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Abstract

Obesity is an important health problem worldwide. Adipose tissue acts as an endocrine organ that secretes various bioactive substances, called adipokines, including pro-inflammatory biomarkers such as TNF-α, IL-6, leptin and C-reactive protein (CRP) and anti-inflammatory molecules such as adiponectin. The deregulated production of adipokines in obesity is linked to the pathogenesis of various disease processes and monitoring their variation is critical to understand metabolic diseases.

The aim of this study was to determine the plasma concentration of adipokines in healthy subjects by multiplexed measurements and the effect of anticoagulants on their levels.

Plasma samples from 10 healthy donors were collected in two different anticoagulants (sodium citrate or heparin).

All markers, excluding TNF-α, showed significantly higher concentrations in heparinized compared to citrate plasma. However, levels of adipokines in different plasma samples highly correlated for most of these markers.

We reported that different anticoagulants used in the preparation of the plasma samples affected the measurements of some adipokines. The importance of the present results in epidemiology is relevant when comparing different studies in which blood samples were collected with different anticoagulants.

Keywords: adipokines, anticoagulants, cytokines, microbead assay, plasma

1. Introduction

Adipokines are defined as cell signaling mediators secreted by the adipose tissue. They have both pro-inflammatory and anti-inflammatory activities, and the imbalance between the different factors secreted by adipose tissue contributes to metabolic dysfunction. [1]. When adipocyte dysfunction is developed as a result of adipose tissue expansion, the deregulation of adipokine levels can produce several effects on inflammatory responses, thereby contributing to the initiation and progression of obesity-induced metabolic and cardiovascular complications [1]. Therefore, further elucidation of the functions and mechanisms of key adipokines will lead to a better understanding of the pathogenesis of obesity-linked disorders.

Adipokines are involved in the regulation of metabolism and insulin sensitivity. Moreover, inflammation and blood concentrations of various adipokines are associated with obesity, metabolic and cardiovascular diseases. It is, therefore, emerging that serum adipokine levels may serve as biomarkers of obesity-related illnesses [2].

Recent papers demonstrated the effect of anticoagulants on multiplexed measurement of cytokines/chemokines in healthy subjects [3-7], highlighting the importance of sample preparation in biomarkers measurements in plasma. The Luminex multiplex platform system is a highly efficient fluorescent (or magnetic) bead-based capture/detection sandwich immunoassay that allows for measurements of multiple analytes simultaneously in a single reaction with small sample volumes [8, 9]. This technology can measure up to 100 different analytes using as little as 50 µl sample volume, making it an assay very useful in clinical trials or epidemiology studies, especially when volumes are limited.

The aim of this study was to evaluate the effect of two different anticoagulants (lithium heparin (LiEP) and sodium citrate (NaCitr)) on the levels of adipokines in plasma collected from 10 healthy subjects. Multiplex microbead immunoassay was performed for measuring IL-6, TNF-α and leptin while adiponectin and C-reactive protein (CRP) were measured as a single measurement.

2. Materials and Methods

2.1 Samples

Samples were obtained concurrently from 10 healthy donors. Twenty milliliters of peripheral venous blood were drawn into vacutainer tubes containing two different anticoagulants (LiEp or NaCitr). Samples were centrifuged immediately after the blood withdrawal, and plasma was analysed within 1 hour. Cells were removed from plasma by centrifugation for 15 minutes at 2,000 x g at room temperature. Written informed consent was achieved from all subjects. The study protocol was approved by the ethics committee of the Fondazione IRCCS (Istituto per la Ricerca e la Cura del Cancro, National Institute for Research and Treatment of Cancer) Istituto Nazionale dei Tumori (Milan, Italy).

Aliquots of the samples were stored at -80^{0}C and analysed ten days later.

2.2 Experimental measurements

The adipokine analysis was performed with the Luminex technology, which combines the principle of a sandwich immunoassay with fluorescent bead-based technology. In this way, individual and multiplex analysis of different analytes in a single microwell plate are allowed [10]. Capture antibodies directed against the

biomarker of interest are covalently coupled to fluorescently dyed magnetic microspheres, each with a different color code or spectral address to allow discrimination of individual tests within a multiplex suspension. Coupled beads react with the sample containing the analyte of interest. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed by the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin serves as a fluorescent indicator.

The assay for human IL-6, TNF-α, leptin, adiponectin (Bio-Rad Laboratories, Hercules California, USA) and CRP (Merk Millipore, Darmstadt, Germany) was carried out on fresh plasma samples using 96-well microplates accordingly to the recommendations of manufacturers. The contents of each well were moved into the Bio-Plex 100 System array reader (Bio-Rad Laboratories Bio-Rad Laboratories, Hercules California, USA), which identifies and quantifies each specific reaction based on bead color and fluorescent signal intensity. The data were finally processed using Bio-Plex Manager software (version 6.1) using five-parametric curve fitting and converted in pg/ml.

2.3 Statistical analyses

All results are showed as mean ± standard deviation (SD). Data were compared by nonparametric analyses with Wilcoxon's matched pairs test, in which the median was used to calculate significant differences. Spearman correlation coefficients were calculated to investigate the correlation between measurements obtained with the two anticoagulants. All statistical analyses were performed using the

statistical software GraphPad Prism 5, version 5.04 (GraphPad Software, San Diego, CA).

When measurements were below the lower limit of detection (LLD, <5% of measurements), we assigned a value equal to the midpoint between the LLD and zero.

3. Results and Discussion

Significant differences in the measurements of all adipokines, except TNF-α, were observed in plasma samples collected in different anticoagulants (Fig. 1). IL-6, leptin, adiponectin and CRP showed a significantly higher concentration in heparin plasma compared to citrate.

However, despite these differences in absolute levels, the measurements of all adipokines were highly correlated each other, except for TNF-α (Fig. 2). We can speculate that, even though the measured levels differ, the same variation between individuals can be detected, as demonstrated by the significant correlations.

TNF-α measurements differed from the other mediators both with regard to the effect of anticoagulant and the lack of correlation between the levels in the paired samples. These differences could be due to the very low levels, quite undetectable, of this cytokine in our samples. To explain these results we performed also an ELISA assay for TNF-α on the same samples: by this way we confirmed the absence of significant differences in the levels of TNF-α in plasma samples obtained using different anticoagulants. On the contrary, correlation analysis of ELISA results obtained with the two anticoagulants was statistically significant ($r = 0.811$; $p =$

0.004), confirming the results obtained with the other adipokines (data not shown).

We carried out this study with explorative purposes only on ten subjects, with the aim to understand if the cross-comparison of levels or profiles of adipokines performed in different anticoagulants (e.g. results collected from various studies) could be performed.

Adipokines are involved in the regulation of metabolism, insulin sensitivity, and inflammation and serum concentrations of various adipokines are associated with obesity, metabolic and cardiovascular diseases [1, 2]. It has been therefore hypothesized that serum adipokine levels may serve as predictors of obesity-related diseases or the individual disease outcomes [11].

Our study focused the attention on the measurement of five adipokines. Anticoagulants effect on IL-6 and TNF-α measurements has been already described in recent papers [3-6], while only a few of them described the effect on leptin [3, 5] or CRP [5], and none, to our knowledge, reported anticoagulants effect on adiponectin measurement. However, the importance of such a study is underlined by the proposal of using adiponectin as a clinical biomarker for several diseases [12]. Lifestyle modification with visceral fat reduction combined with targeted therapeutic interventions designed to improve adiponectinemia seem to be potential clinically useful strategies to prevent obesity-related diseases, including type 2 diabetes, cardiovascular diseases and malignancies [12].

Significant differences observed between different plasma samples in IL-6, leptin and CRP levels were not in agreement with previous data reported by Biancotto et al. [5], however, we measured analyte levels in fresh sample while they performed

160 the assay on frozen specimens. When we did the same test on frozen samples,
161 statistically significant differences between plasma samples (LiEp vs. NaCitr) were
162 no more observed for IL-6 measurements but were still significant for leptin
163 measurements ($p=0.002$, Supplementary Materials Fig. S1). However, we observed
164 a significant difference between fresh and frozen plasma samples with Li Ep for IL-
165 6 measurements ($p<0.001$, Fig. S1) and between fresh and frozen plasma samples
166 both with LiEp and NaCitr for leptin measurements ($p=0.002$, Fig. S1). Moreover,
167 these statistically significant results always showed a significant correlation (IL-6
168 LiEp fresh vs. frozen $r=0.988$, $p<0.0001$; Leptin LiEp fresh vs. frozen $r=1$,
169 $p<0.0001$; Leptin NaCitr fresh vs. frozen $r=0.939$, $p<0.002$; Supplementary
170 Materials Fig. S2). Previous papers on anticoagulant effect on cytokine
171 measurements often showed undetectable levels of IL-6, TNF-α or leptin, probably
172 because samples were stored at -80^{0}C for a different time and affected by multiple
173 freeze-thawing cycles [3, 5, 6].
174 We dedicated our attention only at plasma samples, but differences between
175 plasma and serum cytokine measurements have been also described. However,
176 results are often discordant: for many analytes, high correlation were observed
177 between serum and plasma levels even when absolute levels differed. In fact, the
178 removal of fibrinogen, platelets and other circulating proteins from the plasma
179 during serum preparation could influence the presence or detection of an analyte.
180 Also during the cascade of coagulation, activation of cellular elements can release
181 inflammatory mediators, that may affect cytokine levels. This kind of information
182 are available for IL-6 and TNF-α [3-6], leptin [3, 5] or CRP [5], but are not available
183 for adiponectin. Possible differences between serum and plasma samples should

be taken into consideration also when measuring adipokine levels and further evaluations are needed.

We used Luminex multiplex bead-based technology, with the antibody kits purchased from Bio-Rad or Merk (see Section 2). Previous studies compared the performance of multiplex microbead assays between various vendors, and it was noted that the consistency between the vendors was high only for some analytes [13, 14]. Though our conclusions are broadly applicable, specific care must be taken when other kits or suppliers are used.

4. **Conclusions**

Our results emphasize, as already demonstrated for cytokines/chemokines [3-7], the need to consider sample collection methods carefully when a study requires measurement of adipokines in the peripheral blood. Cross comparison of levels or profiles of adipokines performed in different anticoagulants should be avoided. However, we observed a correlation between levels in plasma samples obtained with different anticoagulants, and a possible solution might be the normalization of values from one type of samples to an alternative type. Despite this possibility, Jung and Wu demonstrated that this kind of conversion is complicated, and calculations based on correlation analyses alone are not sufficient [15].

Acknowledgements

This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (project number IG13273, V.K.). Partial funding for this project has also been received from the Compagnia di San Paolo (www.compagniadisanpaolo.it)

(G.M.) and by the Human Genetics Foundation-Torino (www.hugef-torino.org) (G.M.).

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