INTRODUCTION

Carbohydrate-deficient transferrin (CDT) is considered the most specific biochemical marker for detecting chronic alcohol abuse and for monitoring abstinence during treatment (Arndt, 2001; Golka et al., 2004; Salaspuro, 1999). Moreover, CDT may be useful in early identification of surreptitious alcohol abuse in pregnant women and therefore in possibly preventing foetal alcohol syndrome (FAS) and foetal alcohol effects, which are otherwise permanent and irreversible (Cook, 2003). However, although widely used in clinical and forensic medicine, much controversy exists regarding which CDT glycoform should be measured, how to express the results and which analytical method should be preferred (Bianchi et al., 2008).

Contrasting data are also available in the literature on the diagnostic accuracy of CDT during pregnancy. While some authors have shown an increase of CDT values as a function of gestational age (Stauber et al., 1996a), others have indeed demonstrated a negative correlation, potentially leading, at least theoretically, to both false-negative or false-positive results in pregnant women with alcohol abuse. These differences may depend in part on how CDT was evaluated and expressed. Here, we report on variations of CDT levels in pregnant women using the high performance liquid chromatography (HPLC) candidate reference method. METHODS: Alamine aminotransferase, aspartate aminotransferase, gamma-glutamyltransferase, mean corpuscular volume, serum transferrin, urine and serum ethyl glucuronide and CDT were measured in 64 women, self-reporting as non-alcohol abusers (age: median 34, IQR: 28–38), at different stages of normal pregnancy (gestational weeks: median 28, IQR: 8–33). CDT was expressed as percentage of disialotransferrin to total transferrin (%CDT). Results: Transferrin was associated with both CDT (r = 0.66; P < 0.001) and gestational week (r = 0.68; P < 0.001). Interestingly, %CDT was highly correlated with gestational week (r = 0.77; P < 0.001), even after controlling for the effect of transferrin. Moreover, statistically significant differences in %CDT were also evident between women grouped for pregnancy trimester (first trimester: mean 1.01% (SD 0.19); second trimester: 1.30% (SD 0.14); third trimester: 1.53% (SD 0.22); ANOVA P < 0.001). Trend analysis confirmed a proportional increase of %CDT along with pregnancy trimesters (P < 0.001).

Conclusions: %CDT, measured with the HPLC candidate reference method, is independently associated with gestational week. Differently from what has been previously reported or expected, the relationship between pregnancy and CDT could be more complex. The diagnostic accuracy of CDT for detecting alcohol abuse in a legal context may be limited in pregnant women and the effect of gestational age should be considered.

MATERIALS AND METHODS

Subjects and samples

The blood and urine samples from 64 pregnant women were collected at the outpatient department and analysed at the Regional Reference Laboratory of Toxicology of SS. Antonio e Biagio e C. Arrigo Hospital in Alessandria (Northern Italy). Blood samples were collected in Becton Dickinson Vacutainer Plastic Serum tubes (CDT, Ethyl glucuronide and clinical chemistry parameters) and K$_2$EDTA tubes (blood cell count). After clotting, serum was separated by centrifugation at 3500 g for 5 min and immediately analysed or stored at −20°C until further CDT analysis. Urine samples were collected in sterile plastic tubes. After
centrifugation at 3500 g for 5 min, a clear aliquot was immediately processed or stored at −20°C until EtG analysis. All women were interviewed about their age, gestational week and their drinking habits by trained medical personnel. The study was planned according to the guidelines of the local ethical committee in conformity with the principles of the Declaration of Helsinki.

Methods
%CDT was measured using the original method published by Helander (Helander et al., 2003). Briefly, we saturated 100 µl of serum with iron by adding 20 µl of ferric nitrolitratetic acid (final concentration 1.7 mmol/l). After the addition of 20 µl of dextran sulfate (20 g/l)-CaCl₂ (1 M) solution (equal volumes), samples were left in the cold (4°C) for 30–60 min and then centrifuged at 3500 g at 5°C for 5 min. Then 100 µl of the clear supernatant was diluted with 400 µl of water and then transferred to glass HPLC vials; 200 µl of this was injected into an Agilent 1200 HPLC system with a SOURCE® 15Q PE 4.6/100 anion-exchange chromatography column (GE Healthcare Bio-Science, Uppsala, Sweden) kept at 25°C, using a ternary buffer gradient, as reported by the authors, with a flow of 1.0 ml/min, detection at 470 nm and a total run time of 40 min. Transferrin glycoform peaks were measured by baseline integration, using the Chemstation software Rev. B. 03.01, with CDT expressed as a percentage of DST (%DST) to the total transferrin area. Although in previous publications CDT indicated collectively a group of minor glycoforms of human transferrin with a lower degree of glycosylation (asialo-, monosialo- and DST), with %CDT used to express the relative amount of these glycoforms to total transferrin, throughout this article, according to the IFCC document, when reporting data in text, tables and figures, %CDT has been expressed as the ratio between the single DST glycoform and total transferrin (indicated also as %DST). In this regard, it should be noted that %DST was directly calculated by chromatogram integration (DST area divided by total transferrin area) and not using transferrin concentration measured by ADVIA 2400.

Two sets of controls (low control: 0.7–2.1%; high control: 2.6–4.4%) supplied by Bio-Rad (Hercules, CA USA) were run as triplicates, and always found within the range declared by the manufacturer. External quality assessment was performed by taking part in the GTFCh (Gesellschaft für Toxikologische und Forensische Chemie) proficiency test formed by taking part in the GTFCh (Gesellschaft für Toxikologische und Forensische Chemie) proficiency test and EQUALIS (External Quality Assurance in Laboratory Medicine in Sweden).

Ethyl glucuronide in serum and urine was determined by the DRI-EtG EIA kit (Microgenics-Thermo Fischer, I.L., Milan, Italy) on an ADVIA 2400 apparatus (Siemens Healthcare, Milan, Italy) in standalone mode (Bianchi et al., 2009). Analytical performances were evaluated by taking part in the GTFCh proficiency test. A cut-off concentration of 0.5 mg/l was used for reporting elevated EtG (Bottcher et al., 2008). The limit of detection of the method was 0.1 mg/l.

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT) and total transferrin were measured on ADVIA 2400 (Siemens Healthcare, Milan, Italy) with its own kit based on IFCC recommendations. The mean corpuscular volume (MCV) was assessed by an ADVIA 2120 apparatus (Siemens Healthcare, Milan, Italy).

Statistical analysis
Statistical analyses and graphs were performed by SPSS statistical software v. 15.0 (SPSS Inc., Chicago, IL, USA), R software v. 2.11.0 (R Foundation for Statistical Computing) and Statistica v. 7 (StatSoft Inc., Tulsa, OK, USA). Continuous variables were reported as the median value and the inter-quartile range. Normality distribution was assessed by the Kolmogorov–Smirnov and the Shapiro–Wilks test, and visually by a histogram and kernel density plot. Differences between groups were estimated by the analysis of variance (ANOVA) and by post hoc multiple comparison tests (Bonferroni). The homogeneity of variances assumption was verified by applying the Levene test. Associations between CDT, %CDT, transferrin and gestational week were evaluated by bivariate analysis (Pearson’s correlation) and partial correlation. Trend analysis was conducted using one-way ANOVA polynomial contrasts (linear degree).

RESULTS
AST, ALT, GGT and MCV were, respectively, 19 IU/L (IQR: 16–23), 15 IU/L (IQR: 12–21), 8 IU/L (IQR: 5–11) and 86.5 fl (IQR: 82.6–90.4; Table 1). Recent alcohol consumption was excluded in all women by the fact that EtG in serum and urine was negative or below the limit of detection. Absolute CDT (concentration of DST), %CDT (%DST) and transferrin were, respectively, 5.2 mg/dl (IQR: 3.6–6.7, min–max: 1.3–10.2), 1.4% (IQR: 1.1–1.6%, min–max: 0.5–2.0%) and 378 mg/dl (IQR: 310–424, min–max: 221–681; Table 1).

Transferrin values were associated with both absolute CDT (r = 0.89; P < 0.001), %CDT (r = 0.66; P < 0.001) and gestational week (r = 0.68; P < 0.001; Fig. 1). Interestingly, %CDT was highly correlated with gestational week (r = 0.77; P < 0.001; Fig. 1); accordingly, the significant correlation between absolute CDT and gestational week remained even after controlling for the effect of transferrin (partial correlation r = 0.61; P < 0.001). Moreover, statistically significant differences in %CDT were also evident between women

Table 1. Age details of the pregnancy and laboratory data of the 64 women investigated

<table>
<thead>
<tr>
<th>Variable (units)</th>
<th>Statistics Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/pregnancy details</td>
<td></td>
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<tr>
<td>Age (years) median, IQR</td>
<td>34, 28–38</td>
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<tr>
<td>Gestational age (weeks) median, IQR</td>
<td>28, 8–33</td>
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<tr>
<td>Gestational age (trimester)</td>
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<tr>
<td>I n (%)</td>
<td>22 (34)</td>
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<tr>
<td>II n (%)</td>
<td>6 (10)</td>
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<tr>
<td>III n (%)</td>
<td>36 (56)</td>
</tr>
<tr>
<td>Laboratory characteristics</td>
<td></td>
</tr>
<tr>
<td>Aspartate aminotransferase (AST), IU/L</td>
<td>19, 16–23</td>
</tr>
<tr>
<td>Alanine aminotransferase (ALT), IU/L</td>
<td>15, 12–21</td>
</tr>
<tr>
<td>Gamma-glutamyltransferase (GGT), IU/L</td>
<td>8.5–11</td>
</tr>
<tr>
<td>Mean corpuscular volume, (fL)</td>
<td>86.5, 82.6–90.4</td>
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<tr>
<td>Total transferrin, (mg/dl)</td>
<td>378, 310–424</td>
</tr>
<tr>
<td>Serum ethyl glucuronide (mg/l)</td>
<td>median, IQR &lt;0.1</td>
</tr>
<tr>
<td>Absolute CDT (mg/dl)²</td>
<td>median, IQR 5.2, 3.6–6.7</td>
</tr>
<tr>
<td>Relative CDT (%CDT) (%)</td>
<td>median, IQR 1.4, 1.1–1.6</td>
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</table>

*CDT and %CDT indicate, respectively, absolute and relative disialotransferrin amount.
grouped for pregnancy trimester (first trimester: mean 1.01% (SD 0.19); second trimester: 1.30% (SD 0.14); third trimester: 1.53% (SD 0.22); ANOVA \( P < 0.001 \); Fig. 2). Mean %CDT differences between trimester groups, estimated by the Bonferroni post hoc test, were (difference and 95%CI): III vs. II 0.23% (95%CI: 0.01–0.45; **\( P = 0.037 \); III vs. I 0.52% (95%CI: 0.38–0.65; *\( P < 0.001 \); II vs. I 0.29% (95%CI: 0.06–0.52; ***\( P = 0.009 \)).

**DISCUSSION**

Despite the increasing use of CDT in the clinical and forensic setting, few and contrasting data have been published in recent years on the diagnostic accuracy of CDT in pregnant women. The positive trend between absolute CDT values and duration of pregnancy, reported initially by Harlin et al. (1994) and Stauber et al. (1996a), has been subsequently ascribed to the general increase in total transferrin observed during pregnancy (Harlin et al., 1994; Stauber et al., 1996a). Indeed, the same author, in reply to Grimsrud (1997), has otherwise shown a decrease of CDT values when normalized for total transferrin (relative CDT values or %CDT; Grimsrud, 1997; Stauber et al., 1996a, 1997). Based only on these clinical data and on the observation that highly sialylated glycoform account for most of the transferrin augmentation in pregnant women (de Jong et al., 1992), it is today expected, as also reported in authoritative reviews (Arndt, 2001; Helander et al., 1998), that relative CDT values (%CDT) should gradually decline with increasing gestational age, thus potentially leading to false-negative results in pregnant women with alcohol abuse. However, it is remarkable that in most of the published studies on the effect of iron status, oestrogens and pregnancy, CDT was expressed mainly as an absolute concentration or evaluated with analytical techniques which were later abandoned because of poor accuracy and specificity (Stauber et al., 1996a).

Recently, in an effort to overcome the lack of standardization still affecting CDT analysis (Bianchi et al., 2008), the IFCC-WG-CDT has suggested the use of DST as the primary target molecule for CDT testing, measured by HPLC and expressed as a relative amount (%CDT) to compensate for variable levels of total transferrin concentration (Jeppsson et al., 2007). Although several commercial %CDT methods by HPLC are now available (Bianchi et al., 2010; Helander and Bergstrom, 2006), in this study, we preferred to use the HPLC method developed by Helander and Jeppsson, which was recommended as the best interim reference method to be applied in CDT confirmatory analysis (Jeppsson et al., 2007) and for use in method comparison.
studies to evaluate new analytical techniques for CDT testing.

Interestingly, our data do not confirm previous reports, showing otherwise an increasing trend of %CDT along with gestational age. The reason for these findings is not clear at the moment but several considerations are possible. Despite denial of drinking and negative EtG values both in serum and urine, discontinuous alcohol intake cannot be definitely excluded. However, it is quite evident that the positive correlation between %CDT and gestational age cannot be ascribed to misreported alcohol consumption. Indeed, a few patients with high alcohol intake, and thus high %CDT, do not account for a trend, but solely for an increase in variability, even more if considering that the sample investigated does not contain %CDT outliers, which could falsely affect regression or correlation coefficients. Moreover, speculating on false reports, it is quite unlikely an increase in the alcohol intake during pregnancy for most of the women investigated, since it is reasonably expected that a pregnant woman will decrease her alcohol consumption along the weeks or, at least, keep it as constant as before (Cook, 2003; Littner and Bearer, 2007). The positive %CDT–gestational age trend cannot be explained by the transferrin increase, as confirmed by partial correlation and considering that in this study CDT was expressed as relative disialotransferrin (%DST).

The huge variability observed in the sample suggests instead that many factors could lead, with different contributions, to a net increase in %CDT values during pregnancy. The identification of these factors is beyond the scope of this article; however, our data indicate that besides partly identified pregnancy-independent factors (method variability, alcohol consumption, transferrin variants and diseases), others, directly related to pregnancy, may influence %CDT (iron deficiency or supplementation, oestrogen levels). Indeed, the extent by which only gestational age increases %CDT should be evident when considering the coefficient of determination ($r^2$), obtained by squaring the coefficient of correlation between %CDT and gestational week ($r = 0.77$), which indicates that up to 59% ($0.77^2 = 0.59$) of the variance of %CDT can be explained by gestational age-related factors. Among them, an important role for oestrogens and iron has been previously suspected. Although their effect on %CDT has been evaluated in several studies, no definitive and convincing conclusion has been drawn.

Although pre-menopausal women were shown to exhibit significantly higher CDT values than post-menopausal women (Gronbaek et al., 1995; Stauber et al., 1996b), and despite increased levels of CDT observed in women taking oral contraceptives (La Grange et al., 1995), subsequent studies were unable to demonstrate a close correlation between CDT and sex steroids (Stauber et al., 1996a).

Anton and Moak (1994) suggested that iron deficiency might lead to a compensatory increase in transferrin and hence CDT, possibly explaining higher absolute CDT values observed in the female population in the fertile age (Anton and Moak, 1994). Conversely, Stauber found no correlation between CDT and serum iron in pregnant women (Stauber et al., 1996b).

Recent HPLC studies have reported that the serum %DST level is not markedly influenced by gender, clearly showing also that previously false-positive CDT values, related to physiological and pharmacological factors, were otherwise the result of unspecific methodologies for CDT analysis (Bergström and Helander, 2008). Since no study is available where the effect of oestrogens and iron on %CDT has been evaluated by a confirmatory method, their contribution to CDT in pregnant women cannot be discarded. Moreover, since little is known on the exact mechanisms by which alcohol increases CDT in chronic abusers, from a theoretically point of view, it is possible that other pregnancy-related factors, presently unknown, may play a role in CDT increase.

Although CDT increased along gestational weeks, no pregnant woman included in this study displayed a %CDT value higher than the cut-off (2.0%) generally used in our laboratory to detect chronic alcohol abuse for traffic or legal medicine. These data suggest that significant ethanol intake (>20 g/day) will likely increase CDT to a larger extent than the variations observed during late pregnancy. However, our findings indicate that the diagnostic accuracy of CDT for detecting alcohol abuse may be limited in pregnant women and that the effect of gestational age should be considered for women having CDT close to the cut-off, particularly in a legal medicine context where positivity to %CDT testing could translate into personal freedom restriction. Indeed, based on this study, a low–moderate alcohol consumption in late pregnancy could theoretically increase %CDT above the legal cut-off value. More studies are needed with larger sample sizes to establish the population distribution of %CDT levels and to allow calculation of a more appropriate cut-off, to minimize the risk for false-positive results among pregnant women.

Detecting alcohol use among pregnant women represents an important step towards preventing alcohol-related birth defects; however, problems arise in the selection of suitable biomarkers which could allow earlier identification and hence intervention. Besides alcohol abuse, even low–moderate alcohol consumption and binge drinking have been associated with an increased risk of spontaneous abortion, stillbirth and infant mortality (Cook, 2003; Littner and Bearer, 2007; Streissguth et al., 1990). %CDT may be useful in detecting heavy alcohol intake, even in the presence of the confounding increasing trend shown here; however, in the authors’ opinion, %CDT does not have sufficient sensitivity to detect moderate alcohol consumption, when risk or even damage could be already present. To our knowledge, no study has been conducted on the comparison between %CDT, measured according IFCC recommendations, and other alcohol biomarkers in detecting pregnant women with low–moderate alcohol intake. From a speculative point of view, a reasonable diagnostic strategy could be ordering %CDT (baseline, 3rd month, 6th month), followed by serial monitoring by urinary EtG when %CDT is found initially elevated or further increased beyond the trend shown here (Fig. 2) or beyond method variability. Recent studies (Hoiseth et al., 2009; Morini et al., 2009) have suggested that ethyl glucuronide in hair correlates better with alcohol intake and displays higher sensitivity than %CDT; however, these promising data must be confirmed, in the general population and in a context of FAS diagnosis, by studies with a larger sample size, where EtG is compared with CDT measured by HPLC, using accurate statistics and non-arbitrarily chosen CDT and EtG cut-offs.
In conclusion, this study shows a positive correlation between gestational age and %CDT, measured by a confirmatory method and expressed as relative values (%DST). These data suggest that, in contrast to what has been previously reported or expected, the relationship between pregnancy and CDT could be more complex. Moreover, when assessed in a legal context elevated CDT during pregnancy should be carefully evaluated and possibly integrated with serial monitoring of EtG in urine.

REFERENCES


