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# Quantitative Estimation of Abnormal Microheterogeneity of Serum Transferrin in Alcoholics

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STIBLER, H., O. SYDOW AND S. BORG. *Quantitative estimation of abnormal microheterogeneity of serum transferrin in alcoholics.* PHARMAC. BIOCHEM. BEHAV. **13**: Suppl. 1, 47–51, 1980.—A qualitative abnormality of the microheterogeneity of serum transferrin, demonstrated by isoelectric focusing, has previously been shown to be a highly specific indication of chronic alcoholism. The abnormality consists of a selective increase of a cathodal transferrin component which is probably caused by a reduction of the sialic acid content. The present study describes a method for quantitative estimation of the abnormal transferrin. The technique was based on analytical isoelectric focusing as the first step followed by direct immunofixation. The immunofixed transferrin was quantified by computerized on-line densitometry, and the transferrin abnormality was calculated as a quotient, where the amount of the cathodal component was expressed as a percentage of the relative total immunofixed transferrin quantity. This method was shown to possess high sensitivity and good reproducibility. In the controls the mean value of the quotient was 3.7%, while in the alcoholics it was 9.5% which was a highly significant difference ($p<0.001$). The possible functional significance of a disturbed sialic acid metabolism in alcoholism is discussed.

Alcoholism Transferrin Isoelectric focusing Immunofixation Densitometry Sialic acid

A qualitative abnormality of serum transferrin has recently been described in connection with chronic alcoholism [19, 20, 21]. Transferrin thereby changes its microheterogeneity with a selective and often marked increase of mainly one of its normally minor cathodal components. This protein alteration has a high specificity and sensitivity to prolonged alcohol consumption [20]. To demonstrate this phenomenon, it has so far been necessary to employ isoelectric focusing, since this method has the resolving power to allow separation of components with extremely small charge differences [27]. The abnormal transferrin has an isoelectric point (pI) of 5.7 and differs from the normal main component by only 0.3 pH unit [20]. This difference is most probably due to the loss of two sialic acid residues [12, 17, 20].

Isoelectric focusing with conventional staining of the serum proteins is, however, not adequate for evaluating the transferrin heterogeneity quantitatively, mainly due to the presence of other proteins at the same position or in close proximity to transferrin e.g. IgA and C'3 complement [17,20]. Elevated lipoproteins, commonly found in alcoholics [5] may be another source of error [20].

Immunofixation following isoelectric focusing offers the conditions required for specific identification of proteins coupled with excellent resolution [1, 3, 6]. Immunofixation has also been shown to be very well suited for subsequent quantitative evaluation of proteins in agarose as well as polyacrylamide gels [7,15]. Direct immunofixation has previously been employed for qualitative demonstration of the transferrin change in alcoholics [20]. This paper describes a computerized densitometric method for the calculation of a quotient of the immunofixed transferrin components following isoelectric focusing in polyacrylamide gel in alcoholic patients and controls.

## STUDY POPULATION

### *Controls*

The controls consisted of 28 persons, 19 males within the age range 20–66 years, mean 39 years, and 9 females within

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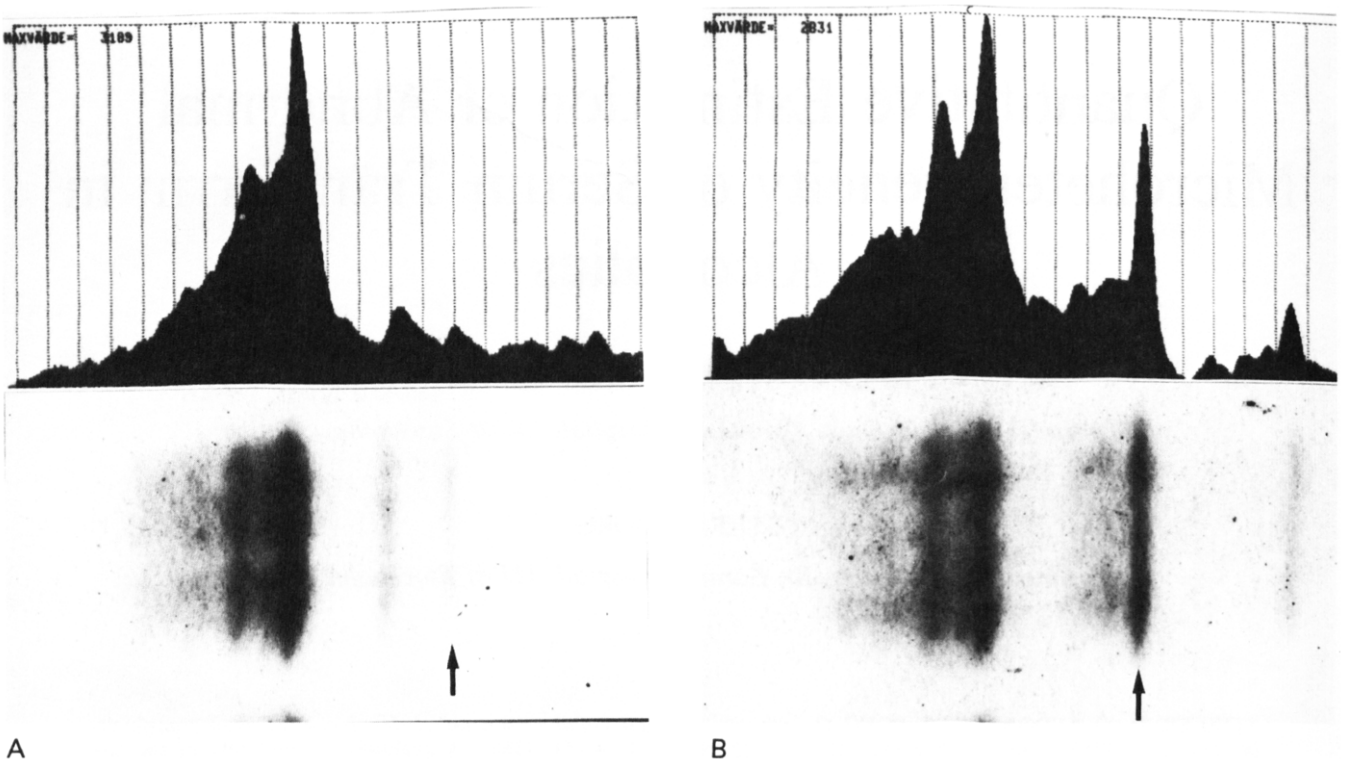

FIG. 1. Densitometric tracings (top) of the serum transferrin pattern after direct immunofixation following isoelectric focusing (bottom) of (A): a control sample with a "transferrin quotient" of 4% and (B): a sample from an alcoholic patient with a "transferrin quotient" of 11%. The quotient was calculated as the peak area of the component with pI 5.7 (indicated by arrows) relative to the total transferrin area. The anode is to the left.

the age range 21–63 years with a mean age of 39 years. All were considered healthy on the basis of medical history and physical examination. In no instance was any regular, excessive alcohol consumption suspected.

*Alcoholic Patients*

These were 31 individuals of which 22 were males within the age range of 27–59 years, mean age 46 years, and 9 were females 27–57 years of age, mean age 38 years. The patients were treated at the Clinical Department of Alcohol and Drug Research, Karolinska Hospital, Stockholm and all fulfilled the criteria of dependence according to WHO [28]. The duration of alcohol abuse ranged from 1 to 23 years with a mean of 14 years. The admitted average consumption was in all cases more than 60 g ethanol/day [20]. The subjects' medical histories contained reports of delirium tremens in 5 instances and epileptic fits in 10. Three patients had signs of liver cirrhosis and one had undergone gastroenteroanastomosis surgery. Fourteen subjects had regular jobs and 3 were homeless.

## METHOD

*Sampling*

Serum samples were taken in the morning with the subjects fasting. In the case of the alcoholic patients, the samples were taken on the first day following admittance to the hospital. The samples were frozen immediately at −23°C and stored for a maximum of two months before analysis [19].

*Single Radial Immunodiffusion*

All samples were analyzed for total transferrin concentration by means of single radial immunodiffusion according to Mancini *et al.* [10]. M-Partigen immunodiffusion plates (Behringwerke, Marburg, West Germany) were used. Standard curves were plotted and the reproducibility was determined according to the manufacturer's instructions using Standard Human Serum (Behringwerke).

*Isoelectric Focusing*

Analytic isoelectric focusing was carried out in polyacrylamide gel with a gel concentration of T=6% and C=3% essentially as outlined previously [20, 24, 25]. The pH gradient covered the range 2.2–11.0 with an electrode distance of 8.5 cm. The gel dimensions were 0.1×10×20 cm. Each serum sample was diluted with distilled water to a final transferrin concentration of 0.04–0.08 μg/μl depending on the activity of different antibody batches used in the subsequent immunofixation. Ten μl of the samples were applied on 6×1 mm pieces of surgical lint (Robinson, Chesterfield, England) on the surface of the gel midways between the electrodes. Thus, a constant amount of transferrin was analyzed for each antibody batch. Usually not more than 12 samples were examined on one gel. The separations were terminated after 70 min. Maximum voltage and power were 1000 V and 40 W.

*Direct Immunofixation*

The immunofixation step was carried out essentially according to previous descriptions [18,20]. During the last 10 min of isoelectric focusing the temperature of the cooling plate was lowered to +5°C to avoid diffusion upon antibody application. After focusing, with the gel still on the cooling plate, 25 μl/cm² of monospecific rabbit antibodies against human serum transferrin (Dakopatts, Copenhagen, Den-

mark) were applied directly on the gel surface over the anodal half of each sample track [5,17]. The gel was then transferred to a moist chamber and incubated for 60 min at room temperature. The gel was then washed for 2 days to eliminate excess antibodies, nonprecipitated proteins and ampholytes and stained with Coomassie Brilliant Blue R 250 and destained [18]. The specificity of the antibodies had previously been tested by crossed immunoelectrofocusing and comparison with pure human serum transferrin [17, 18, 19].

*Densitometry*

A Vitatron TLD 100 "Flying spot" densitometer (Dieren, Holland) was used. Transmission, using a tungsten light source, was measured at 590 nm, which corresponds to the maximum absorbance of Coomassie Brilliant Blue R 250. The computer was a PDP 11/34 (Digital Equipment Corporation, Maynard, MA, U.S.A.), equipped with an analog-digital converter.

The immunofixed samples were scanned and the total amount of fixed transferrin in each component was measured by using the "flying spot" mode (oscillation amplitude ± 4 mm, light aperture 0.1 mm ∅, scanning speed 1 cm/min). The analog signal from the densitometer was fed, via an amplifier, on line, into the computer. The densitometer and amplifier gain were set to utilize the full dynamic range of the analog-digital converter. In the computer program, a peak detection algorithm detected the peaks in the transferrin pattern and, after base line subtraction, calculated their location, peak area etc. [26]. The computer produced a listing of the results and a plot of the densitometric curve. The data were stored on a disc for future reference.

A quotient of the peak area of the transferrin component with pI 5.7 relative to the total area of immunofixed transferrin was calculated, expressed as a percentage, and called the "transferrin quotient". The component of interest was identified by the distance from the main transferrin band. If cracks of impurities were present in the gel or destaining was uneven within a sample it was reanalyzed on a different gel [26]. The variation of the method was determined by the SD of the quotient from analysis of 3 normal samples on 3 different gels and triplicate runs within the same gel plate. Quantitative linearity was examined by analysis of control serum samples containing 0.2, 0.3, 0.4, 0.5 and 0.6 μg of transferrin.

## RESULTS

*Single Radial Immunodiffusion*

The variation of the method was 2.5% (intraplate as well as interplate variation). In the controls the total transferrin concentration varied from 1.80 to 4.00 g/l with a mean value of 2.81 g/l. In the alcoholic patients the range was 1.80–3.63 g/l with a mean of 2.42 g/l.

*Direct Immunofixation Following Isoelectric Focusing*

With the above-specified pH gradient, normal serum transferrin separated into 7 components, which is equivalent to the resolution obtained by isoelectric focusing alone of pure human transferrin [17,18] (Fig. 1A). In the alcoholic patients, the abnormal transferrin band was found at a pI of 5.7, while the normal main band has a pI of 5.4 [20]. Its position corresponded to the most cathodal of the normal minor transferrin components and was well separated by 2 mm from the next more anodal band (Fig. 1B).

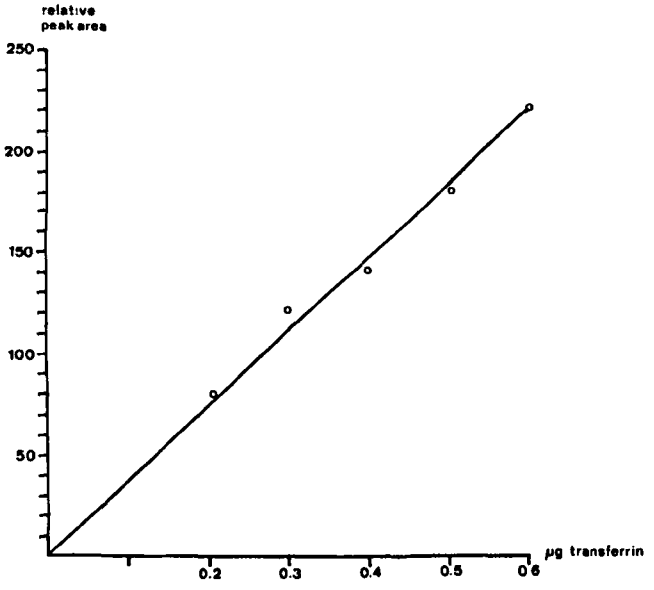


FIG. 2. Plot of the relationship between the relative densitometric peak area of the main transferrin component after immunofixation following isoelectric focusing of 0.2, 0.3, 0.4, 0.5 and 0.6 μg of transferrin from a control person.

Due to the large number of transferrin components containing very different amounts of protein, the optimum total antigen amount had to be determined. Too much transferrin resulted in a clear zone within the main band, which made quantitation impossible, while too little transferrin was insufficient to demonstrate the minor components. It became evident that the activity of different antibody batches differed, although the manufacturer stated the same titer. With the batches used, the appropriate transferrin amount ranged from 0.4 to 0.8 μg. During antibody application and incubation, the gel plates had to be kept horizontal to avoid spreading of the antibody solution outside the desired area, which resulted in antibody deficit. It was also important to carry out antibody application quickly before diffusion of the focused bands started. With 12 samples on the same gel about 5 min were required, during which time diffusion was negligible.

*Densitometry*

The SD of the method was 0.9 for the "transferrin quotient." A linear relationship was revealed between peak area of immunofixed transferrin and the transferrin quantity originally applied, provided that antibody excess prevailed (Fig. 2) [7]. In the controls the "transferrin quotient" ranged from 2 to 6%, with a mean value of 3.7% (SD 1.5). In the alcoholic patients, the range was 3–21% with a mean of 9.5% (SD 3.7) (Fig. 3). The difference between the mean values was highly significant ($p<0.001$). Figure 1 shows 2 representative densitometric tracings. Neither in the controls, nor in the patients could any sex or age difference be detected with this limited material. The 75% percentile for the control persons was < 5%, while for the patients it was > 7%. This corresponds well to the figures obtained previously from qualitative evaluation alone of the transferrin pattern in alcoholics with a daily consumption of 60 g of ethanol or more [20]. On visual assessment of the transferrin heterogeneity in the pa-

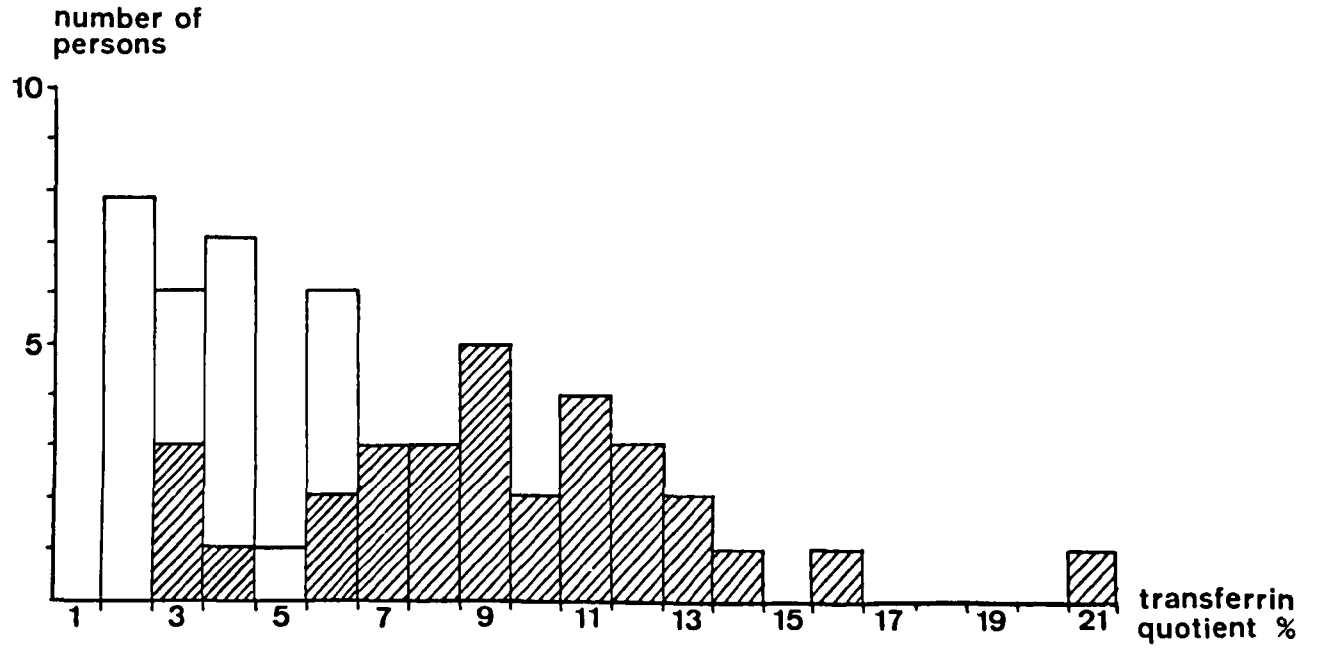


FIG. 3. Histogram showing the distribution of the percentual "transferrin quotient" (see Fig. 1) in controls (open columns) and alcoholic patients (hatched columns).

tients in this investigation, 74% were considered abnormal. When compared to the quantitative data, however, 7% were false negative and 10% false positive.

A quotient of less than 7% was thus obtained for 6 of the alcoholics. Three of these were found to have abstained from alcohol for 5–7 days and 2 had had a relapse for less than one week. It is known that it takes at least 5 days for the abnormality to appear and that it is reversible within 10–14 days of abstinence [20].

The lowest value of the quotient was 2% and with a total applied transferrin amount of 0.6 $\mu$g, this means that 12 ng of transferrin was well detectable. The actual immunofixed amount is probably even lower, since it can be assumed that only the molecules in the superficial part of the gel are fixed by the antibodies during the short incubation time used [7].

## DISCUSSION

Since the abnormal microheterogeneity of serum transferrin in alcoholics seemed to be a promising and highly specific new marker for alcoholism [20,21] a useful quantitative method needed to be developed. The change of the transferrin pattern is purely qualitative and can therefore not be detected by quantitation of the total transferrin concentration alone. By combining isoelectric focusing with direct immunofixation [6,18] followed by computerized densitometry, it was possible to calculate a quotient expressed as a percentage between the cathodal transferrin component of interest and the relative total immunofixed amount of the protein (Fig. 1). This method combined very good sensitivity with high reliability, which could be attributed to the following factors: (1) The high specificity and high titer of the antitransferrin antibodies [7] (2) The sensitivity of Coomassie Brilliant Blue R 250. (3) The good and even destaining of the gels after extensive washing. (4) Calculation of a quotient, which eliminates the error of different degree of staining between the gels [26] and variable activity of the antibodies. (5) Maximum utilization of the densitometer and the analog—digital converter. (6) The small size of the light aperture, permitting separate registration of the very narrow and closely spaced protein bands [26].

There are three main sources of error with this technique. One is that the total amount of transferrin applied is critical. To obtain reliable results, a constant amount should be analyzed and the optimal quantity determined for each antibody batch. The second difficulty is when individuals heterozygous for the rare genetic B or D variants of transferrin are encountered [13]. In this study, no such variant was present, but theoretically the quotient should then be calculated, irrespective of the type of variant, as twice the relative quantity of the most cathodal transferrin component in relation to the total amount. Thirdly, uneven destaining with a sample will give false absorbance values and necessitates reanalysis. The disadvantage with the present method is that it requires 4–5 days from isoelectric focusing to the final results. Most of this time is consumed by the long washing procedure for the immunofixed gels. It is not impossible, however, that the time could be reduced by modification of this step.

Our results showed that up to 21% of the transferrin in the studied alcoholics was present in a form with reduced sialic acid content [20] while in the controls it never exceeded 6% (Fig. 3). The mean value in the patients was nearly 3 times the mean level in the controls ($p<0.001$). With a 75% percentile of > 7% among the alcoholics (controls < 5%) the sensitivity of this abnormality for alcohol abuse was confirmed. A higher percentile could probably have been obtained, if reliable information on alcohol consumption had been available at the time of sampling for 5 of the 6 patients with a "transferrin quotient" of < 7%.

No similar alteration has been found in a considerable number of other sialoproteins in serum from alcoholics [20,21], or in fibrinogen (Stibler, unpublished). Such an effect could, however, not be excluded, since all studied serum

sialoproteins other than transferrin are rapidly eliminated upon sialic acid removal [2].

Whether the sialic acid reduction in transferrin interferes with its function is not yet known. Iron binding capacity and plasma iron exchange are not reduced by sialic acid removal [11]. It might, however, affect its receptor affinity [9] and thereby interfere with iron transport. Studies are currently in progress concerning its possible functional significance, as well as on the mechanism behind its origin. A possible similar effect of ethanol on membrane bound sialocompounds is also being investigated. Some recent experimental data have shown that ethanol interferes with sialic acid metabolism in the nervous system [8,16] which in turn may affect the establishment of normal neuronal pathways, as well as the maintenance of synaptic transmission (see [16] for a review, [4, 22, 23, 29]). Our observation may thus be an indication of an action of ethanol that may be of importance for an understanding of complications of alcoholism.

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