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Assessment of macrophage migration inhibitory factor in humans: protocol for accurate and reproducible levels

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ABSTRACT

The analytical validation of a possible biomarker is the first step in the long translational process from basic science to clinical routine. Although the chemokine-like cytokine macrophage migration inhibitory factor (MIF) has been investigated intensively in experimental approaches to various disease conditions, its transition into clinical research is just at the very beginning. Because of its presence in preformed storage pools, MIF is the first cytokine to be released under various stimulation conditions. In the first proof-of-concept studies, MIF levels correlated with the severity and outcome of various disease states. In a recent small study with acute coronary syndrome patients, elevation of MIF was described as a new factor for risk assessment. When these studies are compared, not only MIF levels in diseased patients differ, but also MIF levels in healthy control groups are inconsistent. Blood MIF concentrations in control groups vary between 0.56 and 95.6 ng/ml, corresponding to a 170-fold difference. MIF concentrations in blood were analyzed by ELISA. Other than the influence of this approach due to method-based variations, the impact of preanalytical processing on MIF concentrations is unclear and has not been systematically studied yet. Before large randomized studies are performed to determine the impact of circulating MIF on prognosis and outcome and before MIF is characterized as a diagnostic marker, an accurate protocol for the determination of reproducible MIF levels needs to be validated. In this study, the measurement of MIF in the blood of healthy volunteers was investigated focusing on the potential influence of critical preanalytical factors such as anticoagulants, storage conditions, freeze/thaw stability, hemolysis, and dilution. We show how to avoid pitfalls in the measurement of MIF and that MIF concentrations are highly susceptible to preanalytical factors. MIF serum concentrations are higher than plasma concentrations and show broader ranges. MIF concentrations are higher in samples processed with latency than in those processed directly and strongly correlate with hemoglobin in plasma. Neither storage temperature nor storage length or dilution or repeated freezing and thawing influenced MIF concentrations in plasma. Preanalytical validation of MIF is essential. In summary, we suggest using plasma and not serum samples when determining circulating MIF and avoiding hemolysis by processing samples immediately after blood drawing.

Many parameters have been discussed as diagnostic markers or even biomarkers. The Institute of Medicine of the National Academies outlines the need for biomarkers in medicine, but also the importance of their evaluation process [1]. Its analytical validation is the first step in the long translational process from basic science to clinical routine. In the case of cardiac biomarkers, the parameter needs to present high myocardial specificity, a well-known time course of occurrence, and a rapid detection method [2]. Whereas some parameters have become biomarkers, others are just at the beginning of the troublesome path to clinical routine.

The macrophage migration inhibitory factor (MIF) has been investigated intensively in experimental settings, numerous clinical models, and several clinical studies. Yet, it is far from being a biomarker in clinical routine. In general, MIF is broadly expressed [3–8], but its secretion is limited to specific stimulation by inflammatory, stress, hypoxic, or hyperoxic triggers [9]. Because of its role in modulation of several cellular signaling pathways, antiapoptotic and redox-regulatory activities, and cellular metabolism, MIF's roles in experimental models of atherosclerosis [6,10], sepsis [3,11], cancer [12], and autoimmune diseases [13,14] have been investigated intensively. A number of studies have suggested MIF has strong

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cardioprotective properties in myocardial ischemia/reperfusion (I/R) injury, enhanced by posttranslational modifications. This effect is based on its CD74/AMPK-regulatory action and antiapoptotic and antioxidative capacity [15–17].

The first proof-of-concept studies showed high amounts of MIF in sepsis, but the impact of MIF on clinical outcome in sepsis seems complex [11,18]. In addition, first investigations have begun to establish MIF as a parameter for genesis and progress of human malignancies [19–22]. Analogous to experimental I/R injury, in a small study investigating patients with acute myocardial infarction, high concentrations of circulating MIF were determined [23]. Because patients with acute coronary syndromes caused by plaque rupture showed higher MIF concentrations than patients with flow-limiting stenosis, MIF was considered as a new possibility for risk assessment in the setting of acute coronary syndromes, even though only 286 patients were included in the study [24].

MIF concentrations were mostly measured using an established commercially available enzyme-linked immunosorbent assay (ELISA); some researchers applied self-made ELISAs based on the same reagents. Yet, observed variances in MIF concentrations of healthy individuals are tremendous. MIF levels in healthy humans described in the literature vary between 0.56 [18] and 95.6 ng/ml [25], correlating to a 170-fold difference, and MIF concentrations in cohorts of diseased patients vary similarly. This obviously hampers the comparability of MIF values between studies and thereby the possible translation into clinical settings.

Factors that might explain the variance in results are the lack of standardization of preanalytical processing. This includes the use of different anticoagulants and the process flow time as well as the sample storage, length of storage, impact of hemolysis, sample dilution, and freezing/thawing of the samples. Before large randomized studies are entered into, measurement of circulating MIF needs to be validated.

The aim of this study was therefore to investigate the effect of preanalytical parameters on MIF concentrations. We examined blood samples of healthy young volunteers considering the influence of various preanalytical factors.

Methods

Study population

Ten healthy volunteers between 18 and 48 years of age gave written informed consent before participating in the study. All volunteers were in excellent general health; none were on regular medication or revealed present or past evidence of cardiovascular or autoimmune diseases or malignancies (Table 1). The investigation was conducted according to the principles of the Declaration of Helsinki and after approval by the local ethics review committee (Study No. 3719).

Blood collection

Venous blood was collected using a 21-gauge sample collection set into four vacuumed tubes (both BD Vacutainer; BD Diagnostic Systems, Heidelberg, Germany). Blood was drawn between 8:00 and 10:00 in the morning.

Table 1
Volunteer characteristics.

Number of volunteers included	10
Female/male	6/4
Mean age (± SD)	27.7 (± 4.7) years
Smokers	None
On regular medication	None
With preexisting conditions	None

Preparation of serum

Serum separator tubes (SST II) with silica as clot accelerator (BD Diagnostic Systems) were used. Blood was kept in the SST II tube at room temperature to clot for at least 30 min. Serum was collected after centrifugation at 1000*g* for 15 min at room temperature. Then, the serum was aliquotted and analyzed directly or stored for later analysis (see Sample storage).

Preparation of plasma

To investigate the effects of various anticoagulation mechanisms, we used three different anticoagulants: heparin, EDTA, and citrate. Blood was collected in vacuumed tubes containing spray-dried lithium–heparin with a final concentration of 17 IU/ml blood, spray-dried K_2-EDTA with a final concentration of 1.8 mg/ml blood, or 0.109 M buffered Na_3 citrate (BD Diagnostic Systems). Heparinized blood and EDTA blood were centrifuged at 1000*g* at 4 °C for 15 min, citrate blood was centrifuged at 2000*g* at 20 °C for 10 min. Then, plasma was aliquotted and analyzed directly or stored for later analysis (see Sample storage).

Sample storage

To determine the effects of storage temperature and storage length on MIF concentrations, plasma and serum samples were kept at −20 °C or snap-frozen in liquid nitrogen and kept at −80 °C for up to 6 months. In addition to the analysis of unfrozen plasma samples, MIF plasma concentrations were measured after 2 days, 7 days, 8 weeks, and 6 months of storage (Fig. 1). Before measurement, the samples were thawed at room temperature and processed immediately without further delay.

Sample dilution

To investigate the effects of dilution on MIF concentrations, plasma samples were diluted 1:20, 1:30, and 1:40 using the assay diluent immediately before being added to the ELISA plate.

Freeze/thaw process

To determine the effects of the freeze and thaw process, plasma aliquots were thawed at room temperature and refrozen at −20 °C for three cycles and MIF levels were measured subsequently. The MIF concentrations in these samples were compared to those of samples that were thawed once immediately before conducting the ELISA.

Time to initial processing

Because initial time to processing plays a role concerning the stability for some analytes, the influence of time to initial processing of the blood was tested. Heparinized whole blood samples were kept at room temperature for 3 h. Then, plasma was obtained after centrifugation and stored at −20 °C or snap-frozen and stored at −80 °C. MIF levels in these samples were measured and compared to those processed immediately without being left at room temperature.

MIF ELISA and recombinant MIF protein

Human MIF standard protein (rMIF) was bought from R&D Systems (Minneapolis, MN, USA). It was used in various concentrations