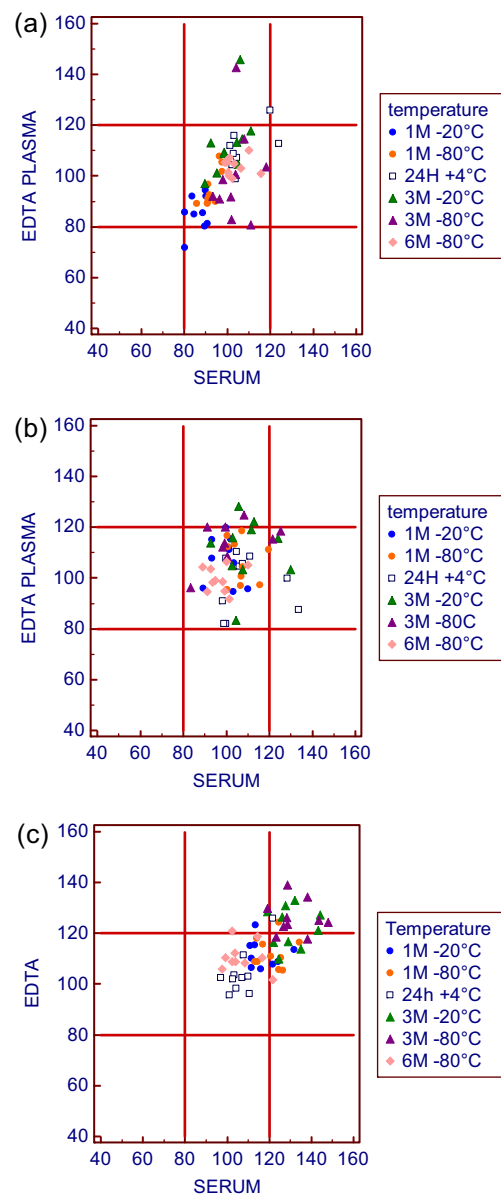


Fig. 1. Stability of activin (a), myostatin (b) and PIIINP (c) at different temperatures and different storage conditions in serum and EDTA plasma. A matrix (serum or EDTA plasma) is considered as unstable if more than 20% of the samples increased or decreased by more than 20% (red lines) compared to T0 (100%).

could not be considered as stable anymore, in serum or EDTA plasma, at -20°C or even at -80°C . Surprisingly, after 6 months of storage at -80°C , results returned in the $\pm 20\%$ for both serum and EDTA plasma.

4. Discussion

Sarcopenia is an emerging disease that remains complete to diagnose and is of uncertain prevalence [2–4]. Biomarkers could thus be of interest to help in the diagnostic, but also in the follow-up of the disease after treatment and in the prediction of the evolution of the disease without treatment. Literature has recently described some interesting biomarkers and we choose to select six of them to evaluate their analytical profile, as nothing was known regarding their coefficient of variation, analytical stability, reference range and renal clearance. We definitely think that these data are of importance before incorporating biomarkers in clinical trials or starting to collect blood samples during long periods for

prospective studies. Our results show that one of the assay completely lacked sensibility. Indeed, OGN levels were undetectable in healthy individuals, but also in subjects selected according to different disease status, like hemodialyzed and diabetic patients as well as obese individuals. We cannot be sure whether this lack of sensitivity was specific to the lot that we tested or if it was related to a problem in the design of the assay (that is also supposed to allow OGN determination in serum and plasma, but also in tissue homogenates and cell lysates, that may contain much higher OGN levels), but we could not go any longer with its evaluation. Two other assays, TMEM119 and IRI presented an analytical variation of more than 15%. We think that, to be used for clinical purposes, the CV of an assay should not be too large to get reliable results (even if there is no universal consensus on the maximal CV that can be allowed). Some doubts have already been raised on IRI determination by different assays [12]. The CV that we obtained here are even larger than those obtained in the literature with the same assay [13,14]. This can be explained by the strength of our protocol, that uses real human plasma and not “standards”, which are run in triplicate during 5 consecutive days (and not in a single batch). There is unfortunately no other data available regarding TMEM119 determination. To go any further with this biomarker, the analytical performances should definitely be improved.

We finally went more deeply in the validation of AA, MYO and PIIINP, which were the only biomarkers that passed our analytical challenge. Compared to the reference range proposed by the manufacturer (on a small subset on “humans”), we found a similar reference range for men, but the values obtained in women were much lower. To the best of our knowledge, this study is the first to report this difference in a well-designed reference population. As AA and MYO are negative regulators of the muscle mass, such finding could be of importance and would certainly deserve extra-investigations to clearly understand the physiological and pathophysiological mechanisms of muscle gain or loss. Reference values for PIIINP were nicely documented by the manufacturer, in a large cohort of adults and children. The results that we found in our population were slightly lower. This could be explained by the relatively younger age of our population, compared to the one selected by Orion Diagnostica. Unfortunately, no data provided was available to confirm this hypothesis.

Finally, our results show that PIIINP and AA are clearly influenced by the renal function of the patients. As aging is associated with a decline in renal function, this finding is of importance for a correct interpretation of these biomarkers in the older old subjects suspected to suffer from sarcopenia – in which creatinine values can be challenging to interpret.

When clinicians plan to prospectively store samples for research purposes, they definitively have to take into account the stability of the analyte, in serum or in EDTA plasma for a short or a longer period of time. In this study, we have shown that, according to our prerequisites, PIIINP was not stable enough to be stored for more than 3 months at -80°C . Surprisingly, after 6 months of storage at -80°C , results returned between the $\pm 20\%$ acceptance criteria. This result was not expected and we have no clear explanation for this phenomenon. We immediately ruled out an analytical error, as all the QC, run in duplicates before and after each series at 1, 3 and 6 months were in the middle of the expected range provided by the manufacturer and were totally comparable. The analyses have been performed by the same experienced and well-trained technician and we did not observe any discrepancies between the calibration curves of the different runs. So, on one hand, this discrepancy can be attributed to an endogenous production of interfering substances that could affect the first 3 months results, and then degradation of these substances – or of the PIIINP molecule itself – or, on the other hand, to a lot-to-lot variation of the PIIINP assays. Indeed, the kits were produced in different batches (RIA assays

are not stable up to 6 months) and it is not impossible that a variation could be observed on native human samples, but not on internal QC, which are generally a simple diluted standard, and not a “real” human matrix. Whatever the reason, it seems difficult to ignore these tremendous variations and we have to consider that PIIINP is not suitable for a long storage period. This is quite unfortunate because this marker had shown interesting analytical features. Researchers that would like to run this marker should thus do it as soon as possible, and, if they do not run all the assays in the same batch, use native human serum as milestones to see if no lot-to-lot variation is observed. On the contrary, AA and MYO were stable at least 6 months.

In conclusion, among the six biomarkers that we tested, myostatin and activin-A are the only ones that passed our validation – even if the latter accumulates in the serum of individuals with decreased renal function. These findings are of interest for clinical studies in sarcopenic individuals in which biomarkers may be needed.

Disclosure of interest

The authors declare that they have no competing interest.

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Some of these results have been presented as a selected oral communication at the World Congress on Osteoporosis, Osteoarthritis, Frailty and Sarcopenia meeting in Milan.

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