Measuring myokines with cardiovascular functions: pre-analytical variables affecting the analytical output

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Abstract: In the last few years, a growing number of molecules have been associated to an endocrine function of the skeletal muscle. Circulating myokine levels, in turn, have been associated with several pathophysiological conditions including the cardiovascular ones. However, data from different studies are often not completely comparable or even discordant. This would be due, at least in part, to the whole set of situations related to the preparation of the patient prior to blood sampling, blood sampling procedure, processing and/or store. This entire process constitutes the pre-analytical phase. The importance of the pre-analytical phase is often not considered. However, in routine diagnostics, the 70% of the errors are in this phase. Moreover, errors during the pre-analytical phase are carried over in the analytical phase and affects the final output. In research, for example, when samples are collected over a long time and by different laboratories, a standardized procedure for sample collecting and the correct procedure for sample storage are acknowledged. In this review, we discuss the pre-analytical variables potentially affecting the measurement of myokines with cardiovascular functions.

Keywords: Pre-analytical variability; sample matrix; stability; sample handling

Introduction

Starting from their classification as an "independent" category of molecule, in 2003 (1), 266 records can be retrieved on PubMed after searching the term "myokines". The ancestor molecule of this family, the first described "exercise factor", was interleukin (IL)-6. Thereafter, a plethora of both already known and newly discovered molecules have been associated with muscle activity, and consequently classified as myokines. The term myokine refers to a protein which if: (I) synthesized and released by the skeletal muscle (SKM); (II) exerts its biological function(s) in an endocrine or paracrine fashion (2). Hence, physical activity (PA) represents the main stimulus to the endocrine functioning of the SKM (3). Other than IL-6, more than 100 myokines have been so far identified (4), among the 300 predicted proteins constituting the SKM secretome (5), and 17 additional novel exercise-responsive transcripts have been recently discovered (3).

PA is directly associated with reduced risk of acute myocardial infarction (AMI), stroke, hypertension, peripheral artery disease, erectile dysfunction, depression, dementia, malignancies, and improved status in diabetes, obesity, sarcopenia, and cognitive functioning and mental health. Thus, regularly exercising helps in slow down aging consequences in association with an improvement of the quality of life, possibly promoting longevity (6). Physical inactivity has been recognized by the World Health

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Organization (WHO) as the fourth cause of death (7). Hence, based on their endocrine action, myokines are key mediators of those mechanisms involved in the exercise-associated beneficial effects. In addition, several myokines are involved in cardiovascular regulation and many of them have protective effects in cardiovascular diseases (8). Despite the increasing interest in these bioactive muscle-derive factors and their possible clinical use, either as diagnostic/prognostic factors or therapeutic targets, only a few studies focused on the pre-analytical warnings in their measurements. Thus, the aim of this review is to collect the information regarding the whole set of knowledge about the pre-analytical phase to be satisfied when a certain myokine, specifically a myokine associated with cardiovascular function, is measured.

Overview of myokines with cardiovascular functions

The effect of myokines on cardiovascular function can be either direct or indirect. The former is directly mediated by a myokine, produced by the activated SKM, which targets cardiomyocytes, neuronal fibers resident within the heart wall, endothelial cells (EC), or vascular smooth muscle cells (VSMC). The indirect effect is mediated by a myokine targeting an organ involved in metabolic functions [e.g., adipose tissue (AT), pancreas, liver] which (dys)functioning can affect cardiovascular function. However, myokines are often able to act through both ways (8). For instance, follistatin-like 1 (FSTL1) is induced, in both SKM and cardiac muscle, by ischemia and hypertrophic response and it has beneficial effects on vasculature (by limiting EC apoptosis, VSMC proliferation, and neointimal formation) and heart (by limiting cardiac ischemic injury, adverse cardiac remodeling, and cardiac rupture) (8,9). On the contrary, irisin induces white-to-brown transition of AT, increasing lipid oxidation-to-storage balance and improving the metabolic profile, finally improving cardiovascular function (10).

Pre-analytical phase in the assessment of myokines involved in cardiovascular function

The pre-analytical phase in laboratory medicine

Regardless of the method of measurement, the concentration an analyte in a biological sample depends on biological, analytical, and pre-analytical variability (11). Indeed, uses and lifestyle in the hours before sampling,

sample drawing execution, handling, processing, and storing can cause unwanted changes in the concentration of certain types of biomolecules (e.g., degradation, release from cellular components or carrier molecules) (12,13). Within the flux of samples, from collection to analysis and final reporting, the whole set of decisions and actions (i.e., sample matrix choice, collection, transportation, treatment and storage) taken during the pre-analytical phase has the biggest impact on the analytical output (70% of all errors) (14-16). Errors in this phase are carried over to the analytical phase affecting measurement accuracy and, ultimately, leading to uncertainty about results (12,17). The effects of these variables can be minimized by carefully following standard operating procedures (SOPs). However, current SOPs are based on "best practices" and not on experimental findings; therefore, studies addressing such procedures under "real-life" conditions are needed (18). While the pre-analytical phase is critical in routine laboratory medicine (19), its importance in research and clinical trials is often underestimated (20,21).

Factors associated with sampling and subjects should be considered. The main sources of variability among sampling-associated factors are represented by the preparation of the patient (timing, environmental conditions, posture), sample collection [identification and labeling, type of disposables (e.g., straight needle, butterfly, cannula, needle caliber), containers (e.g., primary tube), drawing order, phlebotomy procedure (e.g., tourniquet time, tube mixing), contamination], sample transportation (length, temperature, pneumatic tube systems), sample preparation (centrifugation time, speed, and temperature, aliquot preparation), sample storage (length, temperature, freeze/thaw cycles) (14).

Subject-associated variables to be considered when planning blood sampling are: drug administration (timing of drawing and treatments), PA (to be avoided in the 48–24 h before sampling), and menstrual cycle. Likewise, uncontrollable subject-related factors that should be critically considered are: age and gender (e.g., menopause), ethnicity, usual medications (e.g., antihypertensive), dietary supplements, comorbidities/diseases, and circannual and seasonal rhythms (22,23).

Myostatin

Myostatin, also known as growth-differentiation factor-8 (GDF-8), belongs to the transforming growth factor-β (TGF-β) superfamily. It is a negative regulator of SKM

growth, as demonstrated in GDF-8 knock-out mice, which had increased SKM mass (24). Myostatin is secreted by muscle and cardiac myocytes as a prepromyostatin; after cleavage of N-terminal signal-peptide, promyostatin is further cleaved into two active fragments, myostatin-prodomain (N-terminal) and myostatin-ligand (C-terminal). Activation requires the release of myostatin-ligand (25). In type 2 diabetes (T2DM), muscle myostatin mRNA increases 1.4-fold compared to controls; however, plasma myostatin levels do not change, indicating that myostatin release from muscle is well-regulated (26). The attenuation of myostatin expression reduces adiposity and improves cardiac contractility, probably as a consequence of improvements in cardiac Ca^{2+}-handling (27). Myostatin expression is also increased in cardiomyocytes after myocardial infarction (28), although other authors failed in correlating its serum levels with the severity and prognosis of chronic heart failure (29).

Pre-analytical factors

Commercially available enzyme-linked immunosorbent assays (ELISAs) are mainly designed to detect myostatin-ligand, although assays detecting both subunits have been developed (30). Breitbart and colleagues developed an IRMA method for measuring myostatin-prodomain in human serum. According to their findings, the N-terminal domain was stable at room temperature (RT) (25). No information about the sample matrix effect is currently available. Up to four freeze/thaw cycles did not affect myostatin immunoreactivity (25).

In a wide cohort of males, Szulc *et al.* found that myostatin slightly increases until 57 years of age and it subsequently decreases (31) with no association with age-related muscle loss (32,33). Serum myostatin levels follow a circannual rhythm with a spring-time peak, being directly correlated with 25-hydroxy vitamin D levels. Smoke decreases myostatin levels, while it was not dependent on current PA, parathyroid hormone (PTH), testosterone, and 17β-estradiol (31,34). Pro-myostatin is not affected by menstrual cycle (35) and females have slightly lower levels compared to males (25). High plasma levels of myostatin were found in in rats with chronic kidney disease (CKD) (36) and in patients with chronic obstructive pulmonary disease (COPD) (37).

In summary, data are available about subject-specific pre-analytical warnings, but not for sample-specific issues. At this regard, data about myostatin should be contextualized for age and gender, although specific ranges should be still determined.

FSTL1

FSTL1 is a secreted glycoprotein, expressed by mesenchymal cells (38). It belongs to the SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin) protein family and is a myostatin inhibitor. It is increased by cycle ergometer exercise in healthy subjects, and by swimming in mice (39,40). In murine and rat muscle cell lines, FSTL1 was decreased by insulin and during myogenesis (41-43). It is secreted by primary human SKM cells (44) and it promotes function and survival of ECs. FSTL1 overexpression in ECs enhances differentiation and migration and reduces apoptosis (45). Thus, FSTL1 may mediate some of the protective effects of exercise by counteracting the harmful effects of proinflammatory adipokines (46).

High levels of FSTL1 are protective against to cardiovascular diseases (47): beside the revascularization signal, cardiomyocytes themselves express FSTL1 which acts as a survival signal for these cells (9).

Pre-analytical factors

Widera and colleagues found that FSTL1 is stable 48 h at RT in serum and whole blood (WB) (48). In citrate- and He-plasma FSTL1 concentrations were 18% and 17% lower, respectively, than serum (48). Moreover, FSTL1 immunoreactivity was maintained after four freeze/thaw cycles (48). The acute coronary syndrome has been associated with increased serum FSTL1 (48), as well as osteoarthritis with the highest levels in females (49). In summary, FSTL1 should be measured in serum and, precautionary, within 48h from sampling in fresh samples.

Irisin

Irisin is encoded by the FDNC5 gene, highly expressed in SKM and AT. The full-length protein consists of a signal-peptide, a fibronectin III-domain, a hydrophobic transmembrane domain, and a C-terminal domain. The proteolytic cleavage releases irisin which contains most of the fibronectin III-domain. Irisin is secreted during SKM contraction and is associated with increased energy expenditure because of its ability to stimulate the browning of white AT (WAT) (50,51). In myocardium, it is induced by ischemia and it protects against ischemia and reperfusion

heart injury, by improving post-ischemic ventricular function, coronary effluent recovery, and reducing the infarct size. Furthermore, irisin decreases the expression of apoptotic proteins including active caspase-3, cleaved PARP, and annexin V and increases p38 phosphorylation and SOD-1 expression in the post-ischemic myocardium (52).

Pre-analytical factors

Circulating irisin levels vary greatly within species: in human serum or plasma, it ranges between 0.01 and 2,000 ng/mL (53,54). Moreover, irisin immunoreactivity hugely differs among assays. Currently, the most reliable method is mass spectrometry (55).

Serum irisin concentration was unexpectedly inversely correlated with storage length (56). By analyzing the sample matrix effect, specificity and sensitivity were, respectively, 55% and 90% for a cut-off value of 17.2 ng/mL for serum irisin, 90% and 60% for a cut-off value of 19.6 ng/mL for saliva irisin, 90% and 85% for a cut-off value of 17.2 ng/mL for urine irisin. However, these results indicate concerns about the assay method rather than the sample matrix (55).

Circulating irisin is affected by acute exercise [for review (57)], particularly, resistance training more than endurance training (58), although chronic exercising decreases irisin (59). Circulating irisin levels are predicted by biceps circumference, and they correlate with BMI, glucose, ghrelin, and IGF-1 and negatively with age, insulin, cholesterol, and adiponectin levels (60). However, Hecksteden *et al.* demonstrated that, in healthy subjects, only age and total cholesterol predicted irisin levels (56). In CKD patients it correlates with glomerular filtration rate (GFR) and plasma bicarbonate (61) and with nutritional status, body composition (62), and insulin resistance, as recently confirmed by a meta-analysis (62,63), in end-stage renal disease.

48 h fasting decreases blood irisin in normal rats (64). Irisin is lower in amenorrheic than in eumenorrheic athletes and non-athletes (65); however, menstrual cycle does not affect the exercise-induce response of irisin (66). Resting serum irisin does not differ between men and women (lean or obese) but, after acute exercise, it increased significantly more in lean women than in men. In obese, resting irisin is significantly higher than in lean subjects but the response to exercise is blunted (67).

In summary, no indications are available about the sample-related pre-analytical warnings. However, besides the above mentioned analytical issues, irisin concentration should be carefully contextualized within the subject metabolic conditions and the training status.

IL-6, IL-8, IL-15, CXCL2 and other cytokines

IL-6 is a pleiotropic cytokine bridging innate and adaptive immunity. Although synthesized by all cell types, main sources are: hepatocytes, T-cells, macrophages, smooth and SKM cells. It regulates acute-phase response, inflammation, and hematopoiesis (68). IL-6 receptor (IL-6R) is expressed by a limited number of cell types [e.g., bone cells, and myocytes (69)] while the co-receptor gp130 is ubiquitously expressed and is activated by all the IL-6 family members [leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), oncostatin M (OSM), IL-11, ciliary neurotrophic factor (CNTF)] (70). Soluble IL-6R, released by the action of ADAM10 and 17, binds IL-6 and activates gp130; cells expressing these enzymes [cardiomyocytes (71)] modulate IL-6 signaling (72). Activated gp130 induces the JAK/STAT signaling which promotes cardiomyocytes survival and stimulates angiogenesis (70).

Acute and chronic inflammation induced IL-6 in liver (73,74). Instead, SKM contraction generates spiking IL-6 increases [mode-, intensity-, and duration-dependently (75,76)] activating energy metabolism [stimulate glucose sensitization (77,78), insulin signaling (79-81), and lipolysis in WAT (2)] and exerts anti-inflammatory effects (2,82). It is independent of TNF-α (83) and it is not associated with myocyte damage (76) but it drives muscle regeneration and recovery from atrophy (84,85). In AMI, short-term IL-6 signaling has protective effects, while long-term signaling, or IL-6R over-expression, perpetuates the damage (68).

IL-8 belongs to the chemokine CXC family (CXCL-8). Although produced by all cell types, the main sources are monocytes/macrophages and ECs; it acts as a chemoattractant for monocytes and neutrophils and as a pro-angiogenic factor (86,87). It is resistant to enzyme-, temperature-, and acid-dependent proteolysis and, hence, it persists (days/weeks) within the inflammation site (88). As for the other CXC chemokines, IL-8 is induced by IL-6: in mice, CXCL-1 (the murine homolog of IL-8) is induced, in serum, muscle, and liver, by an exercise bout; this response is abolished following IL-6 KO and enhanced by IL-6 overexpression. Muscle IL-8 mRNA, but not plasma IL-8, is increased by a 2.5 h cycling in well-trained cyclists (89) and strenuous endurance activities (i.e., marathons and ultramarathons) markedly induce IL-8 and IL-6 (90,91).

IL-15 is a cytokine supporting survival and proliferation of T lymphocytes (92). It is abundantly expressed by SKM and fairly by AT (93). Although considered a pro-inflammatory cytokine, the negative correlation of circulating IL-15 with obesity, WAT mass and T2DM suggest a role as a myokine (94). IL-15 mRNA, but not protein, and IL-15Rα expression in SKM is increased by endurance and resistance exercising (75,95). It is also expressed at high levels during early myoblast differentiation in culture, indicating a possible role in myogenesis (96).

Pre-analytical factors

IL-6 and TNF-α measured by radioimmunoassay (RIA) were stable in WB stored at 4 ℃ and/or after rapid separation (97). Contrarily, when assayed by high-sensitivity ELISA, they were not altered over time in separated samples while decreased already after 4h in unseparated WB (98). IL-6, IL-8, and TNF-α were stable for up to 8 h in WB collected in EDTA from patients with systemic immune activation. While IL-6 was comparably stable, regardless the matrix (serum, lithium heparin plasma, and ammonium heparin plasma), IL-8 and TNF-α were increased in He-plasma and serum tubes (99). Other authors reported that serum IL-6 was unchanged after storage at 4, 20, and 30 ℃, while it was significantly decreased following storage for 11 days at 40 ℃. sIL-6R was more unstable since it was decreased after 14 days of storage at 20 ℃ and already after 1 day at 30 and 40 ℃. Storage at –20 and –70 ℃ maintained IL-6 and sIL-6R stable for years (100). However, Hardikar demonstrated that IL-6 was slightly decreased after 13 year-long storage at –80 ℃ (101). Hermann *et al.* reported that storage of unseparated WB at RT (25 ℃) for 6 h resulted in a low-to-good recovery of IL-6 (84.5%), while sample processing after 6 and 24 h resulted in an unacceptable increase in IL-8 recovery (197.2% and 1453.3%, respectively) (102). Immediate post-venipuncture centrifugation and storage for 0, 6, 24, and 48 h at 4 ℃ resulted in recovery rates for myokines comprised between 70% and 130%; while storage at RT caused greater changes (102).

In NaHe-plasma, obtained after stimulation with lipopolysaccharide (LPS) and phytohemagglutinin (PHA) of WB, stored at –80 ℃, showed degradation of IL-8 and IL-15 already after 1 year while IL-6 concentration was halved after 3 years of storage (103). CXCL-2 (MIP-2α) concentrations were unaffected by storage over 7 days at 4 ℃ or RT (25 ℃) (104).

Thavasu *et al.* demonstrated that IL-6 and TNF-α measured in EDTA-plasma samples performed most consistently than He-plasma and serum, with no improvement neither after adding Trasylol® nor under sterile, non-pyrogenic conditions. Moreover, recovery of spiked IL-6 and TNF-α was lower in serum than plasma (97). Contrarily, Flower found that serum and EDTA-plasma samples gave comparable results for IL-6, whereas high variability was found in LiHe and Na-citrate tubes (98). Similarly, Friebe and Volk found that IL-6 was stable in separated serum/plasma stored for 24 h at 4, –20, and –70 ℃ with great reproducibility among repeated measures (99). Sample matrix did not affect the IL-6 measurement, tested by ELISA, in plasma (EDTA, He, citrate) and serum samples were drawn from healthy subjects and rheumatoid arthritis (RA) patients (105).

In non-fasting blood, from pre-menopausal women, collected in different tubes [K2-EDTA, plasma preparation tube (PPT), P100, serum separator tube (SST) with clot activator, and no additive serum tube] IL-6 immunoreactivity was decreased by delayed centrifugation (median 5.5 h) whereas it was unaffected by delayed freezing of separated samples for up to 72 h (106). In a multiplex assay, IL-6 concentrations were higher in citrate-plasma, compared to EDTA-plasma, NaHe-plasma and serum, whereas IL-8 was increased in serum. No matrix effect was, instead, observed for IL-15 (103). However, a significantly higher recovery was found in serum for IL8 (113%) whereas slightly lower for IL-6 in serum as compared to plasma. NaHe- and EDTA-plasma showed a stable recovery of all cytokines, with the exception of IL-15 which was lower in EDTA plasma. The recovery of IL-15 was significantly lower compared to the other samples. In citrate-plasma, IL-6 gave the lower recovery whereas NaHe-plasma showed the most constant recovery of all cytokines tested and therefore, the authors, suggested the use of this anticoagulant for cytokine measurement (103). Within a panel of biomarkers, IL-6, IL-8, TNF-α, tested through the immuno-based fluorescent multiplex assay, displayed a relative recovery in EDTA-plasma in relation to serum around 100% indicating a good correlation between the two matrices (102). Delayed processing of WB, from 0 to 24 h at RT, but not a 4 ℃, caused a comparable decrease of IL-6 immunoreactivity in serum and plasma already after 45min, but only in healthy donors. Contrarily, plasma and serum samples from RA patients displayed a greater variability (105).

Clendenen and colleagues demonstrated that IL-6, IL-

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15, and TNF-α, along with many other cytokines and growth factors, measured by Luminex technology, were comparable in EDTA-plasma and serum. Contrarily, IL-6R and IL-8 (but also: IL-7, IL-12p70, sTNFR2) were higher in serum than in EDTA-plasma (107). CXCL-2 levels were comparable in serum and K2EDTA-plasma chronic hepatitis B patients (104). Different studies have demonstrated that up to ten freeze-thaw cycles did not affect the immunoreactivity of IL-6 (97,98,100,105). Whereas TNF-α was increased by 17.0%±3.7% already after three cycles (98).

According to Lee *et al.*, up to five freeze/thaw cycles do not affect plasma and serum IL-8 concentrations measured by multiplex assay; IL-15 was unaffected when measured in plasma, while in serum it was significantly decreased. For comparison, TNF-α was decreased in plasma after five freeze/thaw cycles and increased in serum already after three cycles (108). In multiplex assay, over four freeze/thaw cycles of LPS/PHA-stimulated NaHe-plasma, IL-6 was stable while IL-8 and IL-15 were decreased already after two cycles (103).

CXCL-2 immunoreactivity was unaffected by three freeze/thaw cycles in serum samples stored at –80 ℃ (104).

PA is the main determinant for IL-6, IL-8, and IL-15 (57) although serum IL-6 response to exercise, for instance, was blunted in RA patients (105). IL-6, as the whole cytokine asset, is influenced by long-term dietary regimen (or lifestyle habits) rather than to a single meal or a specific group of nutrients (109). Indeed, fasting does not affect IL-6 concentration (110). Cytokines also follow a circadian rhythm: in serum from healthy subjects, TNF-α peaks at 3 am and IL-6 peaks at 6 am; in RA patients, the peak of both cytokines is shifted forward: at 6 a.m. for TNF-α, at 7 am to IL-6. Furthermore, the IL-6 peak was 10-fold higher in RA patients than in controls (111). Also, a circannual rhythm is present, with a wintertime increase of IL-6, as for many other inflammatory markers (112,113). IL-6 levels, as for the other pro-inflammatory mediators, increase during aging contributing to the biological decline (114,115). Similarly, proinflammatory cytokine increased after menopause, highlighting the importance of estrogens in regulating inflammation (115,116). However, IL-6 does not change during the menstrual cycle (117). IL-6 concentrations are comparable in men and women (118), however increased IL-6 levels are differently associated with different pathologies in women or in men [e.g., hypertension in women and insulin resistance in men (119)].

Summarizing, based on the available data, in order to preserve the stability of IL-6, IL-8, and IL-15, WB collected in serum or EDTA tubes should be separated immediately. Separated plasma/serum samples are more stable and can be stored at –20 ℃ for a decade.

Fibroblast growth factor 2 (FGF2) and FGF21

FGF2, also known as basic FGF, is an angiogenic factor induced by exercise, consequently to the reduction in oxygen tension, within the activated SKM (120). Mechanically-induced FGF2 release is a key autocrine mechanism for transducing the stimulus of mechanical load into a SKM growth response (121). However, the FGF2 response depends on age (122).

FGF21 is mainly produced by the liver (123) and it acts as a nutrient stress sensor (124) regulating the metabolism of carbohydrates and lipids. Circulating FGF21 is associated with insulin resistance, glucose intolerance and dyslipidemia (125). FGF21 is also expressed by the SKM stimulated by insulin via the PI3kinase-Akt pathway (126) and hyperinsulinemia, in youth, increases SKM-derived plasma FGF21 (127). FGF21 activates the βKlotho co-receptor which is highly expressed in AT but not in SKM (128). Also AT expresses FGF21 which might be implicated in CVD since it is associated with cardio-metabolic risk factors, increased intima-media thickness of carotid and iliac arteries, high levels of C-reactive protein, dysglycemia, dyslipidemia, and decreased adiponectin levels (129,130). As for IL-6, the existence of different tissue sources of FGF21 suggests tissue-specific physiological functions (131).

Acute exercise strongly induces FGF21 expression in both SKM and liver, resulting in increased blood FGF21 (132-134). However, this response is blunted in obese (135) and T2DM patients (136). FGF21 increases the thermogenic capacity of the AT by inducing browning of the WAT (137): primary adipocytes treated with FGF21 dose-dependently increased BAT markers expression (i.e., UCP-1α), oxygen consumption and infrared-detected thermogenesis (138). Accordingly, BAT has been demonstrated as an important source of FGF21 in men. FGF21 and irisin have synergistic effects on WAT browning (132,137).

Pre-analytical factors

Hermann *et al.* reported that storage of unseparated WB for 6 and 24 h as well as 48 h long storage of separated serum at either RT or 4 ℃ did not affect FGF2 immunoreactivity (102). In 1998, Webb reported

that FGF2 should be measured in plasma, since it could be released in serum during clotting (139). However, in multiplex assay, the relative recovery in EDTA-plasma in relation to serum was around 100%, indicating a good correlation between the two matrices (102) and confirming previous results obtained by Larsson *et al.* with ELISA (140). Since FGFs are heparin-binding proteins, the use of He-EDTA should be avoided. There is no available information about sample-related pre-analytical issues for FGF21 as well as the effect of freezing/thawing on FGF2 and FGF21 immunoreactivity.

FGF21 is acutely responsive to nutrients intake, hence it should be measured under fasting condition. Moreover, as for IL-6, the established dietary and lifestyle habits affect FGF21 circulating levels (141). FGF21 has been recently depicted as superior to other adipokines in predicting incident diabetes (142).

Circulating FGF21 is associated with several pathophysiological conditions and particularly in metabolic dysfunctions (142), hypertension (143), oxidative stress (144), vascular function (131), atherosclerosis (145), aging (146). Therefore, although reference ranges have not still defined, data about FGF21 levels should be opportunely contextualized.

FGF21 has no precise circadian rhythm, but it displayed oscillations (pulsatile release) during the 24 h. The average duration of oscillation is 2.52 h and the frequency of the oscillations was higher during the light-off period than during the light-on one (2.4 *vs.* 7.3 times). The frequency of oscillation does not differ among lean and obese subjects but the amplitude is greater in obese (147). In contrast, previous findings demonstrated a night-time peak (2:30 am) in female under 72 h fasting (148) and Yu and colleagues demonstrated that regardless from fasting, FGF21 peaked in the first hours of the day with a minimum in early afternoon. The magnitude of the nocturnal rise was blunted in obese subjects. This rhythm resembled that of fatty acids and cortisol and it was opposite to insulin and glucose (149).

A single paper in 2001 described the circadian rhythm of FGF2 in women with breast cancer: acrophase around 1 p.m., a peak-to-trough interval of 18.2%, and a superimposed 12 h frequency (150). Rusnati *et al.* showed that FGF2 was unchanged during the menstrual cycle but it increased significantly after menopause (151). More recently, however, it has been demonstrated that FGF2 had two peaks the first at day 1 and the day 9 (152).

In summary, FGF2 can be measured within 48 h in serum or EDTA-plasma; no information is available for FGF21.

FGF2 and FGF21 concentrations should be carefully contextualized in the pathophysiological conditions of the subjects.

Vascular endothelial growth factor (VEGF)

VEGF is a key mediator in all conditions in which angiogenesis is relevant (153): e.g., tumor growth, through asthma, exudative age-related macular degeneration (154).

A single exercise bout transiently increases VEGF mRNA and protein expression (155-157) with levels returning to baseline after 4–6 h of recovery (158,159). In young healthy individuals and animals, VEGF protein increases during the first few weeks of aerobic training (157,160) and returns to baseline after 4-week-training (161). However, the exercise-dependent response of VEGF differs in individuals with lifestyle-related diseases (162) and aging (163,164). Muscle VEGF content is lower in elderly than in youth but it is enhanced by training (165). During SKM activation, also EC, pericytes, and fibroblasts produce VEGF (165). In myofibers, high amounts of VEGF are stored in vesicles; synthesis of VEGF is not required for secretion but likely occurs after contraction to replenish muscle stores (166). Currently, mechanisms underlying exercise-dependent VEGF secretion from SKM have yet to be fully determined. However, VEGF cannot act alone, and other factors are essential and need to be co-regulated to elicit angiogenesis. Furthermore, VEGF exists in different homodimeric isoforms: VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206. They all play a role in angiogenesis, but VEGF165 has been found to be the most important proangiogenic factor in SKM (165).

Pre-analytical factors

In 2001, Dittadi *et al.* reported that clotting time increased the release of VEGF, which reached the maximum within 2–4 h [median increase: 327% (range, 118–4515%)] compared to samples centrifuged within 10 min from drawn (167). Hermann *et al.* reported that storage of unseparated WB for 6 and 24 h before centrifugation as well as 48 h long storage of separated serum at either RT or 4 ℃ did not affect VEGF immunoreactivity (102).

The degradation rate of VEGF in serum samples increased at higher temperatures although a good recovery is obtained after 40 days at 4 ℃. The degradation rate of VEGF-R2 was temperature-dependent: after 35 days there was a 12% decrease in sera stored at 4 ℃ and a

77% decrease after 21 days at 40 ℃. VEGF-R2 was stable under cryogenic conditions (up to decades at –75 ℃, but only 3 months at –20 ℃) while VEGF was unstable even at –75 ℃. Therefore, serum stored for long periods is not suitable for assessing VEGF (168). Hetland *et al.* observed an increase in VEGF in serum stored at RT but not in EDTA-plasma (169).

With very low platelet activation, VEGF was not different in Edinburgh-plasma (a mixture of EDTA, PGE1, theophylline) and CTAD-plasma (citrate, theophylline, adenosine, and dipyridamole), while it was significantly higher in Na-citrate-plasma. In CTAD-plasma, VEGF levels were not correlated with platelets or leukocytes counts while in serum VEGF correlated with platelet count (167). In a multiplex assay, VEGF was unaffected by sample matrix when EDTA-plasma was compared to serum (mean recovery of about 100%) (102). These results were in line with those previously published by Larsson *et al.* by using ELISA (140).

Recently, Walz and coworkers found that, compared to other anticoagulants (CTAD, PECT), EDTA gave significantly higher results and up to 6-h-storage before centrifugation had no effects (154). They suggested that the type of centrifuge (fixed-angle *vs.* swing-out rotor) could be an important pre-analytic parameter for VEGF measurement: pellet formation and pellet density in fixed angle *vs.* swing-out rotor centrifuges may lead to different degrees of contamination of plasma with cell components containing VEGF: slightly higher VEGF-A levels in samples centrifuged with a fixed angle *vs.* a swing-out rotor centrifuge in association with higher PF-4 levels, indicative of thrombocyte activation (154).

VEGF-R2 was stable in both EDTA-plasma and serum over five freeze/thaw cycles. On the contrary, VEGF concentrations increased by 15% in both matrices (108). Similarly, Hetland *et al.* found that up to ten freeze/thaw cycles of serum and EDTA-plasma samples from patients with RA, after 2 years of storage at –80 ℃, had no effects on VEGF immunoreactivity (169). These findings were in contrast with previous observations by Kisand and colleagues who showed that VEGF is very sensible to freeze/thaw: 67% decrease already after the first cycle. Contrarily, VEGF-R2 was slightly but significantly increased after three freeze/thaw cycles (168). Similar results were obtained by Bünger and colleagues who used a biochip-based measurement in biobanked sera from colorectal cancer patients (170).

VEGF is affected by exercise, however, but depending on the pathological status: plasma VEGF was increased immediately after bicycling in the healthy persons (16%) and in patients with erosive RA (64%) but not in early RA (169). VEGF is not affected by age and gender (140). During the menstrual cycle VEGF peaks between days –1 and 1 and on day 9, suggesting a role in the preparation of the endometrium to reproduction (152). Hetland and colleagues showed that VEGF has a peak at 7 a.m. and two nadirs at 1 and 4 p.m. with no differences among healthy subjects and RA patients. No circannual rhythm was, instead, found (169).

In summary, it has been suggested that VEGF levels should be measured in plasma, possibly CTAD (154), since platelets activation during clotting could potentially contribute to VEGF release (139). Moreover, since the evidenced instability, WB should be processed immediately and plasma should be stored at –80 ℃. Finally, VEGF should be assayed within a few months since the possible instability also under cryogenic conditions.

Brain-, neuron-, glial cell line-derived neurotrophic factors (GDNFs)

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophins subfamily which exerts various effects on the nervous system. Particularly, BDNF plays a role in the development of the nervous system and influences many aspects of neuronal function in the adult brain (171). Other than from neurons, BDNF is produced at consistent levels by SKM cells. Indeed, exercise promotes the release of BDNF from brain (through the blood-brain barrier) and from muscles and increases BDNF blood levels in healthy individuals. The increase of circulating BDNF is directly proportional to exercise intensity (172-174). BDNF increases induced by exercise accounts for at least a portion of the neuroprotective effects of exercise (175).

BDNF is considered a biomarker for mental disorders such as bipolar disorder; as these patients have a high risk of developing cardiovascular disease, BDNF could be a link between the two diseases (176). Manni and colleagues demonstrated that serum BDNF was reduced in patients with acute coronary disease (ACD), hence, this neurotrophin may be implicated in the pathogenesis of cardiovascular disease (177); low serum BDNF were also found in patients with ACD during an oral glucose tolerance test (OGTT) (178).

Neuron-derived neurotrophic factor (NDNF) is a neurotrophic factor containing a fibronectin type III

domain. NDNF is expressed in neurons and may act through an auto- or paracrine loop. NDNF is also expressed at high level in Cajal Retzius cells, neurons contributing to the formation of the cerebral cortex and promotes differentiation and migration of neurons (179). Recently, NDNF has been found to promote vascularization since expressed at high levels by EC of the ischemic muscle in mice. Intramuscular NDNF overexpression increases perfusion and capillary formation in a murine model of ischemia, while NDNF knock-out resulted in an overall reduction of the EC network (180).

GDNF belongs to the TGF-β superfamily and it is mainly expressed by astrocytes. It is necessary for maintenance and development of the nigrostriatal dopaminergic neurons but it may have several functions since is detected in many brain structures (181). It is induced by PA: after 2 weeks of involuntary exercise, GDNF protein content was upregulated in rat Soleus hind limb SKM and down-regulated in extensor digitorum longus, compared to controls. The contrary happened following 4 h of ex-vivo field stimulation of muscles (182). Higher levels of GDNF were also found in glomus cells of the carotid body of hypertensive rats compared to control, indicating a possible involvement in the onset of hypertension (183).

Pre-analytical factors

In plasma (EDTA, He, citrate) BDNF concentrations increased over 48 h of storage at 4 or 25 ℃, while in serum they were stable (184). BDNF has a short half-life in plasma of rats (0.92 min) (185). According to Lommatzsch *et al.* BDNF displayed a wide range of concentrations in serum (median: 22.6 ng/mL), platelets (median: 92.7 pg/10^6 platelets) and plasma (median: 92.5 pg/mL). Maximal and moderate exercise increased serum BDNF in sedentary and trained subjects, although the return to baseline was faster in the latter (186). At this purpose, Pareja-Galeano and colleagues highlighted the need to adjust BDNF by hemoconcentration in exercise studies. Moreover, they also showed that the effects of exercise on BDNF levels is associated with length of clotting: in serum coagulated for 24 h, corrected by hemoconcentration, BDNF was increased by exercise but in serum coagulated for 10 min and in plasma. Plasma showed the most variable results regardless platelets presence (187). Resting BDNF levels were inversely associated with the cardiorespiratory fitness (174).

Plasma, but not platelet, BDNF levels in plasma were inversely associated with age and weight. There were no gender-associated differences in plasma BDNF when matched for weight; however, women displayed significantly lower platelet BDNF than men. Moreover, platelet BDNF levels changed during the menstrual cycle with highest levels during the luteal phase (188). The difference between serum and plasma were confirmed by other authors (189,190).

In healthy males, plasma BDNF follows a circadian rhythm, similarly to cortisol (191): peak in the morning (8:00) and nadir in the night with a minimum between 10:00 pm and midnight (191-193). Interestingly, this rhythm was significant only in plasma in men; diurnal variation was found neither in plasma BDNF of women, during the menstrual cycle, nor in serum BDNF level in both men and women (192).

Diet affects BDNF concentrations: raising free fatty acids levels decreased both serum (43%) and plasma (35%) BDNF levels after 360 min; parallel, at the same time-point, a high-fat meal decrease plasma BDNF levels by 28% (194). On the contrary, a weekly oral assumption of α-linoleic acid increased plasma BDNF levels, particularly in women (195). OGTT-induced hyperglycemia also decreased plasma but not serum BDNF and return to baseline was faster in normoglycemic subjects than in borderline/diabetic ones (190).

Plasma and partially serum, BDNF levels were modified in several diseases and conditions such as: fibromyalgia [increased (196)], hemodialysis [increased, especially in diabetic patients (197)], metabolic syndrome and T2DM [increased (198)], bipolar disorder [decreased (199)], as well as with mood, cognition and motor function (200), alcohol consumption [decreased (201)], antidepressant treatments [increased (202)].

A recent meta-analysis suggested that serum, but not plasma, GDNF concentrations are associated with depression (203). GDNF stability was assessed by Wang and colleagues who tested GDNF immobilized onto electrospun scaffold in different conditions: GDNF is stable for prolonged periods without degradation, even in presence of protease. GDNF is also resistant to repeated freeze/thaw cycles (204). Plasma GDNF concentrations have been associated with mood state, bipolar disorder and age [increased (205)], late-life depression [reduced (206)], adolescent depression [decreased (207)], attention deficit [increased (208)].

In summary, BDNF should be measured in serum within a few hours after sampling, or immediately stored at –20 ℃.

Also, GDNF should be assayed in serum since the revealed association with mental disorders. Overnight fasting should be suggested. However, since the association with mood, care should be taken in interpreting the data. No pre-analytical warnings are available for NDNF.

Conclusions

The issue of pre-analytical management of the sample in the field of myokines is still far to be solved. However, as emerged from this review article it is clear that there are several variables that can affect the measurement of the superclass of molecules.

A very important issue derives from the fact that several studies focused on myokine measurements are based on biobanked samples. In biobanking and longitudinal studies, samples are collected over time, stored freezers (–20 or –80 ℃), and then simultaneously analyzed in order to minimize inter-assay variability (209,210). However, as described in this article, the biomarkers are subject to variability arising from sampling and storage procedures (169). Erroneous sampling or handling and improper storage may lead to degradation of these markers, limiting their clinical reliability (211). The more is the interval between collecting and testing, the more is the weight of this problem. Importantly, such pre-analytical variation, when not appropriately considered, already during experimental planning, would reduce or overestimate the diagnostic power of a biomarker with serious implications in its translation in clinics (212).

In conclusion, if from one hand care should be taken when literature data about myokines are considered, on the other hand stronger effort should be done in order to make more reliable the measurement of these future biomarkers.

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Footnote

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