Reproducibility of novel immune-inflammatory biomarkers over 4 months: an analysis with repeated measures design

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Aim: Assessment of the feasibility and reliability of immune-inflammatory biomarker measurements.

Methods: The following biomarkers were assessed in 207 predominantly healthy participants at baseline and after 4 months: MMF, TGF- β , suPAR and clusterin. **Results:** Intraclass correlation coefficients (95% CIs) ranged from good for TGF- β (0.75 [95% CI: 0.33–0.90]) to excellent for MMF (0.81 [95% CI: 0.64–0.90]), clusterin (0.83 [95% CI: 0.78–0.87]) and suPAR (0.91 [95% CI: 0.88–0.93]). Measurement of TGF- β was challenged by the large number of values below the detection limit. **Conclusion:** Single measurements of suPAR, clusterin and MMF could serve as feasible and reliable biomarkers of immune-inflammatory pathways in biomedical research.

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Human aging predisposes disease via several mechanisms that in part converge on immune-inflammatory pathways [1]. Recent research has introduced the idea that rather than a general decline in the functions of the immune system with age, immune aging is mainly characterised by a progressive appearance of immune dysregulation throughout life [2]. Correspondingly, striking new data has revealed that HIV-infected children and adults exhibit premature biological aging with accelerated immune senescence, which particularly affects adaptive immune responses [3]. Among the hallmarks of aging, cellular senescence is coupled to phenotypic changes involving alterations in the cell's secretome, such as expression of proinflammatory cytokines [2]. These changes are collectively referred to as the 'senescence-associated secretory phenotype' which was recently implicated as a major contributor to aging and disease development [4]. In aging tissues and most age-related diseases, low-grade chronic inflammation is known to stimulate long-term production of substances at toxic concentrations, triggering pathological processes that damage the body's own tissues. This phenomenon has been coined 'inflammaging' which assumes the absence of overt infection, and refers to chronic, low-grade, systemic inflammation documented in aging phenotypes [5].

The biotechnological boom seen in recent decades has led to the discovery of several novel biomarkers possibly able to provide further answers on issues related to aging and disease development. However, characterisation of

such biomarkers in human research has not been done in a systematic manner (i.e. using large prospective cohort design). Multiple factors could contribute to poor reliability of a biomarker, including time of blood collection, laboratory procedures and storage conditions, as well as biological and metabolic variability [6]. In this vein, testing the methodological utility of biomarker measurements represents an essential first step for biomarker evaluation in large human cohorts. Experimental research has uncovered immune-inflammatory functions of several novel biomarkers among which we selected a set of molecules based on their suggested mechanistic role in immune response and aging phenotypes, availability of assays for biomarker measurements in large population-based studies and lack of previous reproducibility assessment in healthy cohorts. Based on these criteria, we identified TGF- β , suPAR, clusterin and MMF as promising biomarkers for future research [7–10]. Several studies have documented the central role of these biomarkers in a number of inflammatory responses and pathological conditions [11–13]. The intriguing question remains whether they act by promoting the pro-inflammatory immune response, or are merely a result of an increased activation level of the immune system. Further research is warranted to evaluate whether these biomarkers can be used as therapeutic targets in reducing inflammation and disease progression. Furthermore, systemic levels of these biomarkers have been shown to positively correlate with the activation level of the immune system in patient cohorts and therefore could potentially serve as promising clinical markers. Indeed, these biomarkers may reflect a stronger link to immune activation compared with traditional inflammatory biomarkers such as CRP. However, potential clinical utility as biomarkers in population-based epidemiological studies has not been explored.

To assist planning of future population-based research studies, we aimed to assess the reliability of TGF- β , suPAR, clusterin and MMF, repeatedly measured over a period of 4 months in a sample of predominantly healthy participants within the EPIC Potsdam cohort. In addition, we evaluated potential interdependence of biomarker performance with participants' adiposity status and levels of inflammation.

Materials & methods

Study population

Based in Germany, the EPIC-Potsdam study is a prospective cohort of the multicenter EPIC study. EPIC is a Europe-wide investigation of associations between nutrition and chronic diseases such as cancer. Between 1994 and 1998 the Potsdam cohort recruited 27,548 participants, including 16,644 women (aged 35–65 years) and 10,904 men (aged 40–64 years). All participants were subject to a baseline examination which consisted of the following: anthropometric measurements, blood sample collection, a health questionnaire and a personal health interview that included questions about prevalent diseases [14].

The current study has a methodological design suitable for reliability assessment of biomarkers in a random sample of healthy participants with characteristics similar to those recruited in large population-based cohorts. The same study design was used to evaluate the reliability of several novel biomarkers as previously described [15–17].

Study participants were randomly selected from the EPIC-Potsdam study population [18]. Blood samples were obtained on two occasions, 4 months apart. The first measurement was collected from October 2007 to March 2008. The second measurement was collected from February to July 2008. With rare exceptions, the blood samples were collected between 8 am and 11 am, and approximately 10% of participants reported a non-fasted state. Participants were excluded based on the following criteria: history of heart disease and stroke, impaired mobility and present treatment with β -blockers or systolic or diastolic blood pressure above 180 mmHg or 110 mmHg, respectively. 407 participants were invited to participate, but 11 failed to respond, 176 declined and 12 were excluded due to reported treatment with β -blockers. Additionally, one participant did not provide blood samples. These exclusions yielded a total sample size of 207 participants (124 women and 83 men).

From these, 39 participants had missing measurements for suPAR (33 due to values below detection limit and six due to implausibly high values >3750 pg/ml); 35 participants had missing measurements for clusterin (33 due to values below detection limit and two due to insufficient sample volume); and 191 participants had missing measurements for TGF- β due to values below the detection limit. MMF was measured in a random subset of participants in the above described study ($n = 30$) selected based on sample availability due to limited funding. No missing values were generated for this biomarker due to values below the limit of detection or implausibly high values. A note of caution should be given to the low effect size for both MMF and TGF- β , which would require reported analyses for their reliability to be carefully interpreted.

This study was approved by the ethical committee of the state of Brandenburg, Germany, and informed consent was provided by all participants prior to recruitment.

Biomarker measurements

A total of 30 ml of blood was sampled from each participant. Blood fractions were separated and immediately stored at -80°C . Biomarker concentrations were measured in EDTA-plasma, except for high sensitivity CRP which was evaluated in serum. Using sandwich ELISA kits from the commercial supplier BioVendor (Kassel, Germany), biomarkers were measured based on the manufacturer's instructions at the Department of Clinical Nutrition, German Institute of Human Nutrition Potsdam-Rehbrücke, Germany. The manufacturer's manuals reported coefficients of variation from 3.2–6.2% for intra-assay variation and from 4.9–7.8% for interassay variation, but no information was reported for MMF. Both samples obtained from each study participant were measured in the same analytical batch. Biomarker concentration measurements were quantifiable, apart from the notable exception of TGF- β which only produced 16 repeated measurements that were above the lower limit of detection (8.6 pg/ml). Considering the large number of missing values and low effect size, reliability analysis for TGF- β should be interpreted with care. Serum concentrations of hsCRP were measured in repeated samples with sandwich ELISA kits (BioVendor) at the same lab as evaluated biomarkers. The intra- and interindividual CVs of hsCRP were 5.1 and 6.1%, respectively, as reported by the manufacturer. Based on our data, the interindividual CV was 11.0%. The overall ICC depicting reproducibility of hsCRP in this study sample was 0.66 (95% CI: 0.56–0.74) denoting very good reliability of the measurements. Due to insufficient serum volume, hsCRP could only be measured in a reduced number of participants ($n = 151$).

Statistical analysis

To allow the application of parametric tests and the calculation of mean concentrations, non-normally distributed data was transformed using the natural logarithm. Since natural logarithmic transformation did not confer normal distribution for suPAR, Box–Cox transformation was applied for standardizing its distribution.

Paired and unpaired Student's *t*-tests were used to evaluate average biomarker concentrations between the two assessment points and between sexes within one assessment point, respectively. Intraclass correlation coefficients (ICCs) were calculated as ratios of interindividual variance (between-person difference) and total variance (inter-individual variance + intra-individual [within-person] variance) to assess the reliability of biomarker measurements. Using random effects ANOVA, critical variance components were assessed by designating biomarker concentration as the dependent variable and participant as the explanatory factor. To determine the agreement between biomarker measurements taken at both assessment points, Bland–Altman plots were constructed. These plots represent quantification of the agreement between the two biomarker measurements by studying the mean difference and constructing limits of agreement [19].

ICC values for each biomarker were compared across various strata – including sex, body mass index (BMI) and waist circumference – in order to assess potential dependence between these parameters. The reproducibility of ICC values was estimated within the following established cutoff ranges: excellent (≥ 0.75), good (0.74–0.60), fair (0.59–0.40) and poor (< 0.40). Using Spearman partial correlation analyses adjusted for age and sex, the average biomarker concentrations calculated for the first assessment measurements were evaluated for correlations with BMI, waist circumference and CRP. 95% confidence intervals (95% CI) for each correlation coefficient were produced by Fisher's *z* transformation. To visualize correlation networks, we employed the widely used network visualization tool, Cytoscape [20]. The networks were imported into Cytoscape as tables based on absolute values of the partial correlations.

Statistical analyses were conducted using the following software: SAS 9.4, SAS Enterprise Guide 6.1 (SAS Institute Inc., NC, USA). Only two-sided statistical tests were used. ICCs for the sensitivity analyses were determined following the exclusion of participants who were not fasted at blood draw and outlier biomarker measurements which are defined here as extreme values within the 1st or above the 99th percentile.

Results

The mean age of the study participants was 56.5 ± 4.2 years. On average, participants had a BMI of 26.0 ± 4.0 kg/m² and a waist circumference of 93.0 ± 12.8 cm. Mean systolic and diastolic blood pressure values were 135.3 ± 14.2 mmHg and 87.5 ± 9.3 mmHg, respectively. The majority of study participants were fasted at blood draw with 10% of the samples taken in non-fasted participants.

Table 1 presents the geometric means of biomarker concentrations for repeated biomarker measurements taken four months apart. Overall, no statistically significant differences were observed between the measurements in the two time points.

Table 1. Geometric means and 95% confidence intervals of biomarker concentrations for repeated measurements taken 4 months apart.

Biomarker	n	First measurement; geometric mean (95% CI)	n	Second measurement; geometric mean (95% CI)	n overlap	P difference [†]
SuPAR (pg/ml)	168	1153 (1087–1223)	168	1165 (1106–1227)	168	0.34
Clusterin (ng/ml)	172	74.9 (72.0–78.0)	172	74.3 (71.6–77.2)	168	0.45
MMF (ng/ml)	32	5.38 (4.69–6.16)	30	5.51 (4.66–6.51)	30	0.64
TGF-β (pg/ml)	24	181.1 (102.0–321.6)	16	270.7 (103.4–709.0)	9	0.78

[†] Paired t-test to compare concentrations between both assessment points.

Figure 1. Intraclass correlation coefficients and 95% CIs for biomarker measurements taken 4 months apart. Intraclass correlation coefficient is applied as a measure of the reliability between the two biomarker measurements calculated by dividing the between-subject variance by the total variance (sum of between- and within-subject variances). An intraclass correlation coefficient greater than 0.74 would suggest excellent reliability; good reliability when ranging from 0.74 to 0.60; fair reliability when ranging from 0.59 to 0.40; poor reliability when lower than 0.40.

Overall ICCs for each of evaluated biomarkers depicting their reproducibility over a four month period are illustrated in Figure 1. Biomarker ICCs (95% CIs) ranged from good for TGF-β (0.75 [95% CI: 0.33–0.90]) to excellent for MMF (0.81 [95% CI: 0.64–0.90]), clusterin (0.83 [95% CI: 0.78–0.87]) and suPAR (0.91 [95% CI: 0.88–0.93]).

Additionally, when analysing the ICCs according to strata of study participants by sex, BMI, waist circumference and CRP values, overall, no substantial differences in ICCs were observed for men compared with women, with the exception for the ICC of clusterin (Figure 2; Supplementary Table 1). Visual inspection of the Bland–Altman plots supported a high level of agreement between the repeated biomarker measurements (Figure 3).

Table 2 presents the sex- and age-adjusted correlation coefficients between biomarkers and BMI, waist circumference and CRP. Overall, no pronounced correlations with these phenotypes have been observed for any of the biomarkers. Moderate positive correlations could be seen for suPAR and TGF-β, whereas no correlations were revealed for clusterin and MMF.

The network analysis reflected close correlations between suPAR, clusterin and CRP as opposed to a lack of substantial correlation with BMI and waist circumference (Figure 4). In sensitivity analyses, excluding from the analysis participants with extreme biomarker measurement values (values within first or above 99th percentile) and non-fasting participants did not substantially alter the ICC estimates (data not shown).

Figure 2. Intraclass correlation coefficients by sex, body mass index, waist circumference and CRP for biomarker measurements taken 4 months apart. Intraclass correlation coefficients defined as ratio of between-person variance and total variance. BMI (<25 kg/m² vs ≥25 kg/m²); waist circumference (men: <94 cm vs ≥94 cm, women: <80 cm vs ≥80 cm); CRP (population median as cutoff). BMI: Body mass index.

Table 2. Spearman partial correlation coefficients (R_{ho-s}) and 95% confidence intervals for biomarker concentrations at the first measurement with body mass index, waist circumference and CRP.

Biomarker	BMI		Waist circumference		CRP	
	R_{ho}	95% CI	R_{ho}	95% CI	R_{ho}	95% CI
suPAR	0.14	-0.01–0.28	0.12	-0.03–0.27	0.18	0.02–0.33
Clusterin	-0.05	-0.20–0.10	-0.01	-0.16–0.14	0.00	-0.16–0.16
MMF	-0.14	-0.48–0.23	0.00	-0.36–0.36	0.07	-0.34–0.46
TGF- β	0.27	-0.18–0.62	0.25	-0.19–0.61	0.25	-0.23–0.63

BMI: Body mass index; R_{ho} : Correlation coefficient.

Discussion

The present study provides first lines of evidence regarding the reproducibility of selected novel biomarkers implicated in immune-inflammatory pathways. Our assessment revealed good to excellent reproducibility over four months for all measured biomarkers with ICCs ranging from 0.75 to 0.91 without evidence of an influence by participants' sex, age and adiposity status on these estimates. However, the measurement of TGF- β in a healthy population seems to be challenging and a large number of values below the limit of detection could be expected. Furthermore, only one batch of MMF was analysed, which limited statistical interpretation of the results associated with this biomarker. Collectively the data suggest that suPAR, clusterin and MMF may serve as feasible and reliable biomarkers closely associated with immune-inflammatory phenotypes that deserve further exploration in aging

Figure 3. Bland–Altman plots representing agreement of both measurements (y-axis) relative to average concentrations (x-axis) per individual. Bland–Altman plot showing the agreement between biomarker concentrations at baseline (t_0) and 4 months later (t_2). The agreement was calculated as difference between both measurements ($t_2 - t_0$) per individual. The difference between t_0 and t_2 are shown on the vertical axis against the average of the two measures on the horizontal axis. 95% confidence intervals represent the expected range of differences based on the average difference. SD: Standard deviation.

Figure 4. Biomarker network of obesity and inflammatory phenotypes based on sex- and age-adjusted Spearman correlation analyses. TGF- β and MMF are not included in the network analysis due to large proportion of missing values. BMI: Body mass index.

research. Given the lack of relevant studies in this context, further understanding of these biomarkers necessitates exploring links between selected biomarkers and age-associated inflammatory diseases in detail.

While the inflammatory biomarkers may play an important role for risk assessment and therapeutic intervention, there is a dearth of evidence concerning their evaluation in human research. The present study provides the first data on the reliability of four emerging biomarkers currently suggested to represent inflammaging phenotypes. Here, we briefly summarize the experimental and epidemiological evidence on the known functional properties of the individual biomarkers, their potential interlinks and the existing evidence for their involvement in age-related disease development.

Among the biomarkers evaluated here, TGF- β is perhaps the most investigated to date. TGF- β is a 25 kDa pleiotropic cytokine known to exert potent effects in the induction and mediation of proinflammatory signaling [12]. While hepatic overexpression of TGF- β has been shown to induce cell cycle arrest and apoptosis during early stage tumor promotion in transgenic mice, tumor progression is characterised by TGF- β signaling that drives acquisition of pathological epithelial-mesenchymal transition, among other processes associated with metastatic progression [21]. Interestingly, TGF- β signaling was shown to increase expression of uPAR, a glycosylphosphatidylinositol-anchored membrane glycoprotein, in human carcinoma cells [13]. When the anchor of uPAR is removed by extracellular proteolytic cleavage or phospholipases, a soluble form of the receptor, suPAR, is produced. SuPAR is a 20–50 kDa cytokine involved in cell migration, chemotaxis, proteolysis, cell adhesion, immune activation, signal transduction and cell invasion [22]. Elevated suPAR levels have been correlated with activation of immune and inflammatory pathways in humans [23]. Moreover, several cohort studies suggested positive associations between elevated plasma suPAR concentrations and a higher risk of several chronic diseases, such as Type 2 diabetes (T2D), cardiovascular disease, chronic kidney disease, cancer and overall mortality independent of CRP levels [24–28]. A recent large population-based study including 3225 individuals, with repeated blood collections and assessments of lifestyle data over 5 years, demonstrated that major lifestyle habits (diet, smoking, alcohol and physical activity) exerted considerable influence on long-term changes of suPAR levels [29]. That study also provided evidence on an association between suPAR with overall mortality independent of lifestyle factors which strengthens the evidence from previous research. As an inflammatory biomarker, suPAR could be superior in predicting outcomes in the general population as compared with other biomarkers with less variability such as CRP [30]. Potential differences between CRP, as a biomarker of adiposity and metabolic inflammation, in contrast to suPAR linked to cellular inflammation and endothelial dysfunction, deserve further evaluation in research [31]. Further studies are also warranted to establish suPAR as a chronic disease risk marker.

In addition to stimulating suPAR production, TGF- β signaling is also known to induce expression of specific glycoproteins such as clusterin [11]. Clusterin is a ubiquitously expressed, 75–80 kDa glycoprotein described as a Golgi chaperone that facilitates the folding of secreted proteins [32]. A number of observational studies have reported on an association between elevated clusterin concentrations and risk of T2D [33], Alzheimer's disease [34] and cancer [35]; however, evidence from large-scale population cohorts is generally lacking.

Experimental research has recently suggested a link between TGF- β and MMF in atrial fibrosis [36]. MMF is a 12.5 kDa proinflammatory cytokine that is ubiquitously expressed in many cell types, including macrophages and T-cells, playing a significant role in regulation of innate immunity [37]. Furthermore, MMF is also released by adipose tissue in obesity, and increasing evidence suggests its role in metabolic and inflammatory processes that underlie the development of obesity-related pathologies, such as T2D, and hepatic lipid accumulation and fibrosis [38]. In population research, elevated MMF concentrations have been associated with Alzheimer's disease [39] and cancer [40].

Despite significant progress in the immunity and aging field, key questions related to biomarker functions in immune responses remain largely unresolved. Elucidation of these pathways would allow improved understanding of the pathogenesis of chronic inflammation, immunity and age-related diseases to foster the development of preventive and therapeutic practices in experimental and ultimately clinical settings.

This study has several strengths. First, the reproducibility of these biomarker measurements over a reasonable period has not been reported, characterising these results as novel and potentially significant contributions to the existing literature on proxies of inflammation with mechanistic links. One vital prerequisite for establishing any suitable biomarker for prospective study is to validate its reliability over a period. Secondly, considering the pioneering nature of this study, adequate sample sizes were available for suPAR and clusterin measurements, permitting assessments by sex, BMI, waist circumference and inflammatory status in sufficient proportions.

However, the present study also has limitations. Given the age range and health status of the EPIC-Potsdam study population, the results of this study may not have been well-suited to detect correlations with age or metabolic status, which may explain the subtle degree of correlation seen here. Due to the absence of gold standard methods of assessing body fat (e.g., calipers), this parameter could have been more accurately estimated; however, measures of adiposity did not differ substantially between the two measurements, so it is unlikely that this would influence reproducibility of the biomarkers. Prior to interpreting the ICCs presented here, researchers are advised to acknowledge details of the current study, such as biosample material, storage time and manufacturer protocols.

Conclusion

Here, we report first lines of evidence for the assessment of the reproducibility of suPAR, clusterin and MMF. Our results largely support the use of these biomarkers as feasible and reliable proxies for assessment of immune-inflammatory status. Investment in TGF- β measurement in epidemiological studies recruiting healthy participants should be done with caution due to expected high number of undetectable values. Further studies are warranted to evaluate roles of these biomarkers as exposures in association with chronic diseases or as surrogate endpoints in intervention trials designed to evaluate strategies for increasing healthy lifespan.

Summary points

- Assessment of the feasibility and reliability of immune-inflammatory biomarker measurements is essential for the planning of large-scale human studies.
- Reliability according to intraclass correlation coefficients ranged from good for TGF- β to excellent for MMF and suPAR.
- Large number of values below lower limit of detection challenged the assessment of TGF- β .
- Further studies are warranted to evaluate roles of these biomarkers as exposures in association with chronic diseases or as surrogate endpoints in intervention trials.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/bmm-2018-0351

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Ethical conduct of research

All participants provided written informed consent prior to recruitment and the study procedures were approved by the Ethics Committee of the Medical Association of the State of Brandenburg.

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