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The fight against doping: back on track with blood

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Introduction

The efficiency of the fight against doping in the future will evolve drastically in several ways. Although, during the last ten years, testing of urine samples out of competition opened the door for intelligent testing, the real shift toward improvement of the system was the adoption of the blood matrix as the biological sample of choice. When collected properly, blood allows the establishment of individual haematological and hormonal profiles, which are currently the basis of the 'biological passport'. A simpler collection procedure also permits the evaluation of the prevalence of doping in specific populations of athletes, as is typically done in epidemiology, to establish a risk assessment.

Anti-doping efforts will need a huge effort by the stakeholders to perform pre-analytical work (such as organization of tests, collection and storage of samples, transportation to the lab) at the level required from WADA-accredited laboratories in the analytical field. A similar system of accreditation should be applied to the partners of this very sensitive pre-analytical work to ensure the validity of the system.

For 'in-competition' tests in which substances that enhance performance during the event as well as some recreational drugs are analysed, blood will be the ultimate biological sample. As in the forensic sciences or in clinical pharmacology, blood permits a safer interpretation than urine regarding the pharmacokinetics of a xenobiotic substance.

Moreover, in the future blood will be the sample of choice to give anti-doping authorities a biological signature of doping. This can be done through the appropriate exploitation of evolving biological fields such as metabolomics or proteomics to allow true monitoring of each athlete.

Urine, a Biological Fluid That Made History in the Fight against Doping

Back in the 1970s and 1980s,[1–4] urine was considered to be the preferred biological fluid in which to examine the most commonly used performance-enhancing substances. This was an obvious choice because urine acts as a waste bin where xenobiotics are deposited by the organism. The first analytical techniques used in the fight against doping were thus tailored to detect drugs or drug metabolites at concentrations that can be found in urine. The use of this biological fluid, which could be collected non-invasively during anti-doping tests, became prevalent.

An additional advantage of urine testing resided in the fact that urinary concentrations of a prohibited substance found shortly after its administration were in the detection range of early anti-doping analytical techniques. Samples could be easily prepared from the urine matrix and the detection range of amphetamines, the most commonly used doping substances in the 1970s and 1980s, was such that it was possible to demonstrate their in-competition use to enhance performance. This was precisely the objective of anti-doping testing at that time.[5,6] Urine collection had no associated legal constraints and was not considered a medical act, so the entire scope of the fight against doping was built on the ability to test this simple fluid.

The Need for Out-Of-Competition Testing

In the 1980s, steroid doping began to take place during training periods. Not surprisingly, out-of-competition doping occurred massively in strength-intensive sports or disciplines requiring explosive quickness.[7–11] This trend developed primarily in sports where there was enough time to 'wash out' the prohibited substances before competition, thus avoiding being caught during a sports event. To improve the efficacy of the fight against steroid abuse, sports authorities responded by introducing out-of-competition testing. Although such testing is taken for granted today, it represented an important paradigm shift in the fight against doping at the time. Out-of-competition testing requires an organization and an infrastructure independent of that needed during competition. Thus, if the Olympic Games are considered to be the reference competition for most sports federations, the International Olympic Committee, as the central authority responsible for fair play, must be able to rely on the efficiency of in-competition (IC) and out-of-competition (OOC) testing organized by the participating sports federations. At the same time, the efficiency of the anti-doping program of any given federation strongly depends on the balance between IC and OOC testing and the specific features of the sport under scrutiny. Indeed, the duration of the athletes' sport season and the frequency of competition events strongly affect each testing program. In addition, the prevalence of doping found in a particular discipline may need to be taken into account when organizing anti-doping controls.

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Blood Samples: Testing Health or Testing Competition?

In the mid-1990s athletes who were inclined to dope themselves to improve endurance rapidly embraced the use of recombinant peptide hormones such as EPO and growth hormone. They quickly realized that EPO, in particular, could drastically increase the mass of erythrocytes and thus enhance athletic performance.

Systematic doping programmes emerged in disciplines such as cycling, athletics and cross-country skiing. Despite various attempts, urinary testing for this type of doping proved completely ineffective, at least initially. This was due to the fact that, unlike xenobiotics of low molecular weight, such as amphetamines or steroids, peptides are only present in the urine at very low concentrations. The bulk of these peptides are reabsorbed and returned to the circulation in the kidneys. Because direct evidence of such doping cannot be found in urine, it was proposed to detect these two hormones indirectly in the blood[12,13] by defining acceptable upper limits for certain haematological parameters affected by EPO. Thus, in an attempt to curb EPO doping, the International Cycling Union (UCI) set an upper limit of 50% for hematocrit (the proportion of red blood cells in the blood). In parallel, the International Ski Federation (FIS) focused on haemoglobin concentrations (limited to 18 g/dl).

The validity of these quantitative threshold values, which were based on population measurements, was intensely debated at the time given the fact that haematological parameters are subject to very strong inter-individual variation. For example, the figure of 50% hematocrit used in cycling was derived from a very simple statistical approach by adding two standard deviations to the average population value.[14,15] Ultimately, the inherent variability in haematological parameters makes it impossible to use chosen limits to implement true anti-doping rules. Nevertheless, it was rapidly realized that EPO abuse could be prevented by setting limits.

The justifications for carrying out the necessary blood tests oscillated between concerns for athletes' health and the need to apply competition rules. This approach contradicted the principle of fairness, which is central to sports. Indeed, it did not matter that a runner with a naturally low hematocrit had the ability to manipulate his parameters and still remain under the limit, in contrast with another runner with a naturally higher hematocrit. Despite such blatant shortcomings, the 'no-start' rule was intuitively understood by the athletes and accepted for a period of at least ten years without any major contests in court. With the advent of a urine-based EPO detection method,[16] even isolated blood tests made it possible to screen effectively for the use of recombinant erythropoietin. A subsequent important step in the evolution of the fight against doping consisted of the development and implementation of the 'biological passport'.

Individual Biological Follow-Up: Haematological and Hormonal Profiling as 'Biological Passports' in the Fight against Doping

International sports authorities and the World Anti-Doping Agency are currently implementing an individual follow-up programme. In the future individual biological profiles shall provide scientifically sound evidence for doping based on a statistical model for data interpretation that is similar those used in forensic sciences for the comparison of traces of DNA.[17–19] This indirect approach relies on the working hypothesis that the biological parameters of a given individual remain relatively stable if a certain context is maintained. If so, the athlete acts as his or her own control, provided that all measurements are carried out in a standardized, longitudinal manner. An abnormal deviation in a specific parameter such as haemoglobin or in a set of biological parameters relative to their own norms may constitute evidence for doping. Such evidence would be admissible only after excluding any pathology that may be related to the observed anomaly or anomalies. In addition, any abnormality in the longitudinal profile of an athlete that belongs to a particular group would need to be assessed with respect to the prevalence of doping measured in the same population. For increased effectiveness, therefore, a 'biological passport' in sports must rely simultaneously on tools that are specific to epidemiology and tools used in forensic sciences.

Two aspects are absolutely essential to meet all of these criteria:

- highest quality of information
- evaluation of the prevalence of doping.

Highest quality of information

To ensure that data can be used for a 'biological passport' all acquisition processes must be standardized. This applies to both transverse population studies and individual longitudinal studies. It is particularly true if the biological parameters under scrutiny depend on the quality of the conservation of samples prior to analysis. Measurements of haematological parameters are very sensitive and depend on the prevailing pre-analytical conditions.[20] A number of environmental variables may influence the recorded values: exercise, altitude, the position of the athlete during blood collection, the characteristics of blood drawing by the phlebotomist, temperature and time of transportation prior to laboratory analysis. The need to ensure pre-analytical quality is well known in the clinical setting, for example in the case of clinical chemistry or haematology. This has not yet been recognized in the context of the fight against doping. Instead, it is the control of the operations of anti-doping laboratories themselves that has been emphasized. The IOC medical commission and, subsequently, WADA have rightfully demanded the implementation of an international quality label that comprises a very dense and demanding auditing system (ISO 17 025 and International Standards for Laboratories) for the testing laboratories. Clearly, pre-analytical aspects are also essential when the sample is a living matrix such as blood and when measurements involve cellular variables. Standards pertaining to this type of testing do exist. They have been introduced by WADA and have received international validation by all of the stakeholders in the fight against doping. In the absence of auditing and a real quality-control system, however, without cooperation and training of the organizations in charge of collecting the samples, it will not be possible to use the obtained blood data in a legal setting. Clearly, blood collection is a medical act and must follow international medical guidelines.

There is also an analytical issue. Anti-doping laboratories have developed extremely sophisticated techniques that can detect xenobiotics in the urine with great specificity and sensitivity. They shall need to adapt, however, to the new era of longitudinal follow-up. If the existing quality control system is efficient with

regard to the analysis of forbidden substances in biological samples, a new dimension of networking and cooperation shall need to be achieved if the data used to establish an individual profile are generated by different laboratories. Such is the case for RTP (registered testing pool) athletes who travel worldwide to participate in training and competition events: all biological measurements obtained with their samples must be independent of the laboratories that carry out the tests. It is thus very likely that in the case of haematological measurements, which must be carried out less than 36 hours after sample collection, different laboratories shall have to adopt a system similar to that used for haematological or clinical chemistry laboratories in hospitals.

Another corrective measure shall need to be implemented for steroid profiling, which classically relies on urine analyses. Differences stemming from analytical methods specific to each laboratory shall have to be resolved. In this case, as in the case of haematological analyses, longitudinal follow-up cannot suffer from a lack of cooperation. This is true for various analytical techniques and is absolutely essential for results obtained in different laboratories.

Evaluation of the prevalence of doping

To fully realize the potential of biological follow-up in the fight against doping, the context in which it operates should be as wide as possible. Indeed, the epidemiological results of such analyses must be interpretable as a function of the populations studied. To understand the evolution of specific biological parameters in an individual who suffers from some metabolic dysfunction or has developed a doping habit, that individual's profile must be compared to data from the population under scrutiny. In other words, a doping habit becomes similar to a disease in the sense that its prevalence in the population needs to be described. It is therefore necessary to carry out doping prevalence studies in all populations of athletes and to make these data available before the tools of longitudinal follow-up can be effectively used. This is particularly important because different sports are affected differently by doping as a function of the physical characteristics required for athletic performance. In public health different pandemics are not distributed evenly around the globe: environmental, social and economic factors play an important role in distribution. The same is true for sports: doping prevalence varies as a function of the sport and the country where it is practiced.

Certain sports federations, sports disciplines and even entire countries may of course fear total transparency in their 'doping cultures', which may hurt their public image. At the same time, it is the only manner in which to proceed if the 'biological passport' is to become an effective tool in the fight against doping.

In-Competition Testing: Stimulants and Recreational Drugs

The prohibited list defined by the international WADA standards and annexed to the 2009 World Anti-doping Code contains the following classes of substances that are prohibited in-competition only:

Class S6: Stimulants
Class S7: Narcotics
Class S8: Cannabinoids
Class S9: Glucocorticoids

Stimulants (S6) constitute a very heterogeneous class. They include amphetamines and other derived products, including some substances used as recreational drugs such as MDMA (also known as Ecstasy). At the same time, S6 substances administered prior to 48 hours before a competition may yield amphetamine metabolites in the urine, which are easily detected by in-competition testing. In such cases the athletes may not have intended to dope themselves at all to enhance their performance. This class also contains sympathomimetics, which have reduced action in the CNS compared to amphetamines. Depending on the dosage and the association with other substances, however, their effects may be comparable. The upper limits for urinary concentrations of certain specific ephedrines are clearly defined (cathine, 5 ug/ml; ephedrine and methylephedrine, 10 ug/ml). These threshold values have been applied because athletes treated for a common cold use OTC medication containing these active compounds (ephedrines have both bronchodilator and vasoconstrictive properties). Consequently, these threshold values were adopted to avoid positive findings in athletes taking such medications several hours or days prior to competition, with residual concentrations that have no impact whatsoever on performance. This approach may lead to difficulties in interpreting results for cathine (5 ug/ml upper limit), which is a metabolite of pseudoephedrine, a substance that is not prohibited. The adoption of a threshold value in urine for this type of substance, however, seems reasonable, unless a better correlation between dose and effect can be established. It is well known in pharmacology that this can only be done when the dose is measured in the blood matrix. The advantages and drawbacks of both urine and blood matrix in anti-doping process are described in Figure 1. Moreover, if this is applicable

	Time of collection	Volume	Risk of Manipulation	IC Recent information	OOC Long term information	"omic" (metabol-, proteo-, gen-)
 **Urine**	**Long** *10' - 6 h*	**High**	**High** *Dilution, Protease*	**Poor**	**High** *Direct/ Indirect*	**Low potential**
 **Blood**	**Short** *1-5 min*	**Limited**	**Low**	**High**	**High** *indirect*	**High potential**

Figure 1. Advantages and drawbacks of respectively urine and blood matrices in the fight against doping.

to ephedrines, it could also be applied to other stimulants when they are absorbed out of competition. This is especially true for recreational drugs.

Recreational Drugs

Of all the substances that are tested in-competition, recreational drugs are certainly among the most problematic for anti-doping authorities. Cannabinoids and stimulants such as cocaine or certain amphetamines are usually consumed outside of sports, essentially as recreational drugs. As such, their use is generally prohibited and represents an offence in most countries that are signatories to the World Anti-doping Code. Accordingly, a positive finding following a competition does not merely result in a sports-specific sanction; it may also lead to criminal sanctions. The World Anti-Doping Code defines which products are prohibited during a competition, implying that their use is prohibited specifically during the period when the event is taking place. This period may vary for different sports disciplines and may begin the day preceding competition. At the Olympic Games, the prohibition period starts at the opening of the Olympic Village. In the case of soccer, it lasts for 48 hours (starting 24 hours before a match and ending 24 hours afterwards). At the same time, the sensitivity of the measuring devices in use today is such that, given a positive result, it is hardly possible to determine whether the drug was administered during the prohibited period or not.

Cannabis and cocaine are good examples of substances that exhibit very long windows of detection in urine. It is extremely difficult to establish the time of administration of either of these two drugs by analysing this matrix. In forensics in general and in 'drugs and driving' testing programs in particular, there is a need to determine the time of initial exposure. The matrix that has been in use for many years to reach this objective, of course, is blood.

We believe that in this context, the anti-doping authorities must redefine the real purpose of in-competition testing and issue directives to the laboratories on how to interpret the results obtained. If the objective is to evaluate the period during which the substance was most likely to have been administered and whether this period corresponds to that of the competition, the matrix of choice must certainly be blood and not urine.

Certain team sports may actually benefit from the developments described above without reducing the efficiency and credibility of their current anti-doping policies. Indeed, the adoption of reference values established in forensics or clinical medicine would actually reinforce decisions already in place for these sports disciplines. Recently, the different stakeholders in the fight against doping accepted raising the volume of a urine sample collected during an anti-doping test from 75 mL to 90 mL. This decision is consistent with WADA international anti-doping test standards[21] and can only be praised because the objective is to allow the laboratories to test for a larger number of prohibited substances in the field. From a more pragmatic point of view, however, this approach is hardly workable. Indeed, it will certainly not be possible to keep increasing the volume of a biological sample collected in-competition to have enough material to screen for all substances on the prohibited list. The logistics are already quite problematic when collecting samples after certain competitions: some athletes are dehydrated and have a hard time producing the required volume of urine. In such cases strict compliance with standards may lead to situations that are extremely difficult to manage (for example, when return travel after the competition has been pre-booked).

The problems outlined above could be solved easily if, instead of urine, blood samples were collected at the end of a competition. On the one hand, blood analysis would result in a more reliable assessment of the athletes' exposure to performance-enhancing substances. This has been already described for many drugs in impaired driving toxicology sciences.[22] On the other hand, there would be no logistical problems in collecting samples, given the fact that the entire procedure of drawing blood usually requires 5 minutes or less per athlete.

Testing Campaigns: Guaranteeing Efficiency Following an Assessment of Prevalence

Many biological control activities that rely on the same analytical tools as the fight against doping operate as campaigns, in two phases. First, testing campaigns are run to determine the prevalence of a phenomenon. Once the prevalence has been correctly assessed, targeted treatment campaigns are conducted. Such an approach is common in public health. In the case of breast cancer, for example, a number of studies have been conducted to determine the prevalence of the disease in specific populations; these are then followed by mammography campaigns targeting the same populations. The rationale is to identify some metabolic dysfunction that may have an adverse impact on health and to monitor its occurrence in a targeted population using adequate diagnostic tools. It should be noted that the populations at risk and the tools employed are quite different for different diseases (for example, HIV and breast cancer).

The same scenario can be applied to the fight against doping. As mentioned earlier, the first step would consist of determining the prevalence of doping in the population under study. Indeed, if anti-doping rules are by definition universal and apply to all athletes, in practice cheating athletes in different disciplines adopt different doping behaviours. A quick glance at the distribution of positive cases among different sports demonstrates the lack of uniformity. For example, the statistics are quite different in athletics and in soccer. In the latter case, recreational drugs (such as cannabis and cocaine) are clearly more prevalent. At the same time, most samples originating from soccer players are collected after the competition. This leads to a higher proportion of stimulants among the positive cases reported in comparison to other sports. This finding can be easily explained by the fact that stimulants act directly on performance.

It is therefore necessary to carry out a large study on the prevalence of doping in different sports disciplines.[23] The approach must be systematic, without any bias stemming from particular practices adopted by an anti-doping authority or sports federation. The data collected must provide a true assessment of the doping habits in a specific population of athletes.

Such an assessment can then be followed by an appropriate campaign screening for one or several specific substances and targeting a specific population. Instead of testing athletes' urine or blood for all substances on the WADA prohibited list, this more rational approach would search for fewer substance that are better targeted (thanks to the prevalence studies) in a larger number of athletes.

Such testing campaigns will prove more efficient, as has already been demonstrated in other areas such as environmental protection, epidemiology and food safety.

Perspectives: Metabolomics, Proteomics and Genomics

Longitudinal haematological profiling ('biological passport') should be expanded to yield a broader metabolic profile. There is no reason to limit the biological signature of a doping substance (drug) to cellular parameters that are at the end of the metabolic chain. Clearly, the main goal of doping is to induce significant biological changes that eventually lead to enhanced performance of a living organism. At the same time, anti-doping analyses can detect or rely on many different biological changes at various stages of the associated metabolic cascades.

In the case of EPO doping, for example, current doping diagnostics focus only on the end result: an increase in the mass of haemoglobin or the number of red blood cells. Upstream events in the cascade, however, can also be studied and yield other parameters, an approach that is used in medical biology when investigating disease markers or the effects of medications. Indeed, regardless of the positive or negative effects of a given drug, it is very likely to alter the metabolic profile or other biological markers of a patient or an athlete. The systematic study of such modifications on a large scale belongs to the realm of metabolomics, defined as the qualitative and quantitative analysis of all metabolites present in an organism. The focus of metabolomics is relatively small molecules such as hormones and amino acids (MW $<$1000). Other molecular entities, however, are also affected by the cascades resulting from the administration of a specific substance or some specific practice that changes the biological balance of the organism (for example, a blood transfusion). Such changes can affect enzymes and proteins in general (proteomics), RNA (transcriptomics) and even DNA (genomics). Thanks to modern research tools that combine analytical techniques (mass spectrometry) with informatics, the biological signatures resulting from the administration of a doping substance can indeed be studied at many different molecular levels.[24] Molecular mapping and fingerprinting will certainly be at the heart of all diagnostic sciences in the future and the fight against doping is no exception.

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