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Individual blood profiling – A means of improving the fight against doping

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Abstract

The use of intra-individual profiling of analytical parameters both in blood and urine (Biological Passport) has recently been given considerable focus. In order to improve the detection of blood doping and the misuse of erythropoietin and analogues, individual recording of different blood parameters over time has been proposed. The parameters chosen, like haemoglobin, percentage reticulocytes or combinations of them, are generally affected after the use of the above-mentioned prohibited substances and methods.

In a collaborative project between the Danish and the Norwegian Anti-Doping Organizations, 52 top athletes from different sports and of both genders participated in and completed the following study: 7 – 8 blood samples from each athlete were drawn over a period of time (1 – 2 years). The majority of the controls were without any notice, while three were announced the day before, because they were to be taken the next morning fasting and without having trained. Otherwise standardization was limited to a ten minutes rest in the sitting or lying position before collection of blood.

The results of this study evaluated using the Bayesian model clearly show the differences between intra- and inter-individual variability.

As a result, Anti-Doping Norway has already implemented the individual profiling into their anti-doping strategy with more than 90 athletes and many more samples per athlete. The increasing knowledge about a single athlete's haematological characteristics has enabled the anti-doping organizations to be more targeted in their testing for blood doping.

1. Introduction

The World Anti-Doping Agency prohibits erythropoiesis-stimulating agents and methods that enhance oxygen transfer [1]. Detection of these agents constitutes a major challenge in the fight against doping. Although great advances towards a direct analytical proof of doping with erythropoietin preparations have been made[2-4] many challenges remain. Because the possible performance enhancing effect of doping with erythropoietin lasts remarkably longer than the detection time in blood and urine[5] the timing of an effective doping control is very important. Although the detection of non-autologous blood transfusion has been developed [6-9], autologous blood transfusion is still very difficult to detect. Therefore, the alternative strategy of monitoring indirect parameters of doping with substances and methods increasing the oxygen transport capacity [10] is still highly relevant.
Increased levels of certain blood parameters as haemoglobin, haematocrit and the percentage of reticulocytes do indicate indirectly the possible use of doping substances or methods increasing the oxygen transport capacity. Haematological parameters are affected by

- the administration of recombinant erythropoietin (epoetins),
- the administration of second and third generation recombinant erythropoietins as Novel Erythropoiesis Stimulating Protein(NESP, darbepoietin), δ-epoetin, ω-epoetin, Continuous Erytropoietin Receptor Activator (CERA) and many biosimilar erythropoietins,
- the use of autologous and non-autologous blood transfusion,
- the use of erythropoietin mimetica,
- the possible application of gene therapeutical methods.

In order to evaluate the relevance of individual blood profiles in this respect we undertook a collaborative study together with Anti-Doping Norway (ADN) and Anti-Doping Denmark (ADD) with the clear aim to implement the evaluation of individual blood profiles into the day-to-day anti-doping programs.

2. Study design

Over a time period of one to two years blood samples were collected from 60 elite athletes from both Norway and Denmark. The athletes were informed about the project and the samples were collected partly as unannounced normal doping controls (mainly out-of-competition) and partly with announcement in order to achieve a more restrict standardization.

The project plans scheduled a minimum of seven blood samples per athlete. In total 188 blood samples (101 from males and 87 from females) and 216 blood samples (152 from males and 64 from females) were taken by ADN and ADD, respectively. In total, 22 athletes (13 males and 9 females) finished the study for ADN and 30 athletes (21 males and 9 females) for ADD.

Blood sampling for normal controls were taken by venipuncture with the sample taking equipment from Berlinger (Ganterschwil, Switzerland) after at least 10 minutes at rest (sitting or lying), while standardized samples (on average 3) were taken fasting in the morning before any physical activity. Two blood containers were filled.

The description of the participants, the sports chosen and the number of observations are indicated in Table 1 and 2.

The different sports were chosen according to their significance in Norway and Denmark, respectively so that only top-level athletes were included. Thus cross country skiing, biathlon and canoeing were included by Norway, and cycling, football and badminton were selected by Denmark.

	ADN	**ADD**
Number of athletes	29 (22 completed)	32 (30 completed)
Gender distribution	13 males (23-36 yr) 9 females (24 -41 yr)	21 males (19 – 30 yr) 9 females (20 -28 yr)
Number of observation	up to 8 per athlete	up to 7 per athlete
Total number of blood samples	101 from males 87 from females	152 from males 64 from females

Table 1: Total number of athletes and samples by AND and ADD.

ADN	**ADD**
Cross country (3m+3f)	Football (5m+ 0f)
Biathlon (4 + 4)	Triathlon (4 +1)
Cycling (2 + 2)	Cycling (5 + 0)
Canoeing (2 + 0)	Canoeing (4 + 0)
Rowing (2 + 0)	Rowing (3 + 3)
	Badminton (0+5)

Table 2: Sports and sexes selected for the blood profile study.

3. Blood sample analysis and data evaluation

The samples were transported to hospitals in each country and the full blood analyses were performed within 24 hours. Serum samples were stored frozen until analysis.
The following haematological parameters were determined in the samples: haemoglobin (HGB) [mmol/L or g/dL), haematocrit (Hct) [fraction], red blood cell count (RBC) [10^6/mL), reticulocytes (Retic) [1/mL or per cent], serum-erythropoietin (S-EPO) [U/L], serum soluble transferrin receptor (S-TfR) [mg/L]. In addition, samples from ADD were also analyzed for serum-iron [µmol/L], and the samples from ADN for ferritin. Only relevant parameters are discussed in this presentation, while a comprehensive analysis will follow in a subsequent publication.
The haematological parameters were measured on two identical Sysmex XE-2100 instruments (Sysmex Europe, Hamburg, Germany) at the Bispebjerg University Hospital in Copenhagen, Denmark, for the ADD-samples and on an ADVIA 120-instrument (Siemens Healthcare Diagnostics, Deerfield, IL, U. S. A.) at Lovisenberg Hospital in Oslo, Norway. S-EPO and S-TfR measurements were performed on an Immulite 2500 (Diagnostic Products Corporation, Los Angeles, U.S.A.) and with an enzyme-linked immunosorbent assay (ELISA) (Orion Diagnostic, Espoo, Finland), respectively.
For the statistic evaluation of the analytical data SPSS 15.0 software (SPSS Inc., Chicago, U. S. A.) was used. The box plots indicate the median and the four quartiles (except outliers). For the analysis of the individual data sets several approaches have been proposed[11, 12], but we chose the application of a Bayesian model[13] proposed by Sottas et al.[14-16]
A software application called *Athlete's Biological Passport* (Version 1.0.2) is available from the Laboratoire Suisse d'Analyse du Dopage, Lausanne, Switzerland, upon request.
The software is based on the Matlab 6.1.0 software (The Mathworks, Inc., Natick, MA, U.S.A.) and it specifies the differentiation of intra-individual and inter-individual variabilities.

4. Results and discussion

4.1 Descriptive statistics of haematological parameters

The study design restricted the sample taking standardization to a minimum and reflects realistic situations one will meet, when haematological parameters are collected during normal doping control both in and out-of-competition. Announced as well as unannounced testing should give the widest personal range for the different parameters. In addition, the fact

that the haematological parameters were measured in different laboratories should also give some indications for the relevance of inter-laboratory variations.

Fig. 1 shows the results of all the blood samples from female athletes for haemoglobin, two different populations measured on two different instruments. It indicates the suitability of haemoglobin with respect to any analytical and biological variability. The comparability of the selected athlete populations is obvious, when the descriptive statistics of the studied male

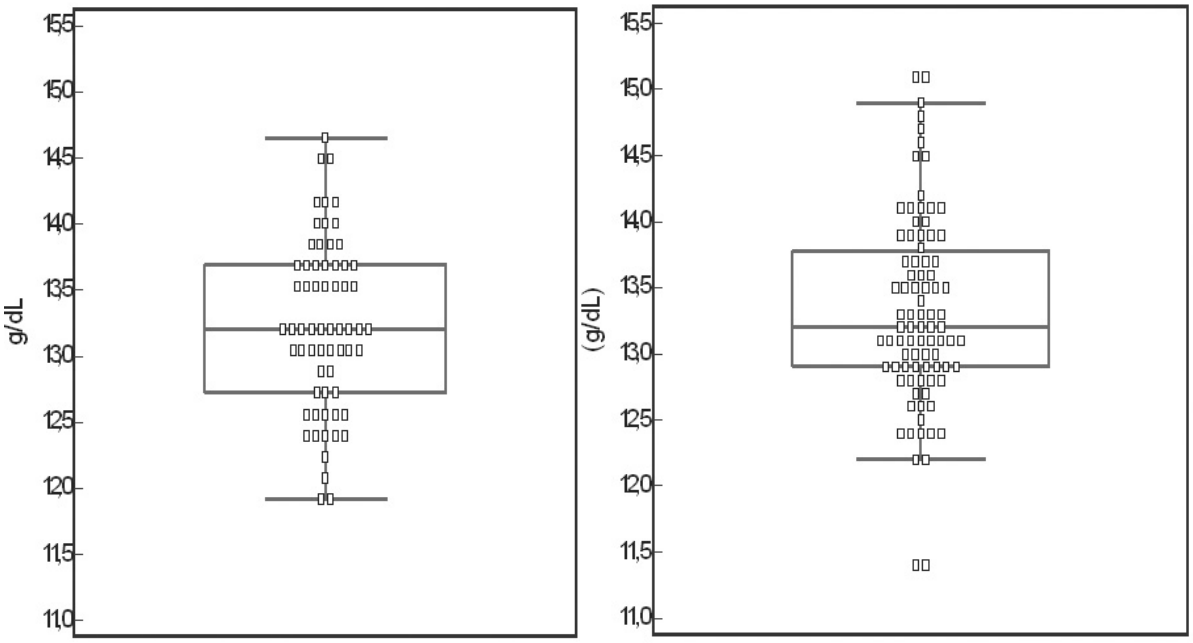

Figure 1: Haemoglobin concentration [g/dL] of all the blood samples from 64 Danish female athletes (left) and of all 87 blood samples from Norwegian female athletes (right).

	Sottas et al. [15] **330 samples /32 males**		**ADD/ADN 251 samples /34 males**	
	95 % reference range	mean	95 % reference range	mean
Hbg (g/dL)	13,4 - 17.0	15,1	13,2 - 16,1	14,6
Hct (x 100)	39,1 - 49,7	43,9	39,0 - 47,0	43,3
%Retic	0,58 - 2,22	1,32		
EPO (IU/L)	5,0 - 26,8	13,2	5,7 - 18,1	11,4
RBC (10^{-6}/mL)	4,12 - 5,93	4,94	4,38 - 5,34	4,82

Table 3: Descriptive statistics for haematological measurements of a Norwegian/Danish athlete male population compared to a study with Swiss athletes[15].

athlete populations is compared to published results from other athlete populations (see table 3).

One of the major aims of the present study was to verify the suitability of individual blood profiles compared to the respective population characteristics. Figure 2 shows clearly the

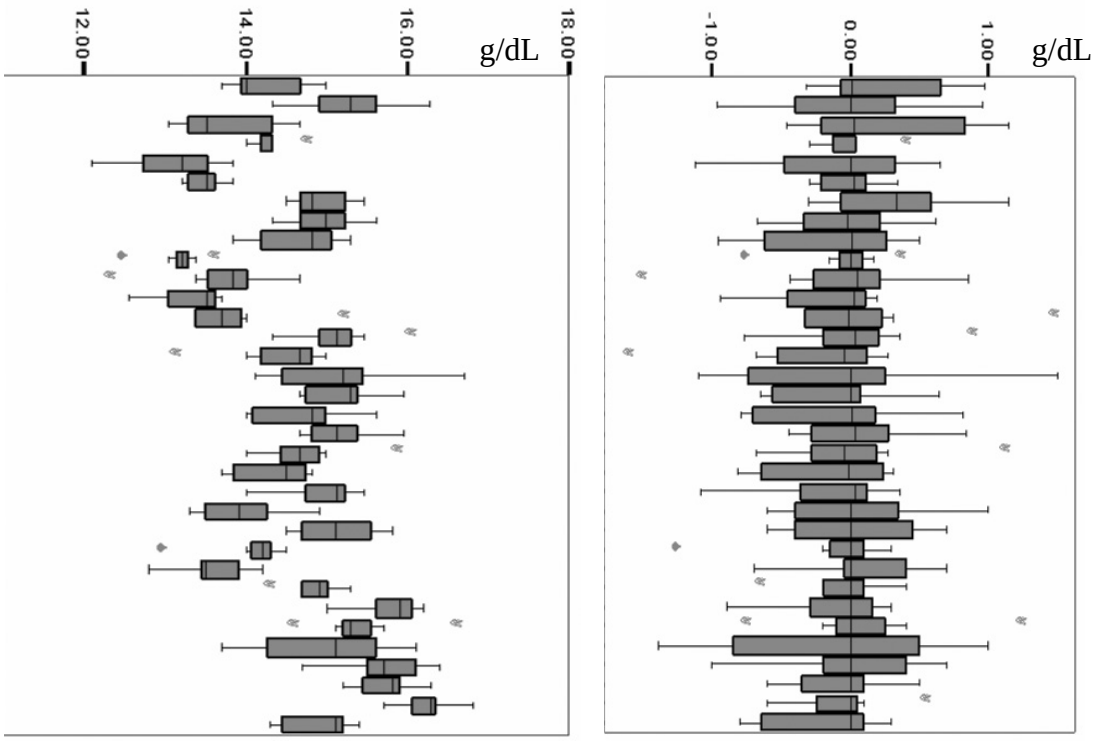

Figure 2: Individual haemoglobin concentration ranges of 34 male athletes (7-8 observations for each), presented as box plots with median and the four quartiles. On the left the results are plotted along a linear axis in g/dL, on the right the respective distributions are lined up at their medians at 0.0, indicating their individual variation.

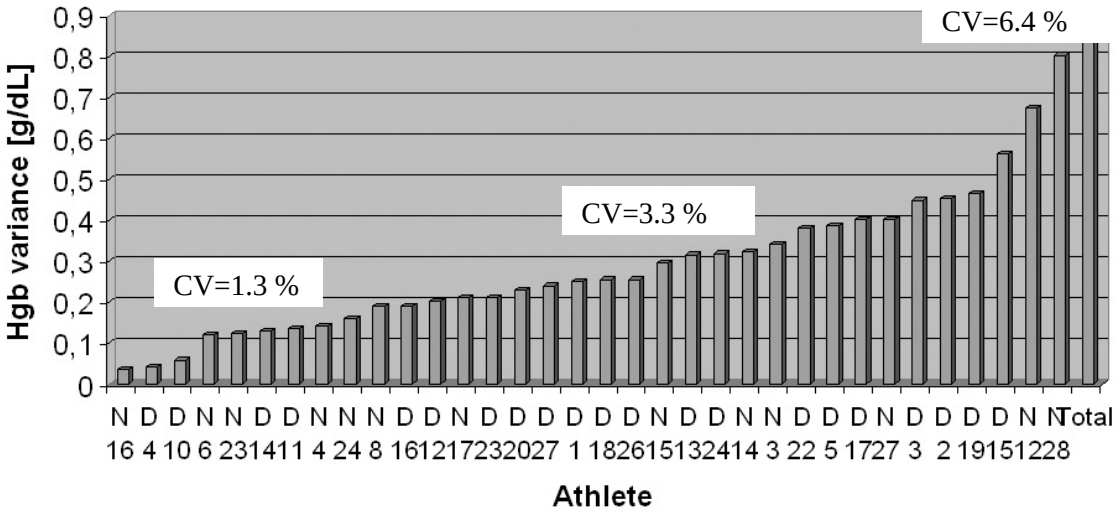

Figure 3: Variances and corresponding coefficient of variations (CV) for haemoglobin concentration measurements in 34 male athletes (7-8 observations each) from Norway (N) and Denmark (D) compared to the variance of the total number of haemoglobin measurements.

differences of measurement ranges for haemoglobin, when the individual variability is compared to the population variability.
It is obvious that the population ranges are greater than the individual variability. An assessment of the result of a haematological parameter measurement like haemoglobin would be more meaningful, when compared to the athlete's own variability.
These results are furthermore confirmed, when presenting the haemoglobin concentration variances of the same population in a sorted graphic (Figure 3).
Although the individual variability is ranging from a variance of 0.04 g/dL, corresponding to a coefficient of variation of 1.3 % to 0.8 g/dL (CV = 6%), all individual variances are smaller than the variance calculated from the total number of observations. (0.85 g/dL, CV = 6.4 %). The mean of individual variances is 0.25 g/dL, corresponding to a CV of 3.3 %.
The evaluation of the different haematological and serum parameters also revealed that the knowledge of the analytical variability is an important pre-requisite, when discussing individual profiles. Because we analyzed the blood samples in two different laboratories, we also were able to confirm the difficulty and complexity of reticulocyte measurements.
The results of the % reticulocyte measurements for 152 Danish male athletes and 101 Norwegian male athlete showed a mean and standard deviation of 0.80 ± 0.20 and 1.27 ± 0.37, respectively. 95 % reference ranges were (0.49 – 1.22) and (0.73 – 2.23). When interpreting these data one should have in mind that it describes two different populations measured on two different instruments.
If individual profiles of haematological parameters are to be evaluated and shall describe the natural biological variability of certain parameters, it is of outmost importance to know about the analytical variability of the analytical measurement. The validation of the reticulocyte measurement indicated an intermediate precision of 5 and 10 % for the Sysmex and the ADVIA-instrumentation, respectively. In our study two different populations were measured. This means that a direct comparison is not possible. However, with more than 100 measurements in each group a systematical bias is obvious for the reticulocyte measurement.

In our opinion, the inclusion of reticulocyte measurements in an individual blood profile requires the harmonization[17] of the analytical measurement, preferably with the use of one type of instrument. In addition, an independent external quality programme will be mandatory, if one wants to include results from different laboratories into one individual profile.

4.2 Statistical evaluation of individual profile data

When applying the *Athlete's Biological Passport* software a series of observations of n haematological parameters, e.g. haemoglobin, generates two-tailed threshold limit values, calculated through the Bayesian network, for the $(n+1)^{th}$ observation to come. The specificity may be determined, e.g. 99 %. So called heterogenous factors like gender, ethnic origin, type of sport, age, altitude and type of analytical instrument can be taken into account in the model. Figure 5 shows an example of an individual haemoglobin profile of a male athlete, calculated by the *Athlete's Biological Passport* software on the basis of seven observations. The Bayesian model calculates an upper threshold of 148 g/L and a lower threshold of 116 g/L for the next observation to come. The sensitivity for the model is set at 99 %. The distribution probabilities indicate clearly, how the characteristics of a population based probability (N=0) change to an individual probability after 7 available results from the athlete. While the first reflects a broad distribution with a mean of 150 g/L, the last distribution is much narrower and indicates a mean of 132 g/L for this athlete. Other athletes may show characteristics shifted to the other side of the population.

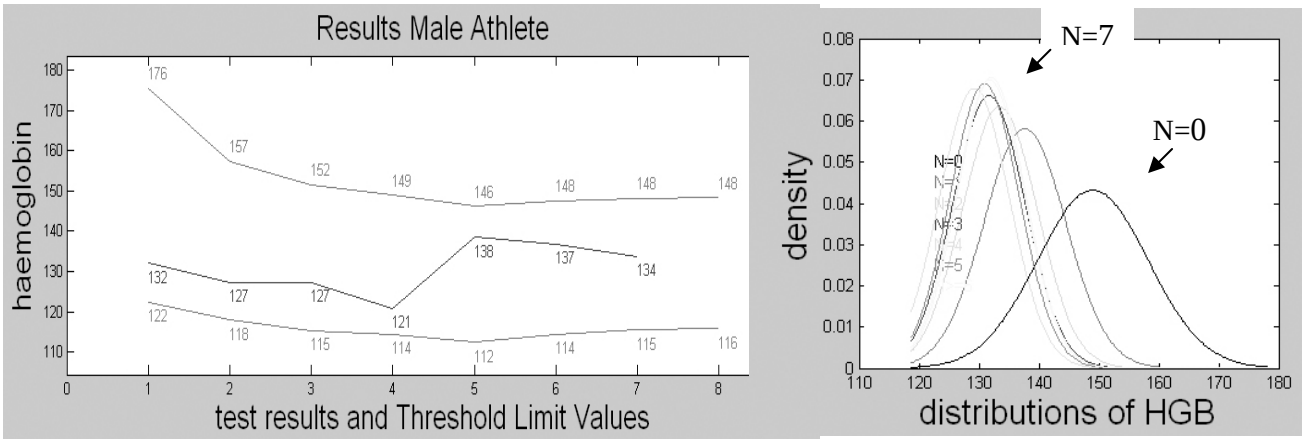

Figure 5: Individual haemoglobin profile (left) of a male athlete with seven observations surrounded by upper and lower threshold limit (99 %) and distribution probabilities (right) changing from the moment, where no observation exists (N=0) to the moment, where 7 observations exist (N=7).

52 profiles of elite athletes in Denmark and Norway were recorded. In addition to haemoglobin, other combined parameters like the OFF-score [18] or the Abnormal Blood Profile Score (ABPS)[15] were calculated and give valuable information about the individual athlete.

5. Consequences in the fight against doping

The range of consequences following an abnormal finding may vary from additional analyses on an actual sample (e. g. EPO-test in urine), target testing of athletes (intelligent testing), no-start/short time suspension to sanctions according to the World Anti-Doping Code provisions. A reading of a haemoglobin concentration above the upper threshold limit might initiate an immediate analysis of the respective urine sample (if available) for recombinant erythropoietin, a reading of the reticulocyte percentage below the lower threshold reading might initiate a target test for non-autologuous blood transfusion, only to mention some examples. A suspicious reading for the OFF-score or the ABPS-score might put the athlete to a state, where he/she is target tested in the future at time periods, where it is likely that he/she use forbidden substances or methods.

All these consequences can be organized and administered confidentially inside the anti-doping agency and have no damaging effect to the athlete. Therefore, it is not critical to put the threshold sensitivities at 99 or even 95 %.

Any consequence including a sanction on the basis of blood profiling information requires, to our opinion, a broad consensus among the stakeholders and WADA on an appropriate legal basis. Full documentation of the analytical measurements including the measurement uncertainty and the sample chain of custody should be made available. Circumstances which may interfere with haematological readings need to be listed.

6. Implementation of a Blood Profiling Programme for Anti-Doping Norway

Based on both the practical and scientific experience from this study, ADN started in January 2008 to implement the individual profiling into their anti-doping strategy.

90 of the best Norwegian endurance athletes where selected for the blood profile program, where the idea was to implement the individual profiles to the day-to-day test planning. At this point of progress, ADN is in a position to closely follow up athletes and conduct target testing of athletes with deviating results and a potential abnormal blood profile. Due to the short detection windows for erythropoietin and the difficulties involved in disclosing the use of blood transfusions, this indirect approach has significantly increased the quality of the target testing. By identifying potential cheats in a blood profile, more resources can also be used for testing on a scientifically based suspicion, using a direct approach. This however implies frequent blood sampling from all athletes in the program, to reveal potential suspicious results.

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