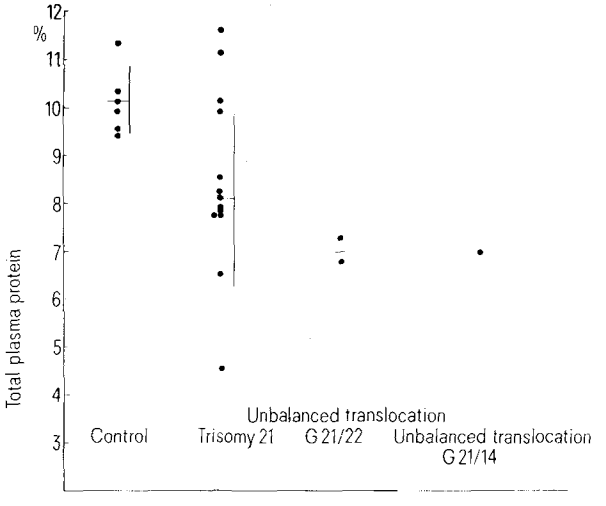
The aim of this investigation was to analyse the ferritin content with respect to the plasma iron concentration in patients with Down syndrome.

Material and methods. Venous blood was taken from fasting donors into heparinised tubes. The 1st group of donors consisted of 13 patients with trisomy 21 aged from 15 up to 23. Brother and sister aged 26 and 25, respectively, constituted the 2nd group. The 3rd group of donors consisted of 6 healthy individuals of similar age. No infectious disease was found during blood testing in all the donors, and the general health status of the patients with Down syndrome was on the borderline of normal.

Blood plasma proteins were separated in 6% polyacrylamide gel, using a Tris-glycine buffer, pH 8.6[6]. 20 µl of plasma diluted with 10% sucrose (1:10) were applied to the gels. The electrophoresis was run for 45 min, at a current of 4 mA per tube. The protein was stained according to Reisner[7]. All samples were run at least in triplicate. The protein fractions on electropherograms were evaluated in a Vitatron TLD-100 densitometer and the percentage of transferrin was estimated in relation to the total serum protein.



Results. The level of transferrin in patients with trisomy 21 was decreased with respect to the control group (8.3±1.8 vs 10.1±0.7% of the total protein, mean ±SD). This difference was statistically significant ($p < 0.05$). Particularly low transferrin levels were observed in patients with translocations 21/22 (6.7 and 7.2%) and 21/14 (6.9%) (figure).

Discussion. In the cases of Down syndrome due to both trisomy 21 and unbalanced translocations (21/22 and 21/14), lowered levels of relative transferrin levels were found. As no significant changes in the total plasma protein exist in Down syndrome[4], these decreases indicate also corresponding changes in absolute ferritin levels. It is interesting to note that a decrease in the plasma iron in trisomy 21 was established in our previous study[5], and even greater decrease in the cases of translocation 21/22. These findings would suggest a possibility of a causative relationship.

An attempt to interprete the low levels of transferrin in Down syndrome on a genetic background would be difficult. The synthesis of transferrin is regulated by a number of allelic genes. Most people have the Tfc/Tfc genotype[8]. A complete lack of transferrin is a very rare phenomenon, described only in few cases[9]. Lowered transferrin levels are not accompanied by clinical symptoms[10]. The observed decrease in the transferrin level in Down syndrome may involve some dependence on the karyotype anomaly and may be due to highly complex disturbances of metabolic processes of protein turnover in this disease.

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Time-qualified reference intervals – chronodesms[1]

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Summary. Methods are presented for qualifying clinical reference intervals, for individuals as well as peer groups, according to circadian and other rhythms, using chronobiologically-defined single samples or time series.

Circadian and other rhythms are known to characterize many variables of clinical interest[9-11,16,21-23] but are almost completely ignored in compilations of 'normal ranges'[6,17,20]. These rhythms may contribute a large fraction of the variability seen in clinical data. Accordingly, diagnosis could be refined if data were time-coded and interpreted against time-qualified reference intervals, herein called 'chronodesms'[2].

The construction of reference intervals qualified by time along a 24-h scale (i.e. of *circadian* chronodesms) will be described but it should be realized that actual evidence on statistically significant rhythms along several time scales in one and the same variable - such as circulating prolactin - is available and, hence, reference intervals may be qualified in this case on the circannual as well as circadian time-scales.

In the simplest case, assume a set of data consisting of single samples obtained from a reasonably homogeneous group of healthy subjects at approximately the same clock hour. (If clock hour is to be used as the time-marker all subjects must be on similar schedules, at least of wakefulness and sleep.) Because the methods to be used assume a normal (Gaussian) distribution of values, the first step is to test this assumption using the Kolmogorov-Smirnov (K-S) test[4]. Values are first ranked in order of increasing magnitude and each *different* value (y_i) is converted to a normalized deviate, Z_i ($Z_i = (y_i - \bar{y})/s$, where $\bar{y}$ = mean of all values and s = standard deviation). The cumulative *expect-*

ed frequency, $\hat{F}_i$, associated with each Z_i-value is obtained from a table of areas under the standard normal curve[5]. The *observed* cumulative frequencies, F_i, are also calculated; i.e. for each y_i the number of values up to and including y_i is divided by the total number of values. The differences, $\hat{F}_i - F_{(i-1)}$, are then determined ($F_0 = 0$) and the maximal difference (d_{max}) - regardless of sign - is compared with a table of critical values for the K-S statistic[4]. If d_{max} is less than the 5% critical value for the size of sample investigated, the null hypothesis (values are normally distributed) is accepted. If the test indicates nonnormality, all values should be transformed (e.g. by taking logarithms or square roots) and again tested.

Given an approximately normally distributed set of data (with or without transformation), a reference interval can be calculated as

$$\bar{y} \pm Ks, \tag{1}$$

where $\bar{y}$ = mean, s = standard deviation and K = multiplier determined by sample size and the kind of reference interval desired. One may construct a 'prediction interval'[8] as one that will include a fixed number of future observations with a desired probability (confidence). Less restrictive is a 'tolerance interval'[5] that will include a specified proportion of the population of values, with a stated confidence[26]. Values of the multiplier K for either kind of reference interval can be obtained from tables in the respective references. If original values have been transformed in order to achieve normality, the calculated reference limits can be converted back to original units for subsequent comparisons.

As an illustration of this first approach to the construction of chronodesms, data on total leukocyte count obtained from 30 healthy young men at approximately 09.00 were subjected to the above methods. The values were normally distributed (K-S test) with a mean of 5877 cells/mm^3 and standard deviation of 1040. A tolerance interval to include 90% of the population with 90% confidence was computed as:

$$5877 \pm (2.025)\ (1040) = 3771 - 7983 \text{ cells/mm}^3.$$

A value outside this interval, if obtained from a comparable subject at about 09.00, could be considered a probable danger signal.

This method could be used to determine reference intervals at any timepoint for which appropriate reference data (single-sample distributions) are available. An alternative is to construct reference intervals based, not just on values obtained at a given time, but on a model describing *all* of the data recorded as a function of time (i.e. along a 24-h scale). The advantages of this alternative approach (assuming an appropriate model) are a) that reference data can be coded with the actual time of sampling and need not be constrained to arbitrary times; and b) that evaluation of future data need not be restricted to the times at which reference samples were actually obtained. In this case the data could consist either of single samples from each member of a reference group (with which any similar subject can later be compared) or of a time series of data from a single subject (serving as a baseline for future assessments of that same subject).

Many rhythms have satisfactorily approximated the cosine model

$$y_i = M + A\cos(\omega t_i + \phi) + e_i, \tag{2}$$

where y_i = measurement at time t_i; M = mesor (average value of cosine curve); A = amplitude (half of difference between minimum and maximum of cosine curve); ω = angular frequency (degrees per unit time, with 360° = complete cycle; i.e. 24 h); ϕ = acrophase (timing of maximum in cosine curve, as a lag given in degrees or time from a selected reference time - e.g. midnight); and e_i = residual (error).

Equation (2) can be rewritten as

$$y_i = M + \beta x_i + \gamma z_i + e_i, \tag{3}$$

where $\beta = A\cos\phi$, $x_i = \cos\omega t_i$, $\gamma = -A\sin\phi$ and $z_i = \sin\omega t_i$. The least squares method[16] can now be used to fit this linear model to the data and obtain estimates for M and the substitute parameters, β and γ. The latter can be converted to estimates of A and ϕ on the basis of the relations given above.

The following computations presuppose that the residual errors e_i are independent random normal deviates with zero mean and a common variance. The K-S test described above can be used to determine whether the residuals are normally distributed about the fitted curve. Before proceeding to the computation of reference intervals, the validity of the cosine model should be examined with a zero-amplitude test[16].

An extension of the method of Wallis[25] is then used to compute a tolerance interval about the fitted model at any time t_I[3]. The predicted value, $\hat{Y}_I$, of the rhythmic variable at time t_I is first computed as:

$$\hat{Y}_I = \hat{M} + \hat{\beta} x_I + \hat{\gamma} z_I, \tag{4}$$

where hats (ˆ) are used to denote estimates.

Equivalently, equation (4) can be rewritten as

$$\hat{Y}_I = \bar{y} + \hat{\beta}(x_I - \bar{x}) + \hat{\gamma}(z_I - \bar{z}), \tag{5}$$

where $\bar{y} = \sum_{i=1}^{n} y_i/n$, $\bar{x} = \sum_{i=1}^{n} x_i/n$ and $\bar{z} = \sum_{i=1}^{n} z_i/n$, with n = total number of values in the actual data analyzed.

The tolerance interval at time t_I is given by

$$(\hat{Y}_I - K_I s,\ \hat{Y}_I + K_I s), \tag{6}$$

in which the factor K_I is determined such that

$$P[W_I \geq p] = 1 - \alpha, \tag{7}$$

where p = specified proportion of the population of residuals, $1 - \alpha$ = confidence coefficient and

$$W_I = P[\hat{Y}_I - K_I s \leq y_I \leq \hat{Y}_I + K_I s] \tag{8}$$

with

$$s = \left[\sum_{i=1}^{n} (y_i - \hat{Y}_i)^2/(n-3)\right]^{1/2}. \tag{9}$$

To determine K_I one first computes the variance of $\hat{Y}_I$ at time t_I, based on equation (5):

$$\begin{aligned}\operatorname{Var}\hat{Y}_I = {} & \operatorname{Var}\bar{y} + (x_I - \bar{x})^2 \operatorname{Var}\hat{\beta} + (z_I - \bar{z})^2 \operatorname{Var}\hat{\gamma} \\ & + 2(x_I - \bar{x})(z_I - \bar{z}) \operatorname{Cov}(\hat{\beta}, \hat{\gamma}).\end{aligned} \tag{10}$$

One next defines a so-called effective number of observations, n*, that depends on $\operatorname{Var}\hat{Y}_I$ (and hence on time, t_I):

$$n^* = s^2/\operatorname{Var}\hat{Y}_I = \left\{\frac{1}{n} + \frac{1}{n(1-r^2)}\left[\frac{(x_I - \bar{x})^2}{s_x^2} + \frac{(z_I - \bar{z})^2}{s_z^2} - \frac{2r(x_I - \bar{x})(z_I - \bar{z})}{s_x s_z}\right]\right\}^{-1}, \tag{11}$$

where

$$s_x^2 = \sum_{i=1}^{n} (x_i - \bar{x})^2/n-1; \qquad s_z^2 = \sum_{i=1}^{n} (z_i - \bar{z})^2/n-1$$

and

$$r = \sum_{i=1}^{n} (x_i - \bar{x})(z_i - \bar{z})/(n-1)s_x s_z.$$

The factor K_I at time t_I is then approximated as:

$$K_I = Z\left(1 + \frac{1}{2n^*} - \frac{2Z^2-3}{24n^{*2}}\right)\left(\frac{n-3}{\chi^2_{1-\alpha}(n-3)}\right)^{1/2}, \quad (12)$$

where $\chi^2_{1-\alpha}(n-3)$ is the chi-square value at probability $(1-\alpha)$ and $(n-3)$ degrees of freedom and Z is determined (from tables of areas under the normal curve) for a standard normal variate y* such that:

$$P[|y^*| \geq Z] = 1-p. \quad (13)$$

A tolerance interval (monodesm[2]) can then be computed according to equation (6) using the value of s calculated with equation (9) and the value of K_I determined with equation (12). Tolerance limits thus computed at any number of different timepoints may be connected by lines to form a chronodesmic tolerance band (about the fitted model).

E.g., construction of a circadian tolerance rhythmo(cosinor)-desm[2] based on a model, a 24-h cosine curve was fitted to data on blood total leukocytes obtained from a young woman at approximately 4-h intervals throughout the usual waking span on each of 20 days during a 10-month period. The cosine model was found to be statistically highly significant ($p < 0.001$), with parameters indicating that the circadian leukocyte rhythm had a mean value of 9664 cells/mm^3 and on the average reached its highest value of 11,039 at 22.04, its lowest value of 8289 at 10.04.

Using the K-S test described above, the distribution of actual data about this fitted curve was found not to deviate significantly from normality. Tolerance intervals (with 90% confidence of including 90% of the distribution of values) were then constructed about the fitted curve at arbitrarily selected timepoints throughout the 24-h period. The resulting *individual* chronodesm for total leukocytes is presented in the table. With a total number of observations = 99, Z = 1.645 and $\chi^2_{90} = 78.7254$, the only difference among timepoints (according to equation (12) is the effective number of observations, n*. As an example, the value computed for n* at the 08.00 timepoint was 24.9294. This yields $K_I = 1.8525$ and, with s = 1648, $\hat{Y}_I = 8.4873$ (equation (4)), tolerance limits are $8487.3 \pm (1.8525)(1648) = 5435, 11{,}540$.

The value of time-qualifying tolerance intervals is clearly evident from this table. Thus, for this particular subject, a leukocyte count of 7700 is below the low tolerance limit at 20.00 or 00.00 but could be considered 'normal' at other times. If this subject were, say, a cancer patient beginning chemotherapy, the circadian stage at which a subsequent blood sample was taken could be an important factor in deciding whether or not myelotoxicity was occurring. Apart from any consideration of rhythms, the importance of *individual* reference intervals is emphasized by the fact that the lower limit of 'normality' for the subject, regardless of timepoint, is considerably above the value often regarded as the lower limit of the normal range[6].

Circadian chronodesm (cosinordesm) for blood total leukocyte count in a young woman

Time (clock hour)	Tolerance interval* (cells/mm^3)
00.00	7820-13,917
04.00	6616-12,767
08.00	5435-11,540
12.00	5430-11,490
16.00	6602-12,671
20.00	7811-13,871

* Interval including 90% of population (of residuals) with 90% confidence; computed about 24-h cosine curve fitted by least-squares method.

For a given sample size, the width of the tolerance interval depends on the choice of proportion and confidence. If, for example, one were content with including only 80% of the distribution with 95% confidence, the limits at 08.00 would be 6040 and 10,934. If one wanted to include as much as 99% of the distribution with 99% confidence, the limits would be 3298 and 13,677. The choice is arbitrary, balanced between an interval so narrow that values from a 'healthy' condition are likely to be considered 'sick', and an interval so broad that values from a 'sick' condition are likely to be considered 'healthy'. However, cost-effectiveness criteria should be applied as work with chronodesms proceeds and the choice may then differ from variable to variable and from goal to goal.

Until experience indicates otherwise, the use of 90% proportion and 90% confidence seems advisable from the standpoint of consistency with the significance level (0.05) commonly used in other statistical tests, at least in the so-called 'one-tailed' evaluation. Thus, a physician will often ask whether a patient's value is too high *or* too low rather than whether it is different from 'normal'. By considering the central 90% of the distribution in constructing tolerance limits, values in the upper 5% (and in the lower 5%) will be judged 'abnormal'. With 90% confidence in, say, the estimated upper limit, there is only a 5% chance that it will lie *below* the upper 5% of the distribution[7]. That is, one can say with 95% confidence that a value above the upper tolerance limit has only a 5% chance of being from a 'healthy' person.

The need for individualizing reference intervals has been noted by Sunderman[24] - without time-qualification, however - and methods for deriving such reference intervals have been described[18,19]. When the collection of time series on an individual entails repeated disturbances (e.g. venipunctures), a methodologic serial-dependency among sampling times may be introduced, apart from existing rhythms[9]. When the collection of time series requires major disturbances or even minor repeated nuisance to the individual (e.g. a biopsy, catheter insertion, venipuncture, rectal temperature or blood pressure measurement) especially during the rest span, the data are almost certainly biased by the measurement, resulting in a so-called serial dependence among samples from the same individual.

Such artifacts from the measurement process in itself would seemingly die out as the individual adjusts to the continuous presence of a catheter, temperature probe (whether telemetered or hard-wired) or other monitoring device which can be sampled as often as is practical without unnecessary awareness of the individual. In this manner an approximation of the serially independent sampling will be obtained for the individual. Prior and simultaneous longitudinal assessment of a number of variables presumably sensitive to loads could increase the understanding of the effect of physiological loads from traumatic or at least nuisance-generating measurement procedures which cannot be replaced by less disturbing procedures.

For example, changes in leukocyte count resulting from catheter insertion may be paralleled by short-lived disturbance in a blood pressure (heart rate or body temperature) rhythm. In this fashion the presence (and, if so, the

extent) of a disturbance in a marker rhythm such as blood pressure could be used to assess the extent of serial dependency of sampling. It is also clear that the absence of an assessable disturbance of a marker rhythm need not indicate a lack of disturbance by measurement of the subject. Moreover, it might be that a specified and (whenever practicable) quantified disturbance may even be preferred for rhythm evaluation in a fashion akin to testing for tolerance to loads. Chronodesms may be established not only for spontaneous changes but also for responses to imposed loads. Both kinds of approaches - direct and indirect periodicity analysis[10] - can be practiced with regard to the state of the rhythm when the stimulant is first applied with regard to the alteration of rhythms (rather than of some imaginary baseline). Possible shortcomings of ambulatory instrumentation for the collection of physiological data (undue size and weight, excessive frequency and 'sophistication' of servicing, reliability and integrity of the transduction-ranging from blunders [e.g. probe off] to excessive disturbances impinging upon record) may further complicate the interference with usual function resulting from annoyance of the monitored subject. These facts deserve careful attention in obtaining data for the construction of chronodesms.

As appropriate time series, relatively free of sampling artifact, become available on individuals (e.g. in automatic monitoring of variables such as body-core or surface temperature[15] or blood pressure[12] reliable reference intervals can be made available not only for single samples obtained at any given stage of a rhythm but also for the rhythm *parameters*. That is, a physician then may be able to judge whether a patient's *rhythms* are 'normal' (euchronic) and need not assume biostationarity[13] in the pattern of time-dependent variation (as in the case when a single value is compared to a previously constructed chronodesm).

Although attention herein has focused on circadian rhythms, the presence of other biologic periodicities (e.g. about weekly, about monthly, about yearly) should not be forgotten. Thus, it is possible that a circadian chronodesm constructed from samples of prolactin obtained in April will not be (completely) appropriate for a sample obtained in December. It seems as yet cost-ineffective to propose that reference intervals be qualified with respect to a complete spectrum of rhythms for each variable of clinical interest and with respect to numerous pertinent factors, such as sex, age, and diet, to cite but a few. A set of criteria (e.g. the relative amplitude of rhythms on different time scales) may be applied in deciding which rhythms and variables merit inclusion in a comprehensive chronodesm. These rhythms could then be represented by a first simplified, yet composite model (e.g. one cosine for each rhythm) fitted to all data before constructing a chronodesm at any stage of the complex waveform. When cost or other factors preclude the preparation of a chronodesm accounting for a spectrum of rhythms in a given individual, information on the dynamics of peer-group chronodesm may serve for adjusting an individual chronodesm prepared for a single rhythm, such as the circadian.

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2 The term is derived from 'chronos', time, and 'desmos', bond, even if 'desmos' is here taken to denote something more like 'bound' or 'band' than like 'bond'. Unless a more appropriate and succinct term can be proposed, the word chronodesm may be used with poetic (if not scientific) license. Names may also be appropriate for different kinds of chronodesms. The term 'monodesm' may indicate that all data used to derive a chronodesm were obtained at a) a single timepoint or b) during a single timespan shorter than the period of a particular rhythm under consideration. Thus, a monodesm would be a conventional reference interval applicable to a single timepoint or timespan. The term 'rhythmodesm' may refer generally to a chronodesm based on any satisfactory mathematical model for a given rhythm, applicable to all stages of the rhythm. If a single cosine curve is an appropriate model, one may use a more specific term: 'cosinordesm'. If a statistically-significant model with normally-distributed residuals cannot be found, with or without data transformation, one may resort to constructing a 'merodesm', in which reference intervals are computed separately at each of several different timepoints or stages of a given rhythm, i.e., as a set of monodesms. The terms may be eventually qualified as to whether it applies to an individual - idiochronodesm - or to a population - panchronodesm. The term may be further qualified as to the model used.

3 The subscript capital i (I) denotes a time used for prediction, which may or may not coincide with an actual sampling time denoted by a small i (i).

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26 On practical grounds, the much more generally applicable tolerance interval is preferred to a prediction interval. However, for unique decisions such as those concerning the surgical removal of an organ (or for some other unique or very rare inference) the prediction interval (estimating the interval in which a very few or only a single future observation might fall) may yield at least theoretically a narrower band[8]. In such rare cases, a prediction interval may be preferable to a tolerance interval. If, then, a time-qualified prediction interval is given the term prediction-chronodesm and the variations of this term described above can be advocated. The term chronodesm, without further qualification, will invariably refer to a time-qualified tolerance (rather than prediction) interval.