Influence of hematocrit on the measurement of lipoproteins demonstrated by the example of lipoprotein(a)

FLORIAN KRONENBERG, EVI TRENKWALDER, MARTINA F. KRONENBERG, PAUL KÖNIG, GERD UTERMANN, and HANS DIEPLINGER

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Background. The measurement of many parameters of human blood is usually performed in plasma or serum. Since lipoproteins or apolipoproteins, for example, are found almost exclusively in the plasma fraction after low-speed centrifugation, these parameters can be expected to be distributed in a different plasma volume depending on the hematocrit value. Therefore, the measured plasma levels might be relatively too low or too high in comparison to the whole blood concentrations in the case of abnormal hematocrit levels. The aim of our experiments was to evaluate the extent of differences between whole blood and plasma concentrations, taking as an example lipoprotein(a) [Lp(a)] in hemodialysis patients with documented decreased hematocrit values.

Methods. Lp(a) was measured in plasma as well as whole blood of 15 hemodialysis patients with low hematocrit values (0.29 ± 0.02) in comparison to 11 control subjects (0.45 ± 0.04).

Results. Plasma concentrations were 27% higher in patients than in controls (19.7 vs. 15.5 mg/dl). The relative difference was twice as high (59%) when measured in whole blood (13.5 vs. 8.5 mg/dl). Similar relative differences were observed when whole blood concentrations of 125 hemodialysis patients and 256 controls were calculated with the formula

[Lp(a)plasma * (1 – hematocrit)].

Conclusions. Our findings clearly demonstrate that hematocrit is a strong confounding variable of lipoprotein measurement in epidemiological studies when concentrations are measured in plasma, especially in cases of abnormal hematocrit values. Furthermore, studies investigating the longitudinal changes of lipoproteins should consider potential hematocrit changes.

Lipoproteins and apolipoproteins are usually measured in plasma or serum [1]. This is convenient and can be done in frozen samples. Whole blood measurements are impossible in many cases since especially colorimetric assays are disturbed by high levels of bilirubin or hemoglobin. Handling of whole blood samples using micropipettes or automated pipetting also often disturbs the measurement due to pipette clogging. Despite the uncontested advantages of measurement in plasma or serum, the possible influence of abnormal hematocrit values in case-control studies and of fluctuating hematocrit values in longitudinal studies remains to be evaluated.

During recent years several studies described an association between high lipoprotein(a) [Lp(a)] plasma concentrations and coronary heart disease [2, 3]. Patients with renal disease have an increased risk for coronary heart disease [4, 5] and high Lp(a) plasma concentrations [6–13; reviewed in 4]. This patient group also suffers from renal anemia with low hematocrit values. The aim of this study was, therefore, to investigate the influence of hematocrit values on the measurement of parameters distributed exclusively in the plasma fraction after low-speed centrifugation. We attempted to illustrate this question by measuring Lp(a) in hemodialysis patients. We demonstrate that Lp(a) from the whole blood of hemodialysis patients is distributed in a higher plasma volume after centrifugation due to the low hematocrit levels in these patients. Therefore, the amount and possibly also the clinical relevance of Lp(a) in these patients is underestimated in relative terms when measured as plasma instead of whole blood concentration (Fig. 1).

METHODS

Patients and controls

In a first step we measured Lp(a) in whole blood as well as in the plasma of 15 hemodialysis patients with hematocrit values below 0.33 and 11 healthy control subjects to investigate the effect of various hematocrit values on Lp(a) concentrations. Additionally, the whole blood concentrations of Lp(a) from 125 randomly selected hemodialysis patients were calculated using the formula [Lp(a)plasma *
Whole blood as well as plasma concentrations of Lp(a) in these patients were compared with those of 256 healthy control subjects.

Laboratory procedures

Nine milliliters of ethylenediaminetetraacetate (EDTA) blood were withdrawn from 15 hemodialysis patients immediately preceding the onset of dialysis therapy and from 11 controls. After blood withdrawal, 2 ml of whole blood were stored in an ice bath, and the rest was centrifuged for 15 minutes at 1500 × g at 4°C. Plasma and 1 ml of cells were separated and stored in an ice bath.

Lp(a) was measured in plasma and whole blood by ELISA exactly as previously described [14]. To determine whether whole blood disturbs the measurement of Lp(a), we added 50 µl of a plasma sample with a high Lp(a) concentration to 150 µl whole blood from six individuals. Lp(a) was then measured in the whole blood with and without added plasma. In parallel, we also measured Lp(a) in the plasma samples from the six individuals above, to which plasma with high Lp(a) concentration had been added. Furthermore, Lp(a) was measured in the cell fraction after resuspending with 150 mM sodium chloride solution in four out of the six subjects. Each sample was diluted and analyzed in triplicate. With this method the intraassay coefficient of variance was 2.5% for plasma and 2.4% for whole blood measurements.

Hematocrit values were determined with a Coulter Counter T660 (Coulter Cooperation, Miami, USA).

Statistical calculations

The Spearman rank correlation coefficient was calculated between the measured whole blood concentrations and those calculated with the formula [Lp(a)plasma * (1 – hematocrit)]. The nonparametric Wilcoxon rank sum test was used to compare the plasma as well as whole blood concentrations of Lp(a) between 125 hemodialysis patients and 256 controls.

RESULTS

In a first step we investigated whether measurement of Lp(a) in whole blood samples is influenced by the presence of blood cells. For this purpose, we added 50 µl plasma with a high Lp(a) concentration to 150 µl whole blood and to 150 µl plasma of six control subjects (A-E). The added amount of Lp(a) was completely recovered in the whole blood as well as in the plasma sample. Abbreviations are: AP, added plasma; ND, not determined.

* Calculated with the formula: 
((150 * [plasma]) + (50 * [AP])/200

b Calculated with the formula: 
((150 * [whole blood]) + (50 * [AP])/200

We added 50 µl plasma with a high Lp(a) concentration to 150 µl whole blood and to 150 µl plasma of six control subjects (A-E). The added amount of Lp(a) was completely recovered in the whole blood as well as in the plasma sample. Abbreviations are: AP, added plasma; ND, not determined.

Table 1. Influence of blood cells on the measurement of Lp(a) (in mg/dl)

<table>
<thead>
<tr>
<th>Subject</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>0.81</td>
<td>7.08</td>
<td>21.7</td>
<td>90.7</td>
<td>2.44</td>
<td>22.8</td>
</tr>
<tr>
<td>Whole blood</td>
<td>0.42</td>
<td>3.76</td>
<td>11.4</td>
<td>49.0</td>
<td>0.95</td>
<td>11.4</td>
</tr>
<tr>
<td>Added plasma (AP)</td>
<td>95.3</td>
<td>95.3</td>
<td>95.3</td>
<td>86.2</td>
<td>58.5</td>
<td>58.5</td>
</tr>
<tr>
<td>150 µl plasma + 50 µl AP</td>
<td>22.7</td>
<td>29.0</td>
<td>40.6</td>
<td>86.3</td>
<td>18.4</td>
<td>32.7</td>
</tr>
<tr>
<td>Calculated*</td>
<td>24.4</td>
<td>29.1</td>
<td>40.1</td>
<td>89.6</td>
<td>16.5</td>
<td>31.7</td>
</tr>
<tr>
<td>150 µl whole blood + 50 µl AP</td>
<td>23.3</td>
<td>26.2</td>
<td>30.8</td>
<td>57.4</td>
<td>14.7</td>
<td>24.9</td>
</tr>
<tr>
<td>Calculatedb</td>
<td>24.1</td>
<td>26.6</td>
<td>32.4</td>
<td>58.3</td>
<td>15.3</td>
<td>23.2</td>
</tr>
<tr>
<td>Cell fraction</td>
<td>0.02</td>
<td>0.2</td>
<td>0.7</td>
<td>3.5</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

We added 50 µl plasma with a high Lp(a) concentration to 150 µl whole blood and to 150 µl plasma of six control subjects (A-E). The added amount of Lp(a) was completely recovered in the whole blood as well as in the plasma sample. Abbreviations are: AP, added plasma; ND, not determined.

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b Calculated with the formula: 
((150 * [whole blood]) + (50 * [AP])/200

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![Fig. 1. Schematic illustration of the effect of hematocrit level on the measurement of a parameter (shown as black dots), which is homogeneously distributed in the plasma fraction after centrifugation. In the event of a decreased hematocrit value, a markedly lower plasma concentration will be measured compared to a subject with the same whole blood concentration but normal hematocrit value.](image-url)
We then measured Lp(a) in plasma as well as whole blood samples from 15 hemodialysis patients with low hematocrit values (0.29 ± 0.02) in comparison to 11 control subjects with normal hematocrit (0.45 ± 0.04). Lp(a) plasma concentrations were 27% higher in patients than in controls (19.7 vs. 15.5 mg/dl). The absolute values of Lp(a) were markedly lower when measured in whole blood of patients and controls (13.5 vs. 8.5 mg/dl) since Lp(a) was then distributed in a larger volume. The relative difference, however, was twice as high (59%) when measured in whole blood (Fig. 2).

In addition, whole blood levels of Lp(a) were also calculated with the formula \[Lp(a)_{\text{plasma}} \times (1 - \text{hematocrit})\]. Measured and calculated values correlated very well \(r = 0.99; \text{Fig. 3}\) with a mean relative difference of 4.4 ± 5.6%. The measured mean concentration, however, was slightly lower than the calculated one: 11.4 versus 11.7 mg/dl in the combined group of patients and controls. This can be explained by the small amount of Lp(a) remaining in the cell fraction due to the “mild” low-speed centrifugation procedure.

Finally, we calculated the whole blood values of Lp(a) in a group of 125 hemodialysis patients with a mean hematocrit value of 0.34 ± 0.07. Similar to an earlier study [7], in hemodialysis patients we observed 38% higher Lp(a) plasma concentrations than in the control group (25.4 ± 28.5 vs. 18.4 ± 22.8 mg/dl). The calculated relative difference in the whole blood concentrations of the same two groups, however, was 70% (16.8 ± 19.6 vs. 9.9 ± 12.4 mg/dl), and therefore nearly twice as high as the relative difference for measured plasma concentrations.

**DISCUSSION**

In this investigation we describe a strong influence of the hematocrit value on lipoprotein concentrations measured in plasma, using Lp(a) as an example. We clearly demonstrate that subjects who have identical plasma concentrations of Lp(a) have markedly different whole blood concentrations in the case of different hematocrit values. This can be explained by the nearly exclusive distribution of Lp(a) and other lipoproteins in the plasma fraction after low-speed centrifugation. In patients with a low hematocrit level, lipoproteins are found in a larger plasma fraction after centrifugation, and therefore show a lower plasma concentration when compared to a subject with normal hematocrit and the same content of Lp(a) in whole blood (Fig. 1). Unfortunately, no study is available, to our knowledge, which systematically investigated whether the whole blood or the plasma concentrations of lipoproteins are...
more decisive for the pathophysiological processes related to lipoproteins. Since the vessel wall is in contact with whole blood and not with the isolated subsystems of plasma or cells, it is highly conceivable that the concentrations measured in whole blood are more informative or predictive for the atherogenic potential of lipoproteins than those measured in plasma.

It was not the intention of this study to investigate whether the concentration of Lp(a) is regulated by the plasma or the whole blood concentration, which would definitely require the use of other methods. Our study questioned the methodological aspects of lipoprotein measurements and the consequences of such measurements in clinical and epidemiological investigations, especially when examining patients with renal disease.

Our findings demonstrate that there is a need to consider hematocrit values in case-control studies investigating patients with renal disease who often have markedly reduced hematocrit levels. Otherwise, these studies may markedly underestimate Lp(a). We emphasize that Lp(a) serves only as an example for a general problem in clinical chemistry. The results can be extrapolated at least to all parameters like lipoproteins or apolipoproteins, which are distributed only in the plasma or serum fraction after centrifugation. We believe that reports on decreased or normal total and LDL cholesterol levels, decreased HDL cholesterol levels [15], or increased apolipoprotein A-IV concentrations [7] in hemodialysis patients should be interpreted in the light of these observations.

In consequence, longitudinal studies measuring concentrations before and after a treatment (illness) must control for possible changes of hematocrit levels caused by the treatment (illness). This is especially true for surgical interventions where the patient may have homeostatical changes or when using drugs that affect the hematocrit directly, where hematocrit must be considered to be a confounding variable when lipoproteins are measured longitudinally in plasma. However, even previous studies investigating drugs that intentionally affect the hematocrit, such as erythropoietin, did not consider the influence of hematocrit changes on the measured lipoprotein concentrations in plasma [16]. The same principle can at least partially explain the 20% increase of proteins and lipoproteins during a hemodialysis session [17]. By therapeutic elimination of the volume overload these plasma components become more concentrated, which is accompanied by a similar increase in hemoglobin.

Finally, it should be pointed out that the hematocrit measurement can be ignored in case-control studies where there are no differences in hematocrit levels between groups or in longitudinal studies when no changes of hematocrit occur.

Our findings are supported by the observation that low molecular weight anticoagulants, such as fluoride, citrate or oxalate, exert osmotic effects that shift rather large amounts of water from erythrocytes to plasma [18]. This results in an artificially-low hematocrit level and dilutes plasma constituents such as cholesterol [19]. Furthermore, posture is an in vivo observation that influences plasma concentrations: when a standing subject sits down or reclines, water is redistributed between the vascular and extravascular compartments. This increases the intravascular volume and thus dilutes the concentrations of nondiffusible components, such as cholesterol [20]. These changes in hematocrit are, however, relatively low in comparison to those occurring in patients with end-stage renal disease.

In principle, the underlying phenomenon for our observation has already been described as “volume displacement effect” [21, 22], which means that deproteinization of serum eliminates the volume fraction of proteins and distributes the remaining small molecular weight constituents in a smaller volume, thus making them more concentrated. Our observation demonstrates the same effect in another system (whole blood) and for a high molecular weight lipoprotein. Although the principle of this phenomenon was already described 60 [21] and 30 years [22] ago, most clinical studies still do not consider this confounding effect [16]. On the other hand, for most epidemiological case-control studies it is uncontested that groups must be comparable for age and sex; otherwise, age and sex are handled as confounding variables. Hematocrit, which can have a very strong influence on the measured parameters, is ignored, even when there is a well known difference between the groups, as is true in renal patients.

Our findings clearly demonstrate that hematocrit levels have a strong influence on the measurement of lipoproteins. Therefore, epidemiological studies in patients with abnormal hematocrit levels or studies investigating longitudinal changes of lipoproteins should consider hematocrit as confounding variable.

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REFERENCES


