Anticoagulants used in plasma collection affect adipokine multiplexed measurements

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1   **Anticoagulants used in plasma collection affect adipokine multiplexed**

2   **measurements**

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4   Alessandra Allione[a,b,*], Cornelia Di Gaetano[a,b], Nadia Dani[c], Davide Barberio[c], Sabina

5   Sieri[d], Vittorio Krogh[d], Giuseppe Matullo[a,b]

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7   [a] Human Genetics Foundation- HuGeF, Via Nizza 52, 10126 Turin, Italy

8   [b] Department of Medical Sciences, University of Turin, Via Santena 19, 10100

9   Turin, Italy

10   [c] Bioclarma Srl, Via Nizza 52, 10126 Turin, Italy

11   [d] IRCCS Foundation, Istituto Nazionale dei Tumori, Via Venezian, 1, 20133 Milan,

12   Italy

13

14   * Corresponding author:

15   Human Genetics Foundation – HuGeF, Via Nizza 52, 10126 Turin, Italy

16   E-mail: alessandra.allione@hugef-torino.org

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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.

65 The aim of this study was to evaluate the effect of two different anticoagulants

66 (lithium heparin (LiEP) and sodium citrate (NaCitr)) on the levels of adipokines in

67 plasma collected from 10 healthy subjects. Multiplex microbead immunoassay was

68 performed for measuring IL-6, TNF-α and leptin while adiponectin and C-reactive

69 protein (CRP) were measured as a single measurement.

70

71 **2. Materials and Methods**

72 **2.1 Samples**

73 Samples were obtained concurrently from 10 healthy donors. Twenty milliliters of

74 peripheral venous blood were drawn into vacutainer tubes containing two

75 different anticoagulants (LiEp or NaCitr). Samples were centrifuged immediately

76 after the blood withdrawal, and plasma was analysed within 1 hour. Cells were

77 removed from plasma by centrifugation for 15 minutes at 2,000 x g at room

78 temperature. Written informed consent was achieved from all subjects. The study

79 protocol was approved by the ethics committee of the Fondazione IRCCS (Istituto

80 per la Ricerca e la Cura del Cancro, National Institute for Research and Treatment

81 of Cancer) Istituto Nazionale dei Tumori (Milan, Italy).

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

83

84 **2.2 Experimental measurements**

85 The adipokine analysis was performed with the Luminex technology, which

86 combines the principle of a sandwich immunoassay with fluorescent bead-based

87 technology. In this way, individual and multiplex analysis of different analytes in a

88 single microwell plate are allowed [10]. Capture antibodies directed against the

89 biomarker of interest are covalently coupled to fluorescently dyed magnetic

90 microspheres, each with a different color code or spectral address to allow

91 discrimination of individual tests within a multiplex suspension. Coupled beads

92 react with the sample containing the analyte of interest. After a series of washes to

93 remove unbound protein, a biotinylated detection antibody is added to create a

94 sandwich complex. The final detection complex is formed by the addition of

95 streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin serves as a

96 fluorescent indicator.

97 The assay for human IL-6, TNF-α, leptin, adiponectin (Bio-Rad Laboratories,

98 Hercules California, USA) and CRP (Merk Millipore, Darmstadt, Germany) was

99 carried out on fresh plasma samples using 96-well microplates accordingly to the

100 recommendations of manufacturers. The contents of each well were moved into

101 the Bio-Plex 100 System array reader (Bio-Rad Laboratories Bio-Rad Laboratories,

102 Hercules California, USA), which identifies and quantifies each specific reaction

103 based on bead color and fluorescent signal intensity. The data were finally

104 processed using Bio-Plex Manager software (version 6.1) using five-parametric

105 curve fitting and converted in pg/ml.

106

107 **2.3 Statistical analyses**

108 All results are showed as mean ± standard deviation (SD). Data were compared by

109 nonparametric analyses with Wilcoxon's matched pairs test, in which the median

110 was used to calculate significant differences. Spearman correlation coefficients

111 were calculated to investigate the correlation between measurements obtained

112 with the two anticoagulants. All statistical analyses were performed using the

113 statistical software GraphPad Prism 5, version 5.04 (GraphPad Software, San

114 Diego, CA).

115 When measurements were below the lower limit of detection (LLD, <5% of

116 measurements), we assigned a value equal to the midpoint between the LLD and

117 zero.

118

119 **3. Results and Discussion**

120 Significant differences in the measurements of all adipokines, except TNF-α, were

121 observed in plasma samples collected in different anticoagulants (Fig. 1). IL-6,

122 leptin, adiponectin and CRP showed a significantly higher concentration in heparin

123 plasma compared to citrate.

124 However, despite these differences in absolute levels, the measurements of all

125 adipokines were highly correlated each other, except for TNF-α (Fig. 2). We can

126 speculate that, even though the measured levels differ, the same variation between

127 individuals can be detected, as demonstrated by the significant correlations.

128 TNF-α measurements differed from the other mediators both with regard to the

129 effect of anticoagulant and the lack of correlation between the levels in the paired

130 samples. These differences could be due to the very low levels, quite undetectable,

131 of this cytokine in our samples. To explain these results we performed also an

132 ELISA assay for TNF-α on the same samples: by this way we confirmed the absence

133 of significant differences in the levels of TNF-α in plasma samples obtained using

134 different anticoagulants. On the contrary, correlation analysis of ELISA results

135 obtained with the two anticoagulants was statistically significant ($r = 0.811$; $p =$

136   0.004), confirming the results obtained with the other adipokines (data not

137   shown).

138   We carried out this study with explorative purposes only on ten subjects, with the

139   aim to understand if the cross-comparison of levels or profiles of adipokines

140   performed in different anticoagulants (e.g. results collected from various studies)

141   could be performed.

142   Adipokines are involved in the regulation of metabolism, insulin sensitivity, and

143   inflammation and serum concentrations of various adipokines are associated with

144   obesity, metabolic and cardiovascular diseases [1, 2]. It has been therefore

145   hypothesized that serum adipokine levels may serve as predictors of obesity-

146   related diseases or the individual disease outcomes [11].

147   Our study focused the attention on the measurement of five adipokines.

148   Anticoagulants effect on IL-6 and TNF-α measurements has been already described

149   in recent papers [3-6], while only a few of them described the effect on leptin [3, 5]

150   or CRP [5], and none, to our knowledge, reported anticoagulants effect on

151   adiponectin measurement. However, the importance of such a study is underlined

152   by the proposal of using adiponectin as a clinical biomarker for several diseases

153   [12]. Lifestyle modification with visceral fat reduction combined with targeted

154   therapeutic interventions designed to improve adiponectinemia seem to be

155   potential clinically useful strategies to prevent obesity-related diseases, including

156   type 2 diabetes, cardiovascular diseases and malignancies [12].

157   Significant differences observed between different plasma samples in IL-6, leptin

158   and CRP levels were not in agreement with previous data reported by Biancotto et

159   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

184 be taken into consideration also when measuring adipokine levels and further

185 evaluations are needed.

186 We used Luminex multiplex bead-based technology, with the antibody kits

187 purchased from Bio-Rad or Merk (see Section 2). Previous studies compared the

188 performance of multiplex microbead assays between various vendors, and it was

189 noted that the consistency between the vendors was high only for some analytes

190 [13, 14]. Though our conclusions are broadly applicable, specific care must be

191 taken when other kits or suppliers are used.

192

193 4. **Conclusions**

194 Our results emphasize, as already demonstrated for cytokines/chemokines [3-7],

195 the need to consider sample collection methods carefully when a study requires

196 measurement of adipokines in the peripheral blood. Cross comparison of levels or

197 profiles of adipokines performed in different anticoagulants should be avoided.

198 However, we observed a correlation between levels in plasma samples obtained

199 with different anticoagulants, and a possible solution might be the normalization

200 of values from one type of samples to an alternative type. Despite this possibility,

201 Jung and Wu demonstrated that this kind of conversion is complicated, and

202 calculations based on correlation analyses alone are not sufficient [15].

203

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209   (G.M.).

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211   **References**

212   [1] N. Ouchi, J.L. Parker, J.J. Lugus, K. Walsh, Adipokines in inflammation and
213   metabolic disease, Nat Rev Immunol, 11 (2011) 85-97.
214   [2] M. Bluher, C.S. Mantzoros, From leptin to other adipokines in health and
215   disease: facts and expectations at the beginning of the 21st century, Metabolism,
216   64 (2015) 131-145.
217   [3] T.H. Tvedt, K.P. Rye, H. Reikvam, A.K. Brenner, O. Bruserud, The importance of
218   sample collection when using single cytokine levels and systemic cytokine profiles
219   as biomarkers--a comparative study of serum versus plasma samples, J Immunol
220   Methods, 418 (2015) 19-28.
221   [4] V.V. Krishnan, R. Ravindran, T. Wun, P.A. Luciw, I.H. Khan, K. Janatpour,
222   Multiplexed measurements of immunomodulator levels in peripheral blood of
223   healthy subjects: Effects of analytical variables based on anticoagulants, age, and
224   gender, Cytometry B Clin Cytom, 86 (2014) 426-435.
225   [5] A. Biancotto, X. Feng, M. Langweiler, N.S. Young, J.P. McCoy, Effect of
226   anticoagulants on multiplexed measurement of cytokine/chemokines in healthy
227   subjects, Cytokine, 60 (2012) 438-446.
228   [6] W. de Jager, K. Bourcier, G.T. Rijkers, B.J. Prakken, V. Seyfert-Margolis,
229   Prerequisites for cytokine measurements in clinical trials with multiplex
230   immunoassays, BMC Immunol, 10 (2009) 52.
231   [7] H.L. Wong, R.M. Pfeiffer, T.R. Fears, R. Vermeulen, S. Ji, C.S. Rabkin,
232   Reproducibility and correlations of multiplex cytokine levels in asymptomatic
233   persons, Cancer Epidemiol Biomarkers Prev, 17 (2008) 3450-3456.
234   [8] C. Belabani, S. Rajasekharan, V. Poupon, T. Johnson, A. Bar-Or, A condensed
235   performance-validation strategy for multiplex detection kits used in studies of
236   human clinical samples, J Immunol Methods, 387 (2013) 1-10.
237   [9] K. Skogstrand, Multiplex assays of inflammatory markers, a description of
238   methods and discussion of precautions - Our experience through the last ten years,
239   Methods, 56 (2012) 204-212.
240   [10] D.A. Vignali, Multiplexed particle-based flow cytometric assays, J Immunol
241   Methods, 243 (2000) 243-255.
242   [11] L.F. Van Gaal, I.L. Mertens, C.E. De Block, Mechanisms linking obesity with
243   cardiovascular disease, Nature, 444 (2006) 875-880.
244   [12] K. Kishida, T. Funahashi, I. Shimomura, Adiponectin as a routine clinical
245   biomarker, Best Pract Res Clin Endocrinol Metab, 28 (2014) 119-130.
246   [13] S.S. Khan, M.S. Smith, D. Reda, A.F. Suffredini, J.P. McCoy, Jr., Multiplex bead
247   array assays for detection of soluble cytokines: comparisons of sensitivity and
248   quantitative values among kits from multiple manufacturers, Cytometry B Clin
249   Cytom, 61 (2004) 35-39.
250   [14] A.M. Dupuy, N. Kuster, G. Lizard, K. Ragot, S. Lehmann, B. Gallix, J.P. Cristol,
251   Performance evaluation of human cytokines profiles obtained by various

multiplexed-based technologies underlines a need for standardization, Clin Chem Lab Med, 51 (2013) 1385-1393.

[15] K. Jung, C.Y. Wu, Methodological weakness in using correlation coefficients for assessing the interchangeability of analyte data between samples collected under different sampling conditions--the example of matrix metalloproteinase 9 determined in serum and plasma samples, Clin Chem Lab Med, 48 (2010) 733-736.