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Assessment of confounding factors influencing the Athlete Biological Passport

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FACULTÉ DES SCIENCES SOCIALES ET POLITIQUES

INSTITUT DES SCIENCES DU SPORT

Assessment of confounding factors influencing the Athlete Biological Passport

THÈSE DE DOCTORAT

présentée à la

Faculté des sciences sociales et politiques

de l'Université de Lausanne

pour l'obtention du grade de

Docteure ès sciences du mouvement et du sport

par

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autorise, sans se prononcer sur les opinions du candidat, l'impression de la thèse de Madame Tiffany RAPILLARD, intitulée :

Assessment of confounding factors influencing the Athlete Biological Passport

Nicky LE FEUVRE
Doyenne

Lausanne, le 20 septembre 2022

ABSTRACT

Performances in endurance sports rely mostly on the capacity of blood to carry oxygen to the working muscles. However, this oxygen-carrying capacity can be exploited by athletes attempting to gain an illegal edge via doping, which seeks to enhance the carrying capacity of the blood mostly by increasing the oxygen carriers, termed hemoglobin. In 2009, the World Anti-Doping Agency (WADA) introduced the hematological module of the Athlete Biological Passport (ABP), which has become a pillar in anti-doping. The ABP functions by longitudinally monitoring hematological biomarkers for an individual athlete. Nevertheless, some relevant biomarkers for anti-doping are reported in concentrations, such as that of hemoglobin [Hb] and hematocrit (HCT), which can be altered by variations in plasma volume (PV) that are induced by many confounding factors (e.g. training). Anti-doping sciences strive to prevent or control confounding factors to better interpret blood variations and to prevent misinterpretation leading to a suspicion of doping. Therefore, the purpose of this thesis was to assess three confounding factors that can potentially alter the ABP reading (i.e. body position, endurance training in elite cyclists and breath-hold training). In an initial study, this thesis demonstrated the influence of body position on hematological values using a total of ten samples taken under different conditions. The second study investigated the influence of training load on the ABP biomarkers in elite cyclists. Finally, the robustness of the ABP was investigated by assessing the hematological values of recreational breath-hold divers in comparison to an active control group. It was demonstrated that a) a short walk (50 m) significantly increased [Hb] and HCT and that the supine position (>10 minutes) decreased significantly [Hb], both due to a shift in PV; b) a higher acute training load (total training load the 5 days before blood samples) significantly reduced [Hb] due to increased PV; and that c) the hematological values from the ABP of breath-hold divers did not differ significantly from a group with a physically active lifestyle, though these values did differ significantly over time for both groups, suggesting a seasonal effect (i.e. summer vs. winter).In conclusion, these results bring new insights regarding the assessment of three confounding factors (i.e. body position, training load in elite cyclists and breath-hold training) in the context of the ABP. The present results demonstrated the robustness of the ABP under these confounding conditions. The results of this thesis will be beneficial for anti-doping sciences and sport hematology knowledges.

RESUME

Les performances dans les sports d'endurance reposent principalement sur la capacité du sang à transporter l'oxygène aux muscles sollicités. C'est pourquoi cette capacité de transport d'oxygène du sang peut être exploitée par des athlètes qui tentent d'obtenir un avantage illégal via le dopage visant à augmenter la capacité de transport de l'oxygène principalement par l'augmentation du transporteur de l'oxygène, nommé l'hémoglobine. En 2009, l'Agence Mondiale Anti-dopage (AMA) a introduit le module hématologique du Passeport Biologique de l'Athlète (PBA), représentant actuellement un des piliers de l'anti-dopage. The PBA fonctionne sur le suivi longitudinal de biomarqueurs hématologiques pour un athlète individuel. Cependant, certains biomarqueurs, pertinents pour l'anti-dopage sont exprimés en concentration, comme la concentration en hémoglobine [Hb] et l'hématocrite (HCT%), qui peuvent être altérés par des variations du volume plasmatique (PV) induites par de nombreux facteurs confondants (e.g. l'entrainement). Les sciences anti-dopage s'efforcent de prévenir et contrôler les facteurs confondants pour mieux interpréter les variations hématologiques et éviter des erreurs d'interprétation qui pourraient conduire à des suspicions de dopage. Par conséquent, le but de cette thèse était d'évaluer trois facteurs confondants qui pouvaient potentiellement modifier la lecture du PBA (i.e. la position du corps, la charge d'entrainement chez des cyclistes élites et l'entrainement en apnée). Dans une première étude, cette thèse a démontré l'influence de la position du corps sur les valeurs hématologiques en utilisant dix prises de sang prélevées dans des conditions différentes. La seconde étude a examiné l'influence de la charge d'entrainement sur les valeurs hématologiques du PBA chez des cyclistes élites. Finalement, la robustesse du PBA a été investiguée en comparant les valeurs hématologiques d'une population d'apnéistes amateurs à un groupe contrôle actif. Il a été démontré que a) une courte marche (50 m) augmentait significativement [Hb] et HCT et que la position couchée (e.g. durant >10 minutes) diminuait [Hb], ces résultats étaient dû au déplacement du PV; b) une charge d'entrainement aiguë plus importante (charge d'entrainement totale les 5 jours précédant la prise de sang) réduisait [Hb] à cause d'une augmentation du PV ; et que c) les valeurs hématologiques du PBA d'apnéistes ne se différenciaient pas significativement d'un groupe physiquement actif, néanmoins, les valeurs hématologiques diffèrent significativement dans le temps pour les deux groupes, suggérant un effet saisonnier (i.e. été vs. hiver). Pour conclure, ces résultats apportent de nouvelles connaissances sur l'évaluation de trois facteurs confondants du PBA (i.e. la position du corps, la charge d'entrainement chez des cyclistes élites et l'entrainement en apnée). Les résultats présentés montrent la robustesse du passeport en regard de ces facteurs confondants. Les résultats de cette thèse seront bénéfiques pour les connaissances en sciences antidopage et en hématologie du sport.

Acknowledgements

I am finally writing these last lines of my thesis. Overall, this thesis has become a patchwork of my interests in life, combining my former work as a nurse with my passion for sport. In the end, I am really proud and happy to share it.

Throughout my life, I did not choose the “easy” way. I was scared of blood, yet choose to be a nurse because I loved understanding how our body and mind work. I was scared of cycling, yet my bicycle became my best mate. I did not even understand the word “statistics”, but I slowly became quite passionate about it.

After my masters degree in Sport Sciences, I realized I really like sciences and research studies, from idea to completion. Along the way towards this thesis, I had many rocks, rivers, and even mountain to climb, but I kept the final mile in mind, though it sometimes seemed very far away for sure. But I truly believe that I met the right person at the right time.

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List of publications

Thesis related

Astolfi, T., Schumacher, Y. O., Crettaz von Roten, F., Saugy, M., & Faiss, R. (2020). Does body position before and during blood sampling influence the Athlete Biological Passport variables? *Int J Lab Hematol, 42*(1), 61-67. doi:10.1111/ijlh.13140.

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Index of abbreviations

ABP	Athlete biological passport
ABPS	Abnormal blood profile score [a.u.]
ABT	Autologous blood transfusion
ADAMS	Administration and management system
APMU	Athlete passport management unit
APP	Athlete performance profile
ATL	Acute training load
ATP	Adenosine triphosphate
ATPF	Atypical passport finding
BHD	Breath-hold divers
BP	Atmospheric barometric pressure [mm Hg]
BV	Blood volume [mL]
CaO_2	Arterial oxygen content
CBC	Complete blood count
CERA	Continuous Erythropoietin Receptor Activator
CTL	Chronic training load
O_2	Oxygen
CO	Carbon monoxide
CO_2	Carbon dioxide
COHb	Carboxyhemoglobin
CV	Coefficient of variation [%]
ELISA	Enzyme-linked immunosorbent assay
EM	Expectation-maximization
EPO	Erythropoietin

ESA	Erythropoiesis-stimulating agents
FiO_2	Inspired pression of oxygen [%]
GH	Growth hormone
[Hb]	Hemoglobin concentration [g/dL]
HBT	Homologous blood transfusion
HCT	Hematocrit [%]
HES	Hydroxyethyl starch
HH	Hypobaric hypoxia
HIF	Hypoxia inducible factor
IF	International Federation
IFT	Isoelectric focusing technique
IRF	Immature reticulocyte fraction [%]
MCH	Mean corpuscular hemoglobin [pg/cell]
MCHC	Mean corpuscular hemoglobin concentration [g/dL]
MCV	Mean corpuscular volume [fL]
MLA	Minimum level of analysis
NADO	National anti-doping organization
NESP	Novel erythropoiesis stimulating protein
NH	Normobaric hypoxia
OFF-Score	$([\mathrm{Hb}]\ 10) - 60\sqrt{\mathrm{Ret}\%}$ [a.u.]
OFFS	OFF-score [a.u.]
OCP	Oral contraceptive pill
PaO_2	Partial pressure of oxygen
PiO_2	Inspired oxygen pressure [%]
PLT	Platelet [1000/µL]
PO_2	Partial pressure of oxygen [mm Hg]

PV	Plasma volume [mL]
PVE	Plasma volume expander
VAS	Visual analogue scale
Ret	Reticulocyte **[%]**
rHuEPO	Recombinant human erythropoietin
RTP	Registered testing pool
RBC	Red blood cell [#]
Ret-He	Reticulocyte hemoglobin equivalent [pg]
Ret	Reticulocyte
RBCV	Red blood cell volume [mL]
SaO_2	Arterial oxygen saturation [%]
STFR	Soluble transferrin receptor
TDSSA	Technical document for sport specific analysis
TE	Typical error [%]
TEM	Typical error of measurement [%]
TBV	Total blood volume [mL]
TPP	Training performance profile
UCI	International Cycling Unit
VO_2 max	Maximal oxygen uptake [mL/kg/min]
WBC	White blood cell [#]
HbCO	Carboxyhemoglobin [%]

CHAPTER ONE

INTRODUCTION

1. Introduction

1.1 Blood Doping in sports

1.1.1 Basics of human aerobic performance

Sport performance relies on a multitude of trainable (e.g. stamina, strength, toughness, etc.) and genetic determinants. However, neither training nor genetic factors alone can account for performance (Thompson & Binder-Macleod, 2006). Instead, an appropriate combination of those determinants is similar to the ingredients of a recipe: just as a good cake can be made with different types of flour or a varying number of eggs, sport performance is complex and multifactorial (Tucker & Collins, 2012). Concurrently, to meet the specific needs of a sport, physiological and biochemical systems (our genetic factors) must function well together and be adequately together and be appropriately trained for the specific nature of the sport.

In endurance sports, one of the major determinants of performance is the capacity to use the oxidative pathway as the main supply of energy, earning these activities the name “aerobic” sports. This pathway uses oxygen (O_2) to produce adenosine triphosphate (ATP) and has the capacity to sustain prolonged exercise (Joyner & Coyle, 2008). Overall, aerobic capacity is highly determined by the capacity of the blood to carry O_2 to the working muscles (di Prampero & Ferretti, 1990; Ekblom et al., 1975; Wagner, 1996). As an unethical way to achieve higher aerobic performances, some athletes use blood doping (e.g., erythropoiesis-stimulating agents [ESA], hypoxia-inducible factor [HIF] stabilizers or blood transfusions) to increase the amount of O_2 transported throughout the body (Jelkmann, 2016) or as a non-physiological increase to the carrying capacity of O_2 in the blood.

As O_2 is a multi-functional energy source required by the entire organism, including for ATP production, it is needed for proper muscle function (Chinopoulos et al., 2014). This means that endurance performance is primarily limited by the maximum capacity of the cardiorespiratory system to transport O_2 from the surrounding air to the mitochondria of the muscle, which is termed the maximum oxygen consumption or VO_2 max (di Prampero & Ferretti, 1990; Ekblom et al., 1975; Wagner, 1996).

This VO_2 max is a trainable factor that varies between individuals (Wagner, 2011). It has been demonstrated that elite endurance champions reach values as high as 70–85 ml of O_2 /kg/min, which is roughly double the values for sedentary men (i.e. 30–40 ml of O_2 /kg/min) (Rogers et al., 1990). Furthermore, the VO_2 max values for women are around 10% lower than for men, mainly due to lower hemoglobin concentrations ([Hb]) and higher levels of body fat (Joyner & Coyle, 2008). Generally, O_2 is transported via hemoglobin (Hb) (97–98%), with a small percentage remaining dissolved in the plasma volume (PV) (2–3%) (Arora & Tantia, 2019). Hb is a determinant of the arterial oxygen content (CaO_2) and is expressed in mL of O_2 per unit of the total blood volume (TBV). This corresponds to the following formula:

$$CaO_2\,(\text{vol}/100\ \text{mL}) = ([\text{Hb}] \times 1.34 \times SaO_2) + (0.003 \times PaO_2),$$

where 1.34 refers to the Hüfner constant representing the oxygen-carrying capacity of Hb in mL of O_2 per g, whereas SaO_2 is the oxygen saturation of the blood. According to Henry's law, $0.003 \times PaO_2$ indicates the amount of dissolved oxygen, which is proportional to the partial pressure of oxygen (PaO_2) and its solubility coefficient (0.0031 mL / mmHg of O_2 / dL of blood). In a physiological state, CaO_2 represents approximately 16–20 mL of O_2 per 100 mL of blood.

As O_2-carrying capacity of blood is one of the pillars of endurance performance, some athletes may be tempted to increase this capacity using blood doping or blood manipulations to thus enhance their performance

1.1.2 Blood doping and the World Anti-Doping Agency

The scandal at the 1998 Tour de France initiated the creation of the World Anti-Doping Agency (WADA) in 1999 (Wagner et al., 2010) to harmonize doping testing programs worldwide and prevent inconsistencies between countries (Ljungqvist, 2014). Doping in competitive sports is defined by WADA, as a transgression of the anti-doping rules. Both the rules and its transgressions are listed in ***The Code*** (WADA, 2021c), the central document in which modern anti-doping is built. Doping is "the occurrence of one or more of the anti-doping rule violations" set forth in articles 2.1 to 2.11 of *The Code* (Table 1) (WADA, 2021c).

Table 1. *Anti-doping rules. Articles 2.1–2.11 of The Code, adapted from WADA, 2021.*

2.1	Presence of a prohibited substance or its metabolites or markers in an athlete's sample
2.2	Use or attempted use by an athlete of a prohibited substance or a prohibited method
2.3	Evading, refusing or failing to submit to sample collection by an athlete
2.4	Whereabouts failures by an athlete
2.5	Tampering or attempted tampering with any part of doping control by an athlete or other person
2.6	Possession of a prohibited substance or a prohibited method by an athlete or athlete support person
2.7	Trafficking or attempted trafficking in any prohibited substance or prohibited method by an athlete or other person
2.8	Administration or attempted administration by an athlete or other person to any athlete in-competition of any prohibited substance or prohibited method, or administration or attempted administration to any athlete out-of-competition of any prohibited substance or any prohibited method that is prohibited out-of-competition
2.9	Complicity or attempted complicity by an athlete or other person
2.10	Prohibited association by an athlete or other person
2.11	Acts by an athlete or other person to discourage or retaliate against reporting to authorities

Blood doping is a specific form of doping related to the use of methods (e.g. transfusion) or substances (e.g. recombinant human erythropoietin [rHuEPO]) to enhance the O_2 transport capacity of the blood. Prohibited substances are listed in ***The Prohibited List***, and the presence of their metabolites or markers in urine or blood samples are forbidden or under strict rules.

This *List* is composed of various sections grouping the prohibited categories (Table 2) and is one of eight mandatory ***International Standards*** (Table 3).

Table 2. *(S0–S5, M1–M3) Substances (S) and methods (M) prohibited at all times (in and out-of-competition), (S6–S9) substances and methods prohibited in competition, (P1) substances prohibited in particular sports. Adapted from WADA, 2021.*

SO	Non-approved substances
S1	Anabolic agents
S2	Peptide hormones, growth factors, related substances and mimetics
S3	Beta-2 agonists
S4	Hormones and metabolic modulators
S5	Diuretics and masking agents
S6	Stimulants
S7	Narcotics
S8	Cannabinoids
S9	Glucocorticoids
M1	Manipulation of blood and blood components
M2	Chemical and physical manipulation
M3	Gene and cell doping
P1	Beta-blockers

Table 3. *List of the International Standards. Adapted from WADA, 2021.*

Code Compliance by Signatories
Education
Prohibited List
Therapeutic Use Exemptions (TUEs)
Testing and Investigations
Laboratories
Results Management
Protection of Privacy and Personal Information

In addition to *International Standards*, WADA also edits ***Technical Documents*** to complement the *International Standard for Laboratories*, one of which is the ***Technical Document for Sport Specific Analysis (TDSSA)***. This document details a minimum level of analysis (MLA) required for specific disciplines and for specific substances (e.g. ESA, growth hormone [GH]). For instance, when a sport has an MLA for ESA of 15%, it is strongly recommended that the sport implement the ABP. When the MLA is 30% or greater, the implementation of the ABP is mandatory (WADA, 2022).

Athletes at the highest level of their sport are part of the registered testing pool (RTP). As per a rule set in *The Code* in 2003, those athletes that are included in a RTP of a National Anti-doping Organizations (NADO) or International Federation (IF) have to report their location throughout the year (Hanstad & Loland, 2009). Nevertheless, the first unannounced pre-competition blood test program was launched in 1997 (Zorzoli, 2005), where athletes could be tested in and out of competition.

1.1.3 Prohibited substances and methods

Endurance performances can be enhanced through several different mechanisms of blood doping, a few of which are described below. This is by far not an exhaustive list of prohibited substances or blood doping methods described in *The List*. Below are examples to demonstrate how blood doping can increase blood O_2 capacity.

a) **The use of rHuEPO**, a biopharmaceutical drug, mimics the hormone erythropoietin (EPO). It is a substance banned by WADA and in the prohibited list falls under the section "S2. Peptide hormones, growth factors and related substances". In its natural state, EPO is an endogenous glycoproteic hormone that is mainly secreted by the kidneys, though the liver also plays a small role, with < 10% of the total EPO production stemming from here (Eckardt, 1996; Robinson et al., 2006). Through their O_2 sensor, the kidneys react by increasing renal production and releasing EPO to increase the release of O_2-carrying transporters (Montero et al., 2017). EPO is known for its pleiotropic effects; indeed, rHuEPO has shown interesting results for many diseases, such as the treatment of patients with anemia due to chronic kidney failure (Cody & Hodson, 2016), human immunodeficiency virus infection, cancer or surgery. This hormone can a) modulate the inflammatory and immune response, b) produce a hemodynamic and vasoactive effect, c) have a proangiogenic factor and d) produce

neuroprotective effects through its local EPO receptor (e.g. EpoR) (Brines et al., 2000; Grasso, 2006; Robinson, 2006).

Global commercialization of the first rHuEPO, epoetin alpha occurred between 1987–1989, and it was placed on *The Prohibited List* of banned substances in 1990 by the International Olympic Committee (IOC) Medical Commission, which was created in 1966 to protect athletes from doping practices. Nevertheless, at this time, no test was able to detect rHuEPO, and this did not become possible until 10 years later when urine testing was developed, based on an isoelectric focusing technique (IFT)[1] (Lasne & de Ceaurriz, 2000). Blood doping in general has been banned by the IOC since 1984.

There are currently three generations of EPO on the market: 1) the first generation of epoetin alpha; 2) the second generation novel erythropoiesis-stimulating protein (NESP), which has a longer half-life to reduce its administration regimen (Egrie & Browne, 2001) and 3) the third generation continuous erythropoietin receptor activator (CERA) consisting of methoxy polyethylene glycol-epoetin beta that can be detected from serum using a specially designed enzyme-linked immunosorbent assay (ELISA). This latter drug is normally used to treat patients with chronic kidney disease (Lamont et al., 2009) and also has a long detection window allowing only once-a-month administration (Salamin et al., 2017).

The effect of rHuEPO in treatment lasts longer than the duration of hematologic changes associated with rHuEPO misuse, which makes it difficult to detect for anti-doping purposes. Its detection is even more difficult when athletes microdose rHuEPO[2]. Microdoses are eliminated more quickly and do not induce large variations in blood

[1] IFT is a technique for separating proteins based on their isoelectric PH vales.

[2] To reduce the detection window of EPO, athletes microdose rHuEPO daily or every second day, with doses corresponding to approximatively <10 IU/kg body weight instead of a regimen with higher doses (approximately 30 IU/kg body weight once or twice a week) (Reichel et al., 2021).

biomarkers. Additionally, it is even harder to detect rHuEPO when the treatment ended several days prior to testing (Varlet-Marie et al., 2004), making EPO testing most accurate when tested outside of competition windows (Puchowicz et al., 2018).

b) **The use of blood transfusions** is banned by WADA under the prohibited methods "M2. Chemical and physical manipulation". Athletes can infuse blood in two ways, with either "autologous" blood transfusions (ABTs), where an athlete withdraws their own blood (withdrawal phase) and reinfuses it or its components (reinfusion phase), or "homologous" blood transfusions (HBTs), where the blood comes from another compatible donor (Lamberti et al., 2018). HBTs were famous in the 70's in endurance athletes, but their use has decreased with the introduction of rHuEPO to the market (Giraud et al., 2009). Both ABTs (Solheim et al., 2019; Berglund & Hemmingson, 1987) and HBTs (Giraud et al., 2009) increase aerobic capacity. For instance, even a small volume of reinfused blood (e.g. low autologous transfusions), as low as 135 mL of stored RBCs (i.e. approximately 225 mL of whole blood[3]), increases exercise performance for varied exercise intensities. These increases range from 70–100% of absolute peak oxygen consumption (VO_2 peak) and duration (between 5–45min), as analyzed by a literature review (Solheim et al., 2019). On the contrary, a blood donation, which leads to subsequent hemodilution to a reduced [Hb], is therefore accompanied by a reduced aerobic performance (Ziegler et al., 2015).

[3] As a comparison, when donating blood, 450 mL of whole blood is taken (Transfusion CRS Suisse).

c) **The use of testosterone**, which is a key male sex hormone produced by the adrenal glands. At a molecular level, the endogenous structure of testosterone is identical to that administered exogenously, which makes its detection more complicated (Solheim et al., 2020). Testosterone regulates many physiological processes: fertility, muscle mass, fat distribution and RBC production (Bain, 2008). Indeed, testosterone stimulates the production of RBCs and suppresses hepcidin, a hormone secreted by the liver that is involved in iron metabolism (Bachman et al., 2010). Hepcidin is the principal regulator of iron absorption and its distribution to tissues (Nemeth & Ganz, 2009). The suppression of hepcidin increases iron absorption and systemic iron transport (Bachman et al., 2010). The hepcidin-suppression effect of testosterone is apparent within one week of testosterone use. It has been shown that the use of testosterone can cause an increase in young red blood cells, termed reticulocytes (Ret%) (Solheim et al., 2020).

d) **The use of hypoxia-inducible factor (HIF) stabilizers,** which are heterodimeric transcription factors composed of α- and β-subunits (Wang & Semenza, 1995). They are a strong regulator in oxygen homeostasis (Semenza, 2012). HIFα is strongly dependent on O_2 tissue concentration. Briefly, when a condition with less O_2 is available for the tissue (i.e. hypoxic conditions at altitude), a stabilization and accumulation of HIFα occurs (Beuck et al., 2012) that stimulates the genes required to compensate for the hypoxic environment (e.g. angiogenesis, erythropoiesis and glucose metabolism) (Bruick & McKnight, 2001). Through those mechanisms, performances are increased mainly by oxygen transport rise related to erythropoiesis. This substance was added to the WADA list in 2011, under the section "S2. 1.2 Hypoxia-inducible factor (HIF) activating agents", which also include agents such as cobalt and xenon. Xenon is a noble gas used in medicine as an anesthetic agent and that also has an important activation effect on HIF-α (Frampas et al., 2017). Cobalt, a naturally present element, has also

been shown to enhance the hypoxia-like response through an increased activation of HIF-α (Lippi, 2006).

e) **The use of genes and cell doping**, included in *The List* under prohibited substances and prohibited methods (M3), is another way for athletes to increase blood O_2 capacity (Haisma & Hon, 2006). Gene doping represents the *"nontherapeutic abuse of cells, genes, genetic elements, or of the modulation of gene expression, having the capacity to enhance athletic performance"* (Haisma & Hon, 2006, p.258).

To mask the use of prohibited substances or prohibited blood manipulations, athletes can use substances such as polysaccharide-based plasma volume expanders (PVEs), including hydroxyethyl starch (HES), dextran, physiogel and albumin. All these compounds impact the ABP by inducing a PV expansion, meaning they can confound anti-doping blood sampling and some blood biomarkers expressed in concentration (Robinson et al., 2003).

1.2 The Athlete Biological Passport (the hematological module)

1.2.1 Emergence of the Athlete Biological Passport (ABP)

Before the introduction of the athlete biological passport (ABP), the primary tool in doping tests was the direct detection of prohibited substances in urine and blood obtained from athletes (Sottas et al., 2006). This method is still used in anti-doping and is related mainly to the first rule of The Code presented above: "2.1. Presence of a prohibited substance or its metabolites or markers in an athlete's sample". However, this method is limited since it is difficult to determine whether a substance was produced by the human body (endogenous) or originated outside of the body (exogenous), hence present due to doping. When ESAs such as rHuEPO arrived on the market, they were quickly adopted by athletes for doping purposes. Initially,

international sports federations such as the International Cycling Union reacted by introducing upper limits for HCT first and then [Hb] (Malcovati et al., 2003). Using the argument that high [Hb] and HCT levels posed a health risk, athletes with values above the limits received a no start ruling (Saugy & Leuenberger, 2020). However, this regulation was unsatisfactory since it implied a tacit acceptance of blood doping up to a defined limit. Moreover, false positives cases were also highlighted (Schumacher et al., 2000).

The statistical notions of specificity and sensitivity play a key role here. For instance, drug tests prioritize specificity over sensitivity, meaning the priority lies in correctly identifying true-negative results. In the context of anti-doping (Table 4), false-negative results lead to underestimating the true values because of a lack of sensitivity (Sottas et al., 2011).

Table 4.*Test result possibilities regarding the condition of the athlete. Sensitivity = [a/(a+c)]×100; Specificity = [d/(b+d)]×100; Positive predictive value (PPV) = [a/(a+b)]×100; Negative predictive value (NPV) = [d/(c+d)]×100. Adapted from Threvethan, 2019.*

Test results	**Athlete doped**	**Athlete undoped**
Positive	a. True positive	b. False positive
Negative	c. False negative	d. True negative
	Sensitivity	**Specificity**

To exclude a potential disease in medicine, for instance, the expected results of a biological marker should be between a certain range that includes 95% of the population. Nevertheless, it has been shown that the smaller the ratio between intra- and inter-individual, the better for early diagnosis or detection (Robinson, 2008), such as the efficient early detection of cancer (McIntosh & Urban, 2003). Nevertheless, this approach is unsatisfactory for anti-doping as, firstly, the population used to set the reference ranges consists of undoped athletes, and secondly, an increase in the number of subjects increases the possibility of obtaining a false positive (Sottas, 2008). More generally, this approach is frequentist therefore one needs a defined number of one needs a defined number of measures (stopping rule), which raises issues

in the context of anti-doping, as we do not know how many times the athletes will be tested. More, in the frequentist approach confidence intervals cannot be interpreted as the probability that the parameter lies within these limits but in terms of replication (Dienes, 2011).

Because of these drawbacks, the Bayesian inference approach has been chosen for testing. Globally, the Bayesian approach overcomes a few of the challenges and misinterpretations related to the frequentist approach of statistics (Dienes, 2011). The Bayesian inference model and a Bayesian network have been chosen and used in the adaptive model of the ABP (Sottas et al., 2010), where prior probability distribution and likelihood are combined to estimate posterior distribution. The following equation represents the probability as proposed by the Bayes theorem (Sottas, Robinson, et al., 2008):

$$\mathrm{P}(\mathrm{D} \mid \mathrm{M}) = \frac{\mathrm{P}(\mathrm{M} \mid \mathrm{D}) \cdot P(D)}{P(M)}$$

where D represents the "doped" state, M represents the blood sample measurement, P(D) represents the prior, $\mathrm{P}(\mathrm{M} \mid \mathrm{D})$ the likelihood, and $\mathrm{P}(\mathrm{D} \mid \mathrm{M})$ the posterior.

The Bayesian approach of the ABP allows the detection of atypical values by confidence intervals of parameter interpreted in term of a probability (i.e. applied specificity that is the chance or less that the deviation from the interval is due to normal physiological variation), explaining why the ABP was built in a Bayesian framework. The Bayesian inference allows us to address the following question: "Given the data, what is the credibility of a model?" The question is answered by re-allocating credibility through the Bayes theorem (i.e. the likelihood principle, which is derived from individual blood marker results), which links conditional probability of a parameter with prior probability of a parameter, as well as conditional probability of data according to a parameter, where the latter shows a re-allocation

of credibility. In the context of anti-doping, the Bayesian inferential technique “switches the focus from comparison with a population to the determination of individual values” (Sottas et al., 2009). To achieve this, the Bayesian model uses reference ranges and inter-individual variability for a specific population (i.e. non-doped endurance athletes) (Malcovati et al., 2003). Bayesian inference then allows for individual limits of each ABP marker to be set for a desired specificity (i.e. 99%) according to several factors. These factors are heterogeneous (i.e. age, sex, genotype and ethnic origin), external confounding (i.e. pharmaceutical preparations use, exposure to altitude and medical conditions) and related to sample collection (i.e. transport and analysis) (Sottas et al., 2009). The Bayesian adaptive model is applied to evaluate the probability that the data is due to normal physiological conditions, considering confounding factors (Zorzoli, 2011b). The ABP requires a specificity of 99%, meaning only one similarly matched individual out of 100 should present a value outside of the limits (Sottas et al., 2009).

Bayesian networks allow for a representation of the causal relationship between blood doping and its effect on hematological biomarkers (Koski & Noble, 2009; Kruschke, 2015). This network involve multiple potential confounding factors (i.e. gender, ethnic, sport, analyzer) (Figure 1). Potentially confounding factors resulting from individual differences are still being integrated.

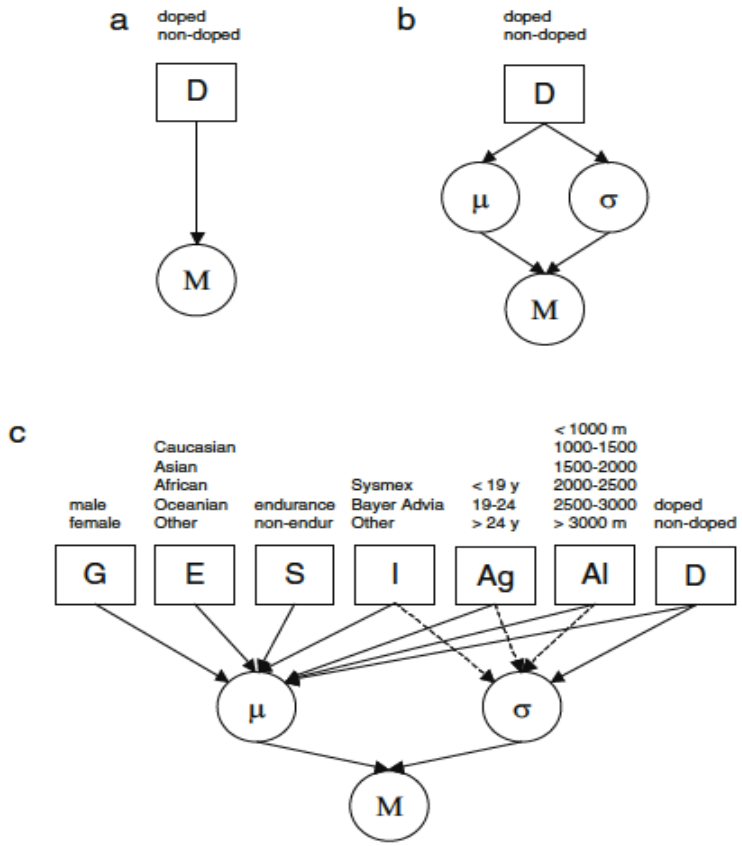

Figure 1. *Bayesian networks for evaluating an indirect marker of blood doping (M). Each rectangle presents a discrete variable, each circle a continuous variable and each arrow a causal relation. (a) "D" represents the doping status of the athlete. Doping (the cause) has an effect on the marker (the effect), and the goal is to know if the athlete is in the category "doped" or "non-doped" in light of the result of the marker M. (b) A longitudinal approach may be modelled by making two variables explicit: the expected mean and standard deviation of the sequence "M" values. (c) Heterogeneous factors known to influence the result of the marker (G = gender, E = ethnic origin, S = sport, I = instrument, Ag = age, Al = altitude), their respective classes and causal relations. Gender, ethnic origin and sport are fixed factors; instrument, age and altitude are time-varying factors. Taken from Sottas et al., 2009.*

In response to highly sophisticated doping regimens, the ABP was first introduced in 2008, after the Puerto scandal in 2006, as an indirect method to reduce doping practices. The ABP can indeed infer doping though repeated biomarker measurement. Instead of directly looking for prohibited substances or their metabolites in biological samples, the ABP uses doping biomarkers (Sottas et al., 2011). The ABP is therefore part of the second rule of *The Code* "2.2. Use or attempted use by an athlete of a prohibited substance or a prohibited method".

The ABP has been proven to be a doping deterrent. Analyses of samples from elite track and field athletes revealed an overall decrease in [Hb] between two major events (World

Championships in Daegu in 2011 vs. in Moscow in 2013), suggesting that the introduction of the ABP in athletics between those two events potentially triggered a change in doping behavior (Robinson et al., 2016). Analyses of indirect blood doping signs also point towards a doping decrease. Faiss et al. estimated an overall blood doping prevalence of 18% among endurance athletes in major international track and field events in 2011 and 2013 (Faiss et al., 2020). This suggests that blood doping continues to be used. Even though this data brings us pertinent information, it needs to be updated with recent events. Other research showed a decrease in the extreme values of Ret% (Zorzoli, 2011). The ABP is now able to highlight the use of ESA (Schumacher & d′Onofrio, 2012) and blood transfusions, including ABT (Pottgiesser et al., 2012), making it a relevant tool in anti-doping systems (Devriendt et al., 2019). Nevertheless, the ABP has also brought numerous challenges, including tracking doping, understanding and following changes in doping behavior among athletes, and distinguishing between exogenous vs endogenous substances, even in small amounts (Aikin et al., 2020). Since its introduction, over 200,000 ABP samples have been collected in Olympic sports (WADA, 2019), and more than 700 athletes were identified and sanctioned for blood doping practices (Faiss et al., 2020). In addition, the ABP has led to targeting accurate test timing for athletes and teams (Vernec, 2014).

The ABP is composed of hematological and steroidal modules (Sottas et al., 2011), with this thesis focusing on the hematological module. The following section provides a description of how it works and its included biomarkers.

1.2.2 How does this ABP works?

The pinnacle of the ABP method is the repetition of biomarker measurement over time for a single athlete such that the athlete becomes their own reference. The first measure of a blood marker is compared to population-derived reference ranges. As an example of the application of the Bayesian approach, Sottas et al. (2008) proposed the case of an elite female Caucasian

endurance athlete who was longitudinally tested for [Hb]. After applying the Bayesian network with a specificity of 99.9% with a population-based threshold fixed at 160 g/dL, the initial value found was 166 g/dL. In other words, there is only 1:1000 chance that the value would be higher than 166 g/dL (for 1000 athletes with the same characteristics).

Each athlete then constitutes their own reference for all subsequent values, with narrowing limits. Those limits are continuously adapted according to all past values. This repeated sampling allows the ABP to track the variation of hematological parameters to define an athlete's individualized hematological profile and identify deviations from the expected values. The adaptive model then predicts an expected range of values for the variables within the individual athlete's ABP profile (Figure 2), while assuming a normal physiological condition.

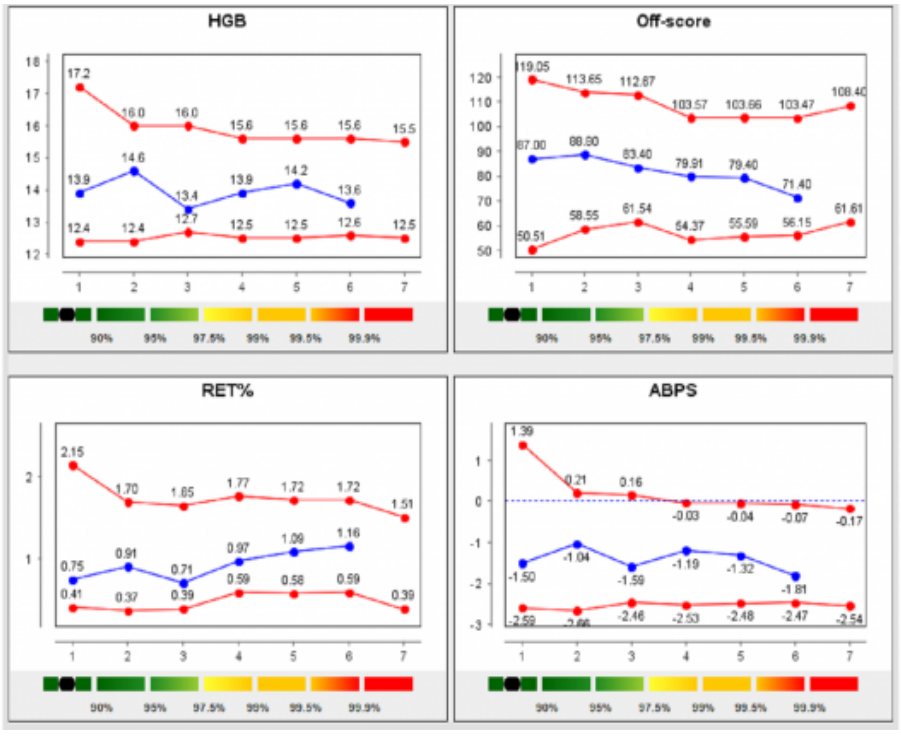

Figure 2. *Example of an athlete biological passport (ABP). Red lines correspond to the individualized range for the athlete, calculated with the Bayesian model. Blue dots represent the actual values of the samples. HBG; hemoglobin concentration, off-score; OFF-score, RET%; reticulocytes percentage, ABPS; abnormal blood profile score. Retrieved from Antidoping.ch.*

1.2.3 Blood biomarkers used in the ABP

A "biomarker" (i.e. "biological marker") refers to "*an objective indication of the medical state outside the patient, which can be measured accurately and reproducibly"* (Strimbu & Tavel, 2010). Monitoring biomarkers in an individual shows the effect of an intervention (e.g. altitude training, changes in training load, nutrition, health status) or treatment on the physiological state of the body (Saugy & Leuenberger, 2020). In other words, we first look at the effect to understand the cause.

For anti-doping, several biomarkers were chosen to be a part of the hematological module of the ABP; they belong to the "biological cascade" influenced by the application of forbidden manipulation and have the potential to increase athletic performance (Saugy et al., 2014). These markers include [Hb], red blood cells (RBCs) and RBC indices (e.g. mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], mean corpuscular hemoglobin concentration [MCHC], red blood cell distribution width [standard deviation] [RDW-SD] and immature RBCs, like reticulocytes [Ret] and immature reticulocyte fraction [IRF]). In addition, the hematological module also includes two composed scores used in anti-doping: the abnormal blood profile score (ABPS) and the OFF-score (OFFS, described further below. Depending on the model of analyzer used, [Hb] and RBC# can be directly measured by an automated analyzer, whereas other biomarkers are sometimes calculated together (e.g. MCV, MCH, MCHC, RDW-SD, OFFS, ABPS).

Biomarker definitions, their values for the general population, and their relevance in sports and in anti-doping are specified below. For the general population, normal ranges of values of these parameters are useful for diagnosing various ailments, notably the different types of anemia as well as some forms of malignancy (see also below). Some reference values found in an athletic population are presented in Table 5.

1.2.3.1 Hemoglobin (Hb)

Definition:

Hemoglobin (Hb) is a tetramer composed of four polypeptide chains with a heme group. Hb is an iron-containing protein, which allows O_2 to be reversibly bound to the heme group and transported to the tissues. Hb can carry up to four oxygen molecules via the four polypeptide chains (Figure 3) and millions of these proteins are present in the RBCs of a human (Tombak, 2019). Hb is often represented as a concentration ([Hb]), which means Hb will be depicted in grams for a given total blood volume (TBV).

$$[\text{Hb}] = \frac{Hb\ (g)}{TBV\ (dL)}.$$

Relevance to sports:

Hb has a key role in aerobic capacity as it is a protein that carries O_2 to the working muscles. Nevertheless, since Hb is expressed in concentration, an alteration of TBV due to changes in plasma volume (PV) or red blood cell volume (RBCV)[4] may alter its value. As an example, low [Hb] concentrations are associated with anemia. "True" anemia is characterized by values of < 14 g/dL (140 g/L) in men and < 12 g/dL (120 g/L) in women (Braunstein, 2021), though "pseudoanemia", characterized by a low [Hb] due to PV expansion, is often present in athletes (Carlson & Mawdsley, 1986).

Hemoglobin; relevance to anti-doping:

As it can be influenced by blood doping, Hb monitoring helps track suspicious practices, though this value is highly sensitivity to changes in PV. [Hb] was first referenced by the International Ski Federation during the season 1996-1997, who authorized athletes to compete when presenting values under 165 g/dL for women and 185 g/dL for men. Here, values above those

[4] These volumes will be described in more detail in Section 1.3 of this thesis.

references introduced the notion of "no start", wherein an athlete was prevented from competition start, which was also adopted by the International Cycling Unit (UCI) in 1997 (Saugy & Leuenberger, 2020). This rule acted as a deterrent for the use of exogenous EPO (i.e. rHuEPO), as it was undetectable at this time. The use of rHuEPO and ABT (reinfusion phase) both increase [Hb] (Salamin et al., 2016). Today, [Hb] has become a primary marker of the ABP.

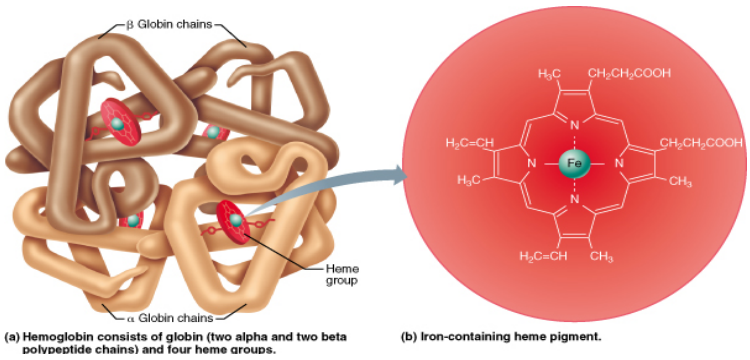

Figure 3. *Hemoglobin molecule. Taken from Marieb & Hoehn, 2007.*

1.2.3.2 Hematocrit (HCT)

Definition:

Hematocrit (HCT) is the ratio between RBCV and TBV (Van Beaumont, 1972). It is expressed in percentage of TBV.

Relevance to sports:

A paradox exists in HCT interpretation: on the one hand, an increase of HCT means a higher [Hb], which induces more O_2-carrying transporters. On the other hand, however, when HCT is lower, microcirculation increases to more easily deliver O_2 to the working muscles (Schumacher et al., 2000). Anemia is defined with an HCT < 42% in men and < 37% in women (Braunstein, 2021).

Hematocrit: relevance to anti-doping:

HCT has been used in anti-doping, along with [Hb], as a cut-off threshold set at 50% for men and 47% for women. This corresponds to the 95th percentiles for both sexes in the general population (Saugy & Leuenberger, 2020). The use of rHuEPO and ABT (reinfusion phase) both increase HCT, whereas ABT (withdrawal phase) decreases HCT (Salamin et al., 2016).

HCT (%) is currently included in the ABP.

1.2.3.3 Reticulocytes (Ret)

Definition:

Ret are immature RBCs. They are transitional cells that are present for 1–4 days in the blood stream (Banfi, 2008). Ret are expressed as a percentage (%) of all RBCs or as a count (10^6/μL). Normal values for adults are between 0.5–1.5%, or as an absolute number from 50,000–150,000/μL, or 50–150 × 10^9/L (Braunstein, 2021).

Relevance to sports:

The higher rate of intravascular hemolysis, or RBCs destruction, occurring in athletes during sport performance increases Ret (Banfi, 2008). Ret are used in the differential diagnosis of regenerative versus non-regenerative anemia (Carlson & Mawdsley, 1986), which shows whether the body is able to compensate for the anemia by stimulating the bone marrow to produce new RBC.

Reticulocytes: relevance to anti-doping:

Ret are the biomarkers that are the most sensitive in identifying bone marrow stimulation (e.g. ESA), making them a useful measurement in anti-doping (Banfi, 2008; Naud et al., 2019). Since 2001, high Ret% values trigger a subsequent urine sample for EPO analysis (Saugy &

Leuenberger, 2020). A cut-off value (> 2.4%) also triggers suspicion of beginning a rHuEPO treatment (e.g., epoetin alfa [Eprex®], epoetin beta [Recormin®]) (Robinson et al., 2003). Indeed, the use of rHuEPO as well as ABT (withdrawal phase) increases Ret%, whereas the ABT (reinfusion phase) decreases Ret% (Salamin et al., 2016).

1.2.3.4 Red blood cell count (RBC#)

Definition:

Forming approximately 45% of the TBV, RBCs are highly deformable, biconcave particles with a diameter of 8 μm. These cells circulate for 100–120 days in the blood stream before being engulfed by macrophages and replaced by reticulocytes (Ret) (Jelkmann, 2016). The turnover is about 1% of the 25×10^{12} cells produced each day. The main function of RBCs is the transport of O_2 from the lungs, where it enters the body, to the tissues. RBC is often expressed in count (i.e. RBC#), represented as $10^6/\mu L$.

Relevance to sports:

RBC#, and more precisely the RBCmass comprising the part of the TBV occupied by the RBCs, is associated with endurance performance. A decrease in RBC# has been observed in impact-sports, such as running, and can cause a so-called “foot-strike hemolysis” (Eichner, 1985; Hunding et al., 2009). Anemia is characterized by values of $< 4.5 \times 10^{12}/L$ in men and $< 4 \times 10^{12}/L$ in women (Braunstein, 2021).

Red blood cell count: relevance to anti-doping:

RBCs are sensitive to various types of blood doping. For example, the use of exogenous EPO promotes the production of RBCs (Jelkmann, 2016) and autologous or homologous transfusion instantly increases the number of RBCs. Thus, an increase or decrease in RBCs, similar to the

withdrawal of blood to be reinfused later on, can be suspicious. It is currently listed as one of the ABP biomarkers.

1.2.3.5 Red blood cells indices

Mean corpuscular volume (MCV)

Definition:

This is the measure of the average volume of a single RBC. It is expressed in femtoliters (10^{-15}; fL). Normal values are between 80–100 fL.

$$\text{MCV} = \frac{HCT\ (\%) \times 10}{RBC\#}.$$

Relevance to sports:

MCV helps to diagnose specific types of anemia: a low MCV (< 80 fL) (i.e. microcytosis) indicates that the RBCs are too small, a high MCV (> 100 fL) (i.e. macrocytosis) indicates that the RBCs are too large or normal values indicate that despite anemia, the RBC are of normal size (i.e. normocytic). In addition, as exercise induces metabolic acidosis, MCV has been shown to increase, but the mechanism for this remains unclear (Banfi, 2008). Interestingly, MCV was also shown to increase after a 10-day stage race in elite cyclists (Lombardi et al., 2013).

Mean corpuscular hemoglobin (MCH)

Definition:

MCH is the amount of Hb per RBC. It is expressed in picograms (pg) per cell. Normal values are 29 ± 1 pg/cell.

$$\text{MCH} = \frac{Hb\ (g/dL)\ x\ 10}{RBC\#}.$$

Relevance to sports:

MCH is used in the diagnosis of anemia (Beyan et al., 2005) and nutriment deficiency, and it aids in the differentiation between hypochromic (< 27 pg/cell) and normochromic anemia.

Mean corpuscular hemoglobin concentration (MCHC)

Definition:

MCHC is the amount of hemoglobin per unit of volume. It is expressed as g/dL of RBC or as a percentage. Normal values are between 32–36 g/dL.

$$\text{MCHC (g/dL)} = \frac{Hb\ (g/dL\ ) x\ 100}{HCT\ (\%)}.$$

Relevance in sports:

Though low levels of MCHC have been shown to be more common in women with an iron deficiency, MCHC nevertheless demonstrates poor sensitivity and specificity (Robertson & MaClean, 1970). Lombardi et al. observed a decrease in MCHC after a 10-day stage race in elite cyclists (Lombardi et al., 2013), highlighting that MCV might not have any physiological influence. Low values of MCHC indicate hypochromic microcytic anemia. For iron-depleted athletes, iron supplementation increased MCHC (Banfi, 2008).

Red blood cell indices (i.e. MCV, MCH and MCHC): relevance to anti-doping:

Red blood cell indices are mostly used to help in the diagnosis of anemia. For anti-doping, MCV, MCH and MCHC, by themselves, may not be sufficient to raise a suspicion of fraudulent practices. In other words, a single value of these indices cannot be taken in isolation as an indication of doping. However, MCV has been shown to be influenced by EPO treatment, as an increase in MCV has been observed on a sample of subjects under EPO treatment compared to a control group (Casoni et al., 1993). After EPO treatment, the macroHypo % also increased (i.e. less content of hemoglobin per RBC). More practically, it has been highlighted that MCV,

MCH and MCHC with the Sysmex XN analyzer (i.e. the same analyzer used for our studies) can also be used to evaluate potential sample deterioration due to problems such as pre-analytical issues (Daves et al., 2015), which is crucial for anti-doping.

1.2.3.6 Red cell distribution width (standard deviation) (RDW-SD)

Definition:

RDW-SD is the variation coefficient of RBC distribution (size) for an individual. It is expressed in fL, with normal values ranging between 40–55 fL.

Relevance to sports:

An increase in RDW-SD is often associated with inflammation (Perlstein et al., 2009) and can also be a sign of a nutrient deficiency, such as folate or B_{12}, which can lead to macrocytic anemia (i.e. fewer but larger RBCs). For iron-depleted athletes, iron supplementation decreased RDW-SD (Banfi, 2008).

Red cell distribution width (standard deviation): relevance to anti-doping:

Any process that releases Ret into circulation will result in an increase in RDW (Bazick et al., 2011). For an increased RDW-SD, experts will suspect a degradation of the blood sampling, as with MCV, which would invalidate the sample. For instance, values of RDW-SD and MCV can be interpreted in terms of the total time between the collection and the analysis of the sample at the laboratory, called the "collection to analysis time" (WADA, 2019).

1.2.3.7 Immature reticulocyte fraction (IRF)

Definition:

IRF is defined as the sum of the high and middle intensity fluorescence fractions and is a highly sensitive biomarker for erythropoietic status (i.e. marrow erythropoietic activity). Automated analyzers can quantify the fraction of each subpopulation of Ret (based on their ribonucleic acid [RNA] content), within regions of low-, middle-, and high-fluorescence intensity (Chang, 1997). Normal values are between 1.6–12.1% (Morkis et al., 2016).

Relevance to sports:

Elite athletes have elevated values of IRF due to the exercise-induced continuous stimulation of their bone marrow as compared to the general population (Nadarajan et al., 2010; Telford et al., 2003). Additionally, IRF measurements have been shown to be a sensitive marker of altitude training (Nadarajan et al., 2010), enabling the monitoring of the athlete's response to altitude. Indeed, IRF% peaks immediately on return from altitude and then decreases within the following days due to the loss of the additional EPO stimuli induced by altitude. IRF also plays an important role in monitoring iron therapy (Geldard et al., 2009) in combination with the monitoring of the reticulocyte hemoglobin equivalent (Ret-He) (Peerschke et al., 2014).

Immature reticulocyte fraction: relevance to anti-doping:

Because IRF and Ret are of great relevance in following the maturation of RBCs in their early stage, they can be a relevant indicator of fraudulent practices. Nevertheless, both sides of these variations (i.e. a decrease or an increase of the values) have to be considered.

1.2.3.8 OFF-score (OFFS)

Definition:

The OFFS is an index of erythropoietic stimulation calculated by combining the [Hb] and Ret%. This stimulation index was introduced in 2004 (Zorzoli, 2005). A range of 85–95 (with a mean of 90) has been found in a population of amateur male athletes (Schütz & Zollinger, 2018).

The OFFS is calculated as followed:

$$\text{OFFS} = ([Hb] \times 10) - 60 \times \sqrt{Ret}\%.$$

OFF-score (OFFS): relevance to anti-doping:

The OFFS is also called the "stimulation index" (Zorzoli, 2011b, p. 206) and is used to indirectly detect blood doping, specifically for rHuEPO administration. The "ON-score" is sensitive to accelerated erythropoiesis, and the "OFF-score" is sensitive to decelerated erythropoiesis (Gore et al., 2003). The function described above was proposed by Gore et al., 2003.

To illustrate how the OFF-score would be influenced, we present an example judged by the Court of Arbitration for Sport in 2017. The athlete presented high values of [Hb] (i.e. 18 g/dL) with a low Ret% (0.20), which leads to a high OFF-score (i.e. 153.20 a.u.). This is typically judged by the panel of experts as a supraphysiologically elevated [Hb] based on the athlete's previous (17.2 g/dL, 0.35% and 136 a.u., respectively) and subsequent (16g/dL, 0.49%, 118 a.u., respectively) blood samplings (TAS, 2017).

1.2.3.9 Abnormal blood profile score (ABPS)

Definition:

ABPS is a score computed from seven biomarkers: HCT (%), [Hb] (g/dL), MCH (pg), MCHC (g/dL), MCV (g/dL), RBC (10^6/μL) and Ret (%). A database of 591 samples (with doped and non-doped athletes) was used to create the algorithm. The score is unit-less and based on two different classification techniques: the Bayesian model and the support vector machine. This creates two different scores that were pooled together as "ensemble averaging" to create the final score (Schütz & Zollinger, 2018). The range is between [-2.35 – 1] (with a mean of 0.67) and has been found in a population of male amateur athletes (Schütz & Zollinger, 2018).

Abnormal blood profile score: relevance to anti-doping:

The ABPS is more sensitive (with an equal specificity level) to doping practices than each biomarker taken separately (Schütz & Zollinger, 2018). It is sensitive to any form of blood doping, including autologous transfusions (Sottas et al., 2006), and is also is sensitive to sample deterioration (time and temperature) (Robinson, 2016). ABPS is considered as a secondary marker in the ABP.

Important note: When blood doping occurs, the temporality of the biomarker responses will not be the same for all biomarkers, as some differences will occur (i.e. an increase in [Hb] and a decrease in Ret% after a re-infusion of an ABT) (Salamin et al., 2106).

Table 5. *Biomarker reference ranges and mean of hematological parameters in amateur male athletes after kernel density estimation. [Hb]: hemoglobin concentration; HCT: hematocrit; RBC#: red blood cell count; Ret%: reticulocyte percentage; Ret#: reticulocyte count; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin content; RDW-SD: red cell distribution width (standard deviation); IRF%: immature reticulocyte fraction; OFFS: OFF-score; ABPS: abnormal blood profile score. Adapted from Sottas et al., 2006.*

Biomarker	Unit	95% Reference range	Mean
[Hb]	g/dL	134–170	151
HCT	%	39.1–49.7	43.9
RBC#	$10^6/\mu L$	4.12–5.93	4.94
Ret	%	0.58–2.22	1.32
Ret#	$10^3/\mu L$	31.2–106.5	64.9
MCV	fL	81.5–98.2	89.1
MCHC	g/dL	21.6–36	34.3
RDW-SD	%	10.9–13.1	11.9
IRF	%	0.13–0.36	0.24
ABPS	a.u	-2.35–1	0.67

Strict protocols are in place to ensure the quality of the blood sample collected and, thus, the determination of biomarker values. The following sections contain descriptions of how blood samples are collected and how the results are interpreted

1.2.4 From sample collection to analysis interpretation

To decrease variability from pre-analytical and analytical factors on the ABP biomarkers, mandatory, standardized protocols have been established (WADA, 2019). The validity of an ABP depends on strict protocols that ensure the quality of the biological material (e.g. blood). WADA defines how samples should be collected, handled and transported to the laboratory, how they should be analyzed and how the results should be evaluated. First, a doping control form (DCF) (WADA, 2021a) gathers personalized athlete information that supports biomarker evaluation (i.e. medication use, hypoxic training/sojourn). Once the blood sample has been drawn from the athlete according to WADA guidelines (WADA, 2019), the Chain of Custody (WADA, 2021b) is responsible for the sample until it is delivered to the laboratory. Each athlete whose has a profile in the Anti-Doping Administration and Management System (ADAMS) must have a Passport Custodian pertaining to one Anti-Doping Organization. Once the blood results have been analyzed by a WADA-accredited laboratory, they are entered into ADAMS. ADAMS then processes the data using a statistical model called the adaptive model, which is described in more detail in the next section. [Hb] and the OFF-score are automatically calculated as primary biomarkers of the ABP, whereas Ret% and the ABPS score are used as secondary biomarkers.

An atypical passport finding (ATPF) is highlighted when at least one primary biomarker falls outside the expected range for the athlete. An "ATPF sequence" can also be identified when the last five values of a primary biomarker deviates from the adaptive model's expected range. Therefore, the [Hb] and/or OFF-score value and the sequence thereof can generate an ATPF. If an ATPF is found, the athlete passport management unit (APMU) sends it (anonymously) to an expert within seven working days. Nevertheless, an ATPF can also be identified by the panel of experts even if it does not have values falling outside the expected ranges.

The experts are carefully chosen by the Anti-Doping Organization (but must be external to the organization) and/or the APMU and must have qualifications in at least one of the following fields: clinical or laboratory hematology, sports medicine or exercise physiology. An Anti-Doping Rule Violation (ADRV), established by the anti-doping tribunal, is the final step in the decision process. ADRVs represent 2% of the tests performed in WADA-accredited laboratories (i.e. not only related to the ABP) (WADA, 2021c).

1.3 Confounding factors

A confounding factor is known as a third variable, or a mixing of effects, which influences the dependent and independent variables. Confounding factors may mask or falsely reveal an association between the cause and effect (Skelly et al., 2012). In scientific studies, distinguishing between the effect of an independent variable versus a confounding one is paramount (Skelly et al., 2012). Thus, in the field of anti-doping, confounding factors such as hypoxic training or blood loss that can alter the ABP could distort the blood results and lead to an ABP misinterpretation. As there are several physiological reasons for hematological change that are not related to doping practices, the ABP's main challenge is the numerous confounding factors that can cause misinterpretation of the blood data (Krumm & Faiss, 2021). In addition to the biomarkers detailed above, an understanding of the various blood volumes is necessary to understand the concept of confounding factors.

1.3.1 Generality on blood volumes

The total blood volume (TBV) of the human body represents the sum of the red blood cell volume (RBCV) and plasma volume (PV), which averages 5–7 L for young male adults (Davy & Seals, 1994). Recently, Falz et al. calculated the TBV for a population of young (20–30 years) men and women to be 6783 ± 903 mL and 4983 ± 640 mL, respectively (2019). With centrifugation, blood can be divided into its various components (Figure 3): a) the plasma or

PV comprising the liquid component of blood and representing approximately 3.5 L for a 70 kg adult (50–55 mL/kg) (Hemmings & Hopkins, 2006), b) the "buffy coat" composed of white blood cells (WBCs) and platelets (PLTs) and representing approximately 1% of the blood and c) the RBCV corresponding to the volume of the blood occupied by RBCs.

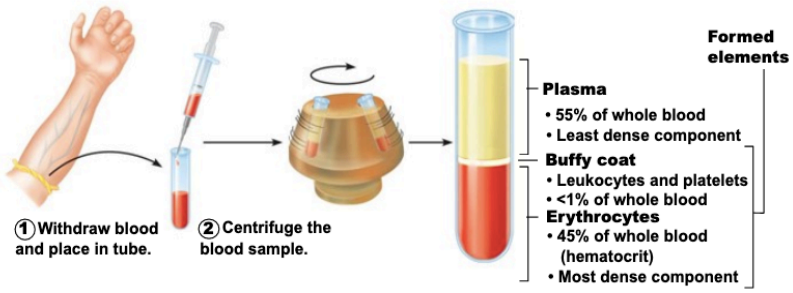

***Figure 3**. The major components of whole blood. Taken from Marieb et Hoehn, 2007.*

Endurance athletes have the advantage of a higher PV compared with team sports athletes, power endurance athletes or untrained subjects (Fellmann, 1992). This induces a higher stroke volume and cardiac output, along with an increase in muscle perfusion, both of which are highly relevant in endurance sports (Convertino, 2007). Additionally, PV helps to regulate body temperature (Sawka et al., 1985). Nevertheless, PV is the most vulnerable component of the blood to acute and chronic variations. Many sources of PV variation are common for athletes (e.g. exercise training, hydration, exposure to extreme environments [heat/cold] or altitude), so analyzing data regarding these possible shifts are paramount for an accurate interpretation of these fluctuations. More importantly, shifts in PV can lead to greater variations in volumetric biomarkers, such as [Hb].

The measurement of hemoglobin mass (Hb_{mass}) is one such way to better comprehend the concentration-based biomarker results, as it provides the exact amount of Hb (in grams) and is

independent from PV variations. A 1 g increase in Hb_{mass} induces an approximate increase of 4 mL/min in VO_2 max (Schmidt & Prommer, 2010). Hb_{mass} is influenced by several factors, such as training, altitude training or the use of doping agents, all of those stimulate RBC production. The opposite effect, or a decrease in Hb_{mass}, has also been shown in the literature following a period of detraining in athletes (Eastwood et al., 2012; Gough et al., 2013). A comprehensive overview of Hb_{mass} and blood volumes across a wide range of sport disciplines has been presented, highlighting the highest values among elite male cyclists with 15.5 ± 0.4 g/kg and 118.9 ± 3.1 mL/kg, respectively (Zelenkova et al., 2019). For women, the highest values were found in cross-country skiers with 11.3 ± 0.2 g/kg and 91.6 ± 1.8 mL/kg, respectively (Zelenkova et al., 2019). In the general population, studies have shown values for men and women aged from 20–30 years of 13.9 ± 1.5 g/kg and 10.3 ± 1.0 mL/kg, respectively (Falz et al., 2019). Nevertheless, the use of Hb_{mass} can be relevant for the ABP in terms of the influence of various confounding factors influencing PV.

1.3.2 Overview of confounding factors for the ABP

This section presents an overview of the possible confounding factors that can be encountered in the population of athletes, covering first the characteristics of the athlete (i.e. origin, pathology) followed by pre-analytical factors for blood samplings. Next, this section covers the specificity of the specific confounding factors of endurance training and hypoxic conditions.

This thesis aims to discuss three confounding factors that have not been fully evaluated in literature. Recently, a systematic narrative review gathering all the confounding factors for the ABP has been proposed (Krumm & Faiss, 2021). The researchers identified several confounding factors that existed prior to June 2020 (Figure 4).

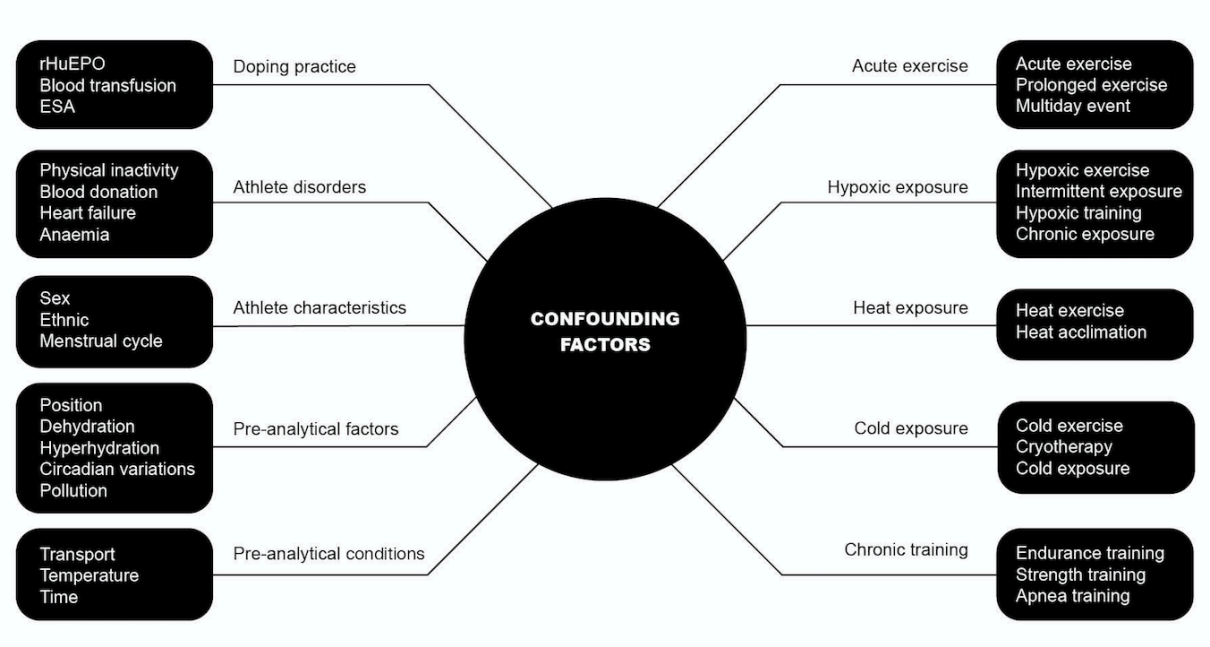

Figure 4. *Illustration of initially identified confounding factors. Adapted from Krumm et Faiss, 2021.*

1.3.2.1 Athlete characteristics

Some confounding factors may be inherent to characteristics specific to an athlete. For instance, ethnicity plays a role in key hematological variables related to both the health and ABP of an athlete (Lobigs, 2013). The author compared five ethnic groups of athletes: Arabic (n = 1145 M, 89 F), Asian (n = 43 M, 4 F), Black (n = 519 M, 43 F), White (n = 302 M, 4 F), and Mixed (n = 73 M, 0 F). Arabic athletes had a significant lower mean for several biomarkers (i.e. MCV, MCH, [Hb], HCT and Ferritin count) and a higher mean for others (i.e. RBC, RDW, total iron binding capacity and soluble transferrin receptor [STFR]). These biomarkers can be related to disorders such as iron deficiency, anemia and/or hemoglobinopathy. In comparison to White athletes, Black athletes had lower mean values of MCV, MCH and [Hb] but higher mean values of Ret% and STFR. Similarly, among 3683 athletes from 6 disciplines at the International Association of Athletics Federations (IAAF) World Championship in Daegu and in Moscow,

Robinson et al. (2019) highlights higher values of [Hb] in African or South African male athletes compared to Oceanian male athletes. For female athletes, Europeans had higher values for [Hb] compared to the others (African, South African and Oceanian female athletes) (Robinson et al., 2019).

In addition to ethnicity, sex also plays a role in interpreting blood values. Recently, the influence of hormones and the menstrual cycle on ABP has been studied. Mullen et al. (2020) found relatively stable ABPs in athletes who were not taking oral contraceptives. Researchers highlighted that menstrual–cycle-related Ret% variations did not alter ABP thresholds. These findings were expanded upon by Moreillon et al. (2022) who studied 15 women taking oral contraceptive pills (ORP) (Moreillon et al., 2022). Blood and serum were collected weekly for 8 weeks, with significant variations found between week 7 and week 1 of an ORP cycle in some of the volume-sensitive biomarkers (i.e. [Hb], HCT and RBC), concomitantly to an increase in PV.

Pregnancy is another state that can alter hematological biomarkers. Often present during pregnancy, anemia is characterized by a decrease in [Hb] and thus a decrease in the oxygen-carrying capacity of the blood. Iron deficiency accounts for 75% of the anemia diagnosed during pregnancy (Sifakis & Pharmakides, 2006). In addition to iron-deficiency anemia, physiologic anemia can also occur during pregnancy. After the 6^{th} week of pregnancy, physiologic anemia can present as an absolute increase in PV that is higher than the RBC mass (Lund & Donovan, 1967).

Anemia is widespread among the athletic population (Garvican et al., 2011). Sports such as running have been shown to induce a higher risk of anemia due to mechanical constraints (Telford et al., 2003), thus affecting RBC (Liu et al., 2018). Iron deficiency alone or in

combination with anemia is often present among endurance athletes (Mechrefe et al., 1997), non-endurance athletes (e.g. basketball players) (Dubnov & Constantini, 2004) and the general population (Sinclair & Hinton, 2005). Multivariate causes of anemia have been highlighted among the athlete population (e.g. hemolysis due to foot strike, iron loss through sweat, inadequate diet and blood loss) (Shaskey & Green, 2000). Anemia is especially high in the second decade of life for the general population (Raunikar, 1992). Though anemia can alter the ABP profile (Malcovati et al., 2003), Parisotto et al. (2013) demonstrated that among a group of 1152 elite athletes, only a few had hematological pathologies, most of which were related to iron store depletion or haemoglobinopathy.

Endurance athletes can also have gastroenteritis, or gastrointestinal tract inflammation, which induces dehydration through fluid loss (i.e. diarrhea, vomiting), temperature increase and sweating. In a case study of five athletes affected by gastroenteritis, Schumacher et al. (2011) investigated if this condition induced variations in the ABP (Schumacher & Pottgiesser, 2011). Interestingly, researchers found that [Hb] was unaffected and PV remained stable despite the dehydration state. This highlights the physiological response of fluid regulation, which stabilizes PV despite illness-induced dehydration.

Blood loss or blood donation can also affect the ABP. When 20 ± 3% of TBV was withdrawn from 10 healthy subjects, representing 1.3 ± 0.2 L, significant changes in blood parameters (e.g. [Hb], HCT, Ret%) were induced (Damsgaard et al., 2006). However, major blood loss is considered to be 20% loss of the TBV (Mannucci & Levi, 2007), which is not likely to be prevalent among athletes. Meurrens et al. (2016) observed that in 24 moderately trained subjects, a single blood draw of 470mL[5] significantly decreased several ABP parameters (i.e.

[5] The blood donation volume was set according to Belgian Law at the time of the study.

RBC, [Hb], HCT) between 10–12%. Hb_{mass} decreased by 7% after a single blood donation. Stangerup et al. found that [Hb] was decreased by 7.6 ± 2.1% and HCT was decreased by 9 ± 2% on the third day after a blood donation of 450 mL in women (Stangerup et al., 2017).

1.3.2.2 Pre-analytical factors

Pre-analytical factors in clinical laboratories, can occur before or during blood withdrawal. These bias-inducing confounding factors (Kouri et al., 2005) can be avoided by following standardized protocols. For this reason, WADA has created specific blood withdrawal guidelines (Damsgaard et al., 2006). Sottas et al. (2009) listed the optimal conditions to minimize pre-analytical error (Table 6).

***Table 6**. List of optimal conditions for blood sampling. Taken from Sottas et al., 2009, p.311-312*

1. The phlebotomist must be qualified, rigorous and possess stress-management skills.
2. Prior to blood collection, the athlete must answer the following questions:
   a) Has he or she had a blood transfusion in the last 3 months? If yes, when and what volumes were transfused?
   b) Has he or she lost blood (hemorrhage) or given blood (blood donation) in the last 3 months? If yes, when and what volume was lost or given?
   c) Has he or she spent any time at high altitudes (>1,000 m) during the last 2 weeks? If yes, when and at what altitude?
   d) Has he or she used a hypoxia tent (>1,000 m) during the last 2 weeks? If yes, when and what PIO_2 was used?
3. The phlebotomist must choose a single type of equipment: one single supplier of materials, identical volume for all tubes, identical type of needle (gauge, model, etc.).
4. The athlete must not have been engaged in a strong physical activity in the last 2 h preceding the blood collection.
5. Eating and drinking excessively must be avoided during the last hour preceding blood drawing.
6. A resting period (sitting position) of at least 10 min must precede blood drawing.
7. The athlete must remain seated during blood drawing.
8. Venous blood must be collected exclusively from the antecubital fossa.
9. Blood drawing time (when the tourniquet is applied) must not exceed 45 s.
10. The tube or tubes containing the blood samples must be homogenized and labelled appropriately.
11. The athlete and the phlebotomist must accept and sign the transcript describing the blood drawing.
12. The phlebotomist must check the site of blood collection (absence of hemorrhage) and must ascertain that the athlete is in good condition and may resume his or her activities.

Some of the conditions listed in the table are related to technical aspects and materials during phlebotomy (i.e. condition 1, 3, 8, 9, 10, 11 and 12). The following conditions are referred to as pre-analytical aspects:

Condition 1: a qualified phlebotomist ensures the quality of the procedure.

Condition 3: material can influence hematological results, notably the size of the needle (Lippi et al., 2006). It is worth noting that using the same material can reduce potential errors.

Condition 8: the antecubital vein is chosen since it is large, accessible and is the most prominent vein in most people.

Condition 9: tourniquet time has also been demonstrated to influence blood results (Cengiz et al., 2009). Kuipers et al., (2005) showed that over 3 min of tourniquet usage can increase [Hb] and HCT values due to venous pressure increase and extravascular fluid shift.

Condition 10: analysis of unhomogenized blood samples can shift blood results (Narayanan, 2003).

Condition 11 and 12: a confident relationship between the phlebotomist and the athlete is paramount to ensure blood procedure quality.

Other pre-analytical factors such as circadian rhythm have been evaluated. Among 24 participants, Sennels et al. (2011) analyzed blood samples every 3 h, observing significant variations during 24 h. The highest [Hb] and HCT values were observed around 12 AM (Sennels et al., 2011). More recently, Robinson et al. (2019) observed a decrease in [Hb] in the afternoon and evening in comparison to the morning (Robinson et al., 2019).

The other conditions (i.e. conditions 2, 4, 5, 6 and 7) are related to physical status, physiological state or position throughout the phlebotomy process.

Condition 2: the athlete should state any recent transfusions or blood loss (i.e. hemorrhage or blood donation), which affect several blood parameters (Zorzoli, 2011a). Altitude training or

sojourn must also be communicated since they can impact both the erythropoiesis process and PV fluctuations (this will be discussed in the next section) (Schmidt, 2002).

Condition 4: blood draw must be at least 2 h after any strong physical activity. Schmidt et al. (2000) showed that a maximum cycle of exercise increased HCT from ~11%, which was due to a 15% decrease in PV (Schmidt et al., 2000). Reduced PV due to fluid shift from intravascular to interstitial space and the intracellular compartment is explained by a) increased hydrostatic pressure accompanied by increased capillary perfusion and b) increased osmotic pressure within the muscle tissue caused by phosphocreatine splitting and metabolite accumulation (e.g. lactic acid) (Schmidt et al., 1990). Nevertheless, PV is restored and increases in the recovery phase, in contrast to the decrease in [Hb] seen in the pre-strenuous exercise condition (Schmidt et al., 1990). In addition, Morici et al. (2005) demonstrated that Ret is affected by acute exercise (i.e. 1000 m rowing at maximum effort). The increase in Ret release was attributed to a probable tissue hypoxia likely caused by progenitor mobilization. (Morici et al., 2005).

Condition 5: the hydration status of the athlete has been shown to affect the OFF-score (Bejder et al., 2016). Some athletes use acute hyperhydration (i.e. large fluid intake in a short time) as a strategy to dilute biomarkers such as [Hb] or HCT, and thus mask prohibited substance use. A study showed that 40 to 80 min after acute ingestion of 1000 mL of water, the OFF-score decreased from 4% to 2% (Bejder et al., 2016). This study also showed that the hyperhydration state can mask over 80% of atypical biomarker levels. Indeed, a decrease in [Hb] of 0.4 g/dL induced a decrease in the OFF-score, while Ret% remained unaffected after ingestion of 1000 mL water when compared to 70 mL/30 min. Nevertheless, a recent study did not come to the same conclusion (Athanasiadou et al., 2020). During a five-week clinical study in which participants received rHuEPO, no differences were shown in the ABP biomarkers. In a recent study, Coffman et al. (2020) showed that induced dehydration (i.e. diuretic administration) decreased body weight by 3.7% and considerably altered the ABP by increasing [Hb] (Coffman

et al., 2020). Contrarily, sweating-induced dehydration is mainly intracellular and did not alter the ABP.

Condition 6 and 7: to guarantee accurate hematological results, body position before and during blood sampling must be controlled. WADA's guidelines require the athlete to be seated for 10 min before sample collection (WADA, 2019). Ahlgrim et al. (2010) confirmed that sitting for 10 min is enough to guarantee [Hb] and HCT stabilization in endurance athletes. However, body position (i.e. supine vs. sitting position) was not assessed even though posture has been shown to have a significant quantitative and qualitative influence on TBV (Jacob et al., 2005). It may be hypothesized that gravity-induced rapid blood pooling in the lower extremities due to positional change (e.g. standing versus lying) may impact the ABP (Jacob et al., 2005). This phenomenon has been largely documented and is likely attributed to an increase in vascular transmural hydrostatic force (Lundvall & Bjerkhoel, 1994). Nevertheless, the influence of various positions, timing and walking a few meters has never been fully investigated.

In this context, Chapter 3 of this thesis presents a study we designed to determine the putative influence of body position and timing on the ABP variables. To carry out this work, 10 blood samplings were performed in supine vs. standing position and after a short walk of 50 meters.

1.3.2.3 Training

As discussed previously, ABP biomarkers are known to be influenced by acute exercise that occurs a few hours before a blood sampling, meaning that training just before a blood sampling can be a confounding factor for the ABP. This explains why there is a requirement that the athlete does not train or compete 2 h before any anti-doping blood sampling (cf. condition 4 above). Nevertheless, training is the main way to achieve performance. Nowadays, a plethora

of training methods have been developed by scientists, and improvements in sciences surrounding athletics also influence training and thus performance (e.g. nutrition, sport psychology, sport medicine). In addition, the quantification of training load becomes a key element for a good follow-up, and multiple models have been developed mostly to prevent fatigue in athletes (Halson, 2014). The third study included in this thesis focuses on the potential of endurance training load to be a confounding factor of the ABP.

Hematological adaptations with endurance training

Endurance training, undertaken for many months or years induces chronic hematological adaptations, mainly by an increase in PV in the hour following the start of the exercise and reaching a peak, for instance, two days after a marathon run (Fellmann, 1992). This PV increase is usually around 9–25%, representing on average an additional 300–700 mL. To better grasp this PV change, Montero et al. (2017) trained untrained volunteers for 8 weeks through 3–4 weekly endurance sessions. Researchers observed a significant increase in the PV of 16% after 2 weeks, 21% after 4 weeks, and 14% after 8 weeks of the intervention. The RBCV also significantly increased, though slightly later, emphasizing the different time responses of adaptations; it was 6% after 4 weeks and doubled to 12% after 8 weeks. Confirming this result, a previous study observed almost the same PV and RBCV adaptation in 8 weeks of endurance training (Green et al., 1991). Therefore, the time course of changes to PV and RBCV differs following endurance training.

This large increase in PV during the two first weeks of endurance training has been noted by several studies (Convertino, 2007). Moreover, the magnitude of changes in PV has been shown to be proportional to the duration of the exercises (Fellmann, 1992). To help explain this increase in PV with endurance training, researchers have observed a transient activation of the renin-angiotensin-aldosterone system that increases plasma proteins and thirst sensitivity

(Convertino, 2007; Warburton et al., 2004). This increase in plasma proteins indicates the involvement of the water-binding principle, wherein the water-binding capacity of PV is increased as plasma proteins increase (i.e. 1 g of protein can bind 14–15 mL of water) (Fellmann, 1992). In addition, an increase in renal and sodium retention reduces the total urine output as much as 20% during the 24 hours of post-exercise recovery. Nevertheless, the glomerular filtration rate is not affected by exercise, suggesting that the mechanism responsible for the reduced urine excretion with physical activity is post glomerular. The increase in RBCV seen in the Montero et al. (2007) study might be due to the decrease in HCT with PV expansion, which decreases tissue O_2 pressure in the juxtamedullary region of the cortical labyrinth of the kidney, thus increasing the release of new RBCs (Donnelly, 2001; Dunn & Donnelly, 2007).

Seasonal influences

Athletes who train throughout the year present seasonal variations in their TBV distributions and thus in blood biomarker concentrations (Mørkeberg et al., 2009). A variation in training load, which is the norm for optimal yearly preparation, can partly account for those variations. [Hb] and HCT were largely impacted by the season, with decreases observed between the competition and off seasons (Mørkeberg et al., 2009). For instance, during the Tour de France, a 11.5% decrease in [Hb] values were observed amongst riders. However, this also differs based on the mechanism, as the hemolysis induced by foot strikes in runners was not seasonal but rather presented only after strenuous exercise (Pizza et al., 1997). Additionally, significant reductions were observed within the taper period in RBC, Hb, MCH and MCHC, whereas Ret% significantly increased (40%) following a 6-day taper (Mujika et al., 2000). This latter increases in Ret% suggests that hematopoiesis increases following tapering, whereas the decrease in the other blood values mentioned was due to a clear PV expansion. Bejder et al., 2017 looked at the influence of acute increase in training load. They found an increased PV along with a concomitant decrease in [Hb] after one week of a 250% increase in training load in elite cyclists

(Bejder et al., 2017). Interestingly, researchers returned to the respective baseline values for PV and [Hb] after only 2 and 4 days, respectively, following the increase training load week.

Endurance competitions

Endurance (or ultra-endurance) competitions (i.e. Ironman, multi-stages cycling events, ultramarathons) have a long duration, sometimes at a relatively lower pace, and can also influence TBV distributions. Decreased values of [Hb] and HCT were observed after the competition, with a major decrease 2 days post-race (Miller et al., 2019); it took 5 days for [Hb] to return to pre-competition values. In addition, the Ret% increased after 5 days. More importantly, 32% of the ABP profiles presented an ATPF, mainly flagged due to [Hb] values outside the calculated thresholds. This phenomenon for long-distance events was also highlighted by Schmidt et al. (2000) after a 10-day stage cycling event. The large overcompensation in PV can be explained by a reduction in central venous pressure after each bout of exercise (i.e. one stage in multi-stage cycling events) and a higher sodium retention due to changes in aldosterone and urodilatin secretion as described elsewhere (Schmidt et al., 1990, 1998). The same decrease in [Hb] and accompanying increase in PV has been highlighted in ultramarathon running (i.e. 16 days in a row for a total of 1600 km) (Fallon et al., 1999). At day 4, an increase in PV was accompanied by an increase in RBC count compared to pre-race values and after correcting for PV expansion. The decrease in [Hb] can be attributed to the well-documented “sport pseudoanemia”, which is characterized by a low [Hb] due to PV expansion (Bärtsch et al., 1998; Carlson & Mawdsley, 1986). Hematological variations in long distance events fluctuate throughout the event, specifically in cycling where there are often stages on multiple consecutive days. In the first half of a competition, Corsetti et al. (2012) showed a decrease in [Hb], RBC count and hematocrit, though an increase in IRF%. During the second half of a competition, [Hb] stabilized and tended to increase. However, it is worth noting that the timing of blood sampling is paramount, as [Hb] measurements taken directly after

endurance races can appear as effectively unchanged from baseline values due to the accompanying strenuous performance (Banfi et al., 2004; Neumayr et al., 2002).

Studying athletes competing in the Tour de France 2007, Mørkerberg et al. (2009) noted the above-mentioned decrease in [Hb] appeared after 12 days (-6% to -12.5%) and after 18 days, where the riders had the 10th day off. Voss et al. (2014) showed a similar significant decrease of -13% in [Hb] in the days after beginning an intense 6-day simulated stage race (Voss et al., 2013). Contrastingly, Banfi et al. (2014) showed that an ultramarathon run at altitude did not significantly change the hematological values (e.g. [Hb], HCT, RBC#, RBCV, MCV, MCH and MCHC). It is worth noting that these athletes competing in such demanding events are trained and prepared for exercise in extreme conditions such that physiological adaptations may have already occurred during training. As an example, a proper acclimatization to altitude can reduce the associated anorexia and weight loss (Vats et al., 2007).

However, an overview of the existing literature on the effect of training on hematological variables showed a lack of focus on the influence of training on the ABP in a longitudinal study. In addition, athletes must state in the DCF if they have participated in a competition over the past three days, which would likely induce a potential hemodilution. Our third study accounted for an increased influence of training load in elite cyclists in the last 5 days before testing.

To study the influence of acute training loads, Chapter 4 of this thesis presents a study we designed that sampled blood and calculated the acute and chronic training loads influences, 5 and 42 days before sampling. This study also addresses seasonal influences (summer vs. winter; high vs. low training load period) on the ABP variables in elite cyclists.

1.3.2.4 Hypoxic conditions

Altitude

Athletes often face extreme conditions, such as temperature or altitude fluctuations during both training and competition, that can alter their physiological homeostasis (Sawka et al., 1983, 1985, 2011). In addition, athletes can use these physiological changes to gain performance improvements using sophisticated training methods that rely on, for instance, altitude and heat (Kissling, Akerman, et Cotter 2020). Indeed, athletes often rely on the use of hypoxia, low oxygen level in the tissues, to induce physiological variations. Usually, normoxic values of inspired pressure of oxygen (PiO_2) are around 160 mm Hg at sea level, which correspond to a barometric pressure (BP) of 760 mm Hg (Conkin & Wessel, 2008). As altitude increases, PiO_2 decreases (Figure 8) (Gallagher & Hackett, 2004). Athletes can also decrease PiO_2 through the use of a device (e.g. hypoxic tent) that simulates a high altitude environment by diluting the air with nitrogen or a combination of gases to produce a low oxygen concentration. This is called normoxic hypoxia (NH), as the BP is constant (Küpper & Schöffl, 2010).

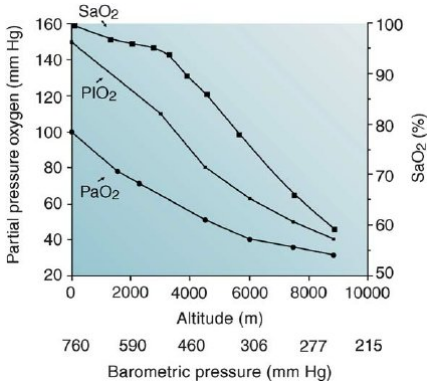

Figure 5. *Relationship between altitude and barometric pressure to partial pressure of arterial oxygen (PaO_2), inspired pressure of oxygen (PiO_2), and arterial oxygen saturation (SaO_2). An increase in altitude results in a decreased barometric pressure, PaO_2), and SaO_2. Oxygen saturation is well maintained up to above 3000 m, despite a significant decrease in arterial PO_2. Above this altitude, small changes in arterial PO_2 result in large changes in arterial saturation. Taken from Gallagher & Hackett., 2004.*

However, NH is not the same as exposure to "real" altitude characterized by a reduced partial pressure in O_2 and leading to hypobaric hypoxia (HH). The effect here is different as homeostasis is maintained by the diffusion of O_2 within tissues and cells, which is controlled by the partial pressure of oxygen in tissue (PO_2). As stated by Dalton's law of pressure, "*the total pressure of a mixture of gases is equal to the sum of partial pressures of individual gases*", which is expressed using the equation:

$$P_{total} = Pgas_1 + Pgas_2 + Pgas_3.$$

With this equation, PO_2 depends upon the atmospheric BP and its fractional concentration. As BP decreases with altitude, the amount of gas molecules in the air decreases; thus, PO_2 is decreased. As a result, the fraction of inspired/breathed-in O_2 (FiO_2) is reduced (Voss et al., 2013). As endurance performances rely heavily on blood capacity to transport O_2, endurance performance is therefore reduced in altitude (Kayser, 2004). Nevertheless, multiple mechanisms exist to compensate for the reduced PO_2, such as an increase in ventilation and cardiac output. However, those mechanisms cannot fully compensate for the decrease of O_2 availability as, for instance, the increase in ventilation comes with increased energy costs. Additionally, the increased cardiac output will reduce the pulmonary transit times such that the same performance in altitude cannot be maintained (Kayser, 2004).

Therefore, the acute response to altitude triggers several compensatory mechanisms, mainly induced by the sympathetic nervous system: a) an increased cardiovascular response (i.e. increased heart rate) to maintain O_2 delivery to the tissues and b) a stimulation of the bone marrow to produce new RBC through the increased release of EPO, which depends on the duration of exposure and degree of hypoxia. The chronic response to altitude (i.e. altitude training camp) depends on multiple elements (i.e. hypoxic dose, training content, training background of athletes and/or individual variability of EPO production) (Płoszczyca et al.,

2018). In addition, altitude induces a reduction in PV, thought to be largely oncotically mediated (Young et al., 2019).

Altogether, this suggests that an athlete residing or training in HH or NH may have several modified ABP biomarkers. This "altitude-exercise" combo induces hematological variations that can produce ATPF cases, which can be explained by the physiological adaptation to altitude (Schumacher et al., 2015). Moreover, Garvican-Lewis et al. (2014) showed that combining two stimuli such as altitude (decreases PV) and exercise (increases PV) eventually generates an increase in PV together with an increase in Hb_{mass} (Garvican-Lewis et al., 2014). As the studied athletes were using the "live-high/train-low" approach, Voss et al. (2020) considered this type of training very unlikely to confuse an expert reading of the ABP (Voss et al., 2020). Nevertheless, the influence of hypoxic stimulation is now being considered by ABP experts, and it is important to know the time required for normalization after hypoxic condition (Voss et al., 2020).

Breath-holding

Other conditions can result in hypoxemia. For instance, breath-hold divers hold their breath as long as possible, sometimes until loss of consciousness. Performance in breath-hold diving relies somewhat on exercise economy and hyperbaric management, but primarily on oxygen conservation. Lindholm et al. (2006) showed that the mean end tidal of PO_2 in 7 divers after an episode of apnea was 26.9 ± 7.5 mm Hg[6] (Lindholm & Lundgren, 2006). In addition to being able to sustain hypoxemia (Henig & Pierson, 2000), breath-hold divers also have to sustain hypercapnia, or an increase in carbon dioxide (CO_2) in the bloodstream (Elia, Gennser, et al., 2021; Ivancev et al., 2007). To delay hypercapnia, breath-hold divers use an extensive

hyperventilation technique, but this is considered as risky as it reduces the CO_2 level by postponing the main trigger for the urge to breathe (Lindholm & Lundgren, 2006).

Due to differing acting mechanisms, breath-hold divers do not have the same physiological responses to counteract hypoxemia as athletes training in altitude. Indeed, the compensatory mechanisms of breath-hold divers rely not on the increase in PiO_2 by ventilatory mechanisms, but rather on to the ability to reduce the oxygen consumption to maintain the breath-holding time as long as possible, which is called the "mammalian diving response". Contrary to other hypoxic responses, this response induces a decrease in heart rate (i.e. bradycardia) that is mediated by parasympathetic nerve activity and a vascularization of the peripheral vascular beds that is mediated by sympathetic nerve activity (Patrician et al., 2021). This leads to a transient decrease in blood flow to the non-vital organs, thus preserving the brain and the heart (Patrician et al., 2021).

Regardless of their differences, more interesting are the hematological variations shared by both breath-hold divers and athletes training at altitude. Here, the spleen plays a key role because it is as a reservoir of RBC released hypoxic stress (Wang et al., 2021). For instance, in divers, the spleen has a blood reserve of approximately 200–250 mL of blood (Stewart & McKenzie, 2002), representing an increase of 2–4% in circulatory RBCs (i.e. approximately 100 mL of concentrated RBC) (Richardson, 2008) that also induces an increase in Ret% (Engan et al., 2013). Indeed, the spleen has been considered for centuries as a dynamic reservoir of RBC (Cesta, 2006). In humans during breath-holding, a decrease in spleen size (18–35%) leads to an increase in HCT of 2–6% (Schagatay et al., 2001, 2012). An indigenous population, the Ama, showed increased HCT values of 10.5% after one hour of repetitive diving. Recently, Patrician et al. (2021) stated that the increase in HCT would increase CaO_2 by 5%, which would protect

the diver when resurfacing (Patrician et al., 2021). Nevertheless, the values returned to baseline after only 2–10 minutes (Prommer et al., 2008; Schagatay et al., 2001, 2005). Breath-hold training lasting 2 and 6 weeks showed an increase in Ret% of 15% and 28%, respectively, with no change in [Hb] (Elia, Barlow, et al., 2021; Engan et al., 2013). However, more hematological data on breath-hold divers over a longer term is still needed, especially as relates to the potential influence of breath-hold diving on the ABP. In the TDSSA, which came into force in 2022, breath-hold diving has a 15% of MLA for EPOs, highlighting the need to question the necessity of an ABP in this sport (WADA, 2021)

To address this, Chapter 5 of this thesis presents a longitudinal study investigating the hematological variables in recreational breath-hold divers compared to an active control group.

This thesis focuses on blood doping in terms of the hematological biomarkers used in the ABP to improve the assessment of confounding factors in anti-doping. To better interpret the ABP biomarkers, Hb_{mass} and PV were used in our studies as additional parameters, as already proposed in other studies (Pottgiesser et al., 2007a, 2012; Prommer et al., 2008).

1.4 Aims of the thesis

Overall, the influence of some factors confounding the accurate measurement of the hematological variables used in ABP profiles has not been fully documented in the literature (i.e. [Hb], OFF-score, Ret% and ABPS). In addition, the use of PV and Hb_{mass} has been proposed to improve the variations or absence of variations in the measurements of the studied confounding factors at stake.

Thus, this thesis aimed to describe the influence of three confounding factors through three main studies:

1. Study 1 (Chapter 5) investigates the influence of body position before and during blood sampling on the ABP variables.
2. Study 2 (Chapter 6) investigates the potential influence of training load on the hematological variables of the ABP over time in elite cyclists.
3. Study 3 (Chapter 7) characterizes the hematological variables over time of breath-hold divers vs. an active control group.

CHAPTER 2

SUMMARY OF EXPERIMENTAL RESULTS

2. Summary of experimental results

2.1 ABP blood values reported in concentration are altered due to body position

The aim of this study was to investigate whether body position (i.e. seated vs supine) during phlebotomy affected [Hb] and HCT measurements due to an altered PV. This study also assessed if a position change (e.g. walking a short distance) influenced these biomarkers used in the ABP to determine whether these changes are acceptable in the context of the normal anti-doping blood sample collection sequence.

We analyzed ten successive venous blood samples from 38 subjects taken in three different groups (breath-hold divers, cyclists and an active control group) in a variety of situations: immediately after 10 minutes of normalized activity (B1), after 10 minutes seated (B2, typical sample in an anti-doping context), after a 50 m walk (B3), after returning to 5 and 10 minutes in a seated position (B4 and B5), and finally after 5–30 minutes supine (B6–B10). To assess these posture-related variations, [Hb] and HCT were determined by flow cytometry.

Our results showed that Ret% was unchanged in all conditions and that [Hb] and HCT were stable after at least 10 minutes in a seated position. Due to shifts in PV, the [Hb] and HCT increased slightly but significantly after a short walk (+0.1 g/dL [$P = 0.008$] and +0.4% [$P = 0.01$], respectively), but readjusted to previous levels after only 5 minutes. A supine position (>10 minutes) decreased [Hb] (average -0.2 g/dL, $P < 0.01$) and HCT (average -1.1%, $P < 0.01$).

In conclusion, body posture may influence the measurement of specific blood values sufficiently to affect sampling in an anti-doping context even if the values are unlikely to affect a clinical context. These results may expect to be broadly useful in antidoping.

2.2 Acute training load induces variations in blood variables in elite cyclists

To investigate the influence of acute and chronic training load changes on the ABP variables, we followed 10 male elite cyclists (25.6 ± 3.4 yrs, 181 ± 4 cm, 71.3 ± 4.9 kg, 6.7 ± 0.8 W/kg, 5-min maximal power output) using monthly blood samples over one year. We used these samples to calculate individual ABP profiles along with the distance to the individual limits. Hb_{mass} and PV were measured, over the last eight months of the study, using the CO-rebreathing method. Acute and chronic training loads, 5 and 42 days before sampling, respectively, were calculated considering duration and intensity (training stress score, TSS™).

Over the course of the study, the [Hb] averaged 14.2 ± 0.0 g/dL (mean ± SD) (range: 13.3 to 15.5 g/dL) with significant changes over time ($P = 0.004$). The ABPS also showed significant changes over time ($P < 0.001$; average -1.32 ± 0.41 a.u.; range: -1.68 to -0.40 a.u.). The Ret% averaged 1.2 ± 0.31% (range: 0.76 to 1.81%), and the OFF-score averaged 76.6 ± 10.0 a.u. (range: 64.0 to 91.7 a.u.) with no significant variations over the course of the study ($P > 0.05$). The Hb_{mass} averaged 1030 ± 87 g (range: 842 to 1116 g) with no significant variations over time ($P = 0.118$), whereas the PV averaged 4309 ± 350 mL (range: 3688 to 4751 mL) with a time-effect observed over the study ($P = 0.014$). Both ATL and CTL varied significantly with time ($P = 0.002$ for ATL and $P < 0.001$ for CTL).

Over the 120 measurements (10 subjects × 12 blood samples), 10 [Hb] values (8.3%) fell within < 0.5 $g \cdot dl^{-1}$ of the individual limit (upper or lower) and 4 fell within < 0.1 $g \cdot dl^{-1}$ (3.3%). Four individually calculated OFF-score values were closer than 5 points (a.u.) to an individual limit (3.3%).

Higher acute, but not chronic, training loads were associated with significantly decreased in [Hb] ($P < 0.001$) and an increase in PV ($P = 0.007$). The OFF-score was thus also significantly affected ($P = 0.04$). A cumulative 3-month period of high vs. low training load did not significantly affect ABP variables, PV or Hb_{mass} ($P > 0.05$). Season (i.e. cumulative 3 months in summer vs. in winter) did not affect the ABP, Hb_{mass} or PV. However, a not significant ($P = 0.06$) increase in PV (+ 4.7%) was observed in the summer season.

Despite the observed variations, the ABP variables remained within the individually calculated limits. In conclusion, this study showed that accounting for acute variations in training load is relevant for interpreting the ABP.

2.3 Hematological values in recreational breath-hold divers

To investigate the potential influence of breath-hold training on hematological variables, we used the ABP to monitor hematological variables in recreational breath-hold divers (BHDs) compared to an active control group over a year, expecting both breath-hold training and seasonal effects. We also hypothesized that BHDs would have a higher Hb_{mass} and PV and more pronounced seasonal variation compared to the controls.

This study compared 11 healthy non-smoking BHDs (8 men and 3 women) to a control group of twelve sports science students over one year with monthly blood sampling and breath-hold

training monitoring. We observed that the BHDs trained mainly in dynamic disciplines, representing 52% of the total of breath-hold training, but that their hematology did not differ significantly from the controls over the study time ($P > 0.05$). However, all the hematological values studied varied significantly over time for both groups, excepting ABPS ($P = 0.99$). Polynomial contrasts were mostly quadratic, suggesting a seasonal effect. Blood samples were taken at least 19 h after a breath-hold training and showed no effect on blood biomarkers between 19–24 h after a breath-hold training nor after 24h, suggesting neither acute nor chronic effects of breath-hold training. Over time, significant interactions were highlighted for Ret% ($P < 0.001$), Ret# ($P = 0.004$) and IRF% ($P = 0.013$), wherein a linear decrease with time was seen for the values for the controls; the values for the BHDs were stable. In conclusion, the hematological variables of BHDs did not significantly change from those of an active population. These results may expect to be useful for further research on blood variables and particular environment.

CHAPITRE 3

ARTICLE I

Does body position before and during blood sampling influence the Athlete Biological variables?[7]

[7] Tables, figures and references follow their own numbering system within this chapter.

3. Article I: Does body position before and during blood sampling influence the Athlete Biological variables?

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Abstract

Introduction: The Athlete's Biological Passport (ABP) is a tool for the indirect detection of blood doping. Guidelines from the World Anti-Doping Agency (WADA) require 2 hours delay after any physical exercise, and to be seated for 10 minutes before collecting an ABP sample. This study investigated posture-related hematological variations with changes in body position during blood sampling.

Methods: Ten successive venous blood samples from 38 subjects were collected in three situations: immediately after 10-minutes of normalized activity (B1), after 10-minutes seated (B2, typical reference sample in an anti-doping context), after a 50 m walk (B3), after 5 and 10-minutes in a seated position again (B4 and B5), and finally after 5-30 minutes supine (B6-B10). Hemoglobin concentration [Hb] and hematocrit (HCT) were determined by flow cytometry to assess putative posture-related variations.

Results: Reticulocytes percentage was unchanged in all conditions, [Hb] and HCT were stable after at least 10-minutes in a seated position. Due to shifts in plasma volume, [Hb] and HCT increased slightly but significantly higher after changing posture for a short walk (+0.1 gr/dL [P = .008] and +0.4% [P = .01] respectively), but readjusted to previous levels after only 5 minutes. Supine position (>10 minutes) induced decreases of [Hb] (−0.2 g/dL in average, P < .01) and HCT (−1.1%, in average, P < .01).

Conclusion: The observed variations in [Hb] and HCT may have minor clinical significance, while they underline the need to follow strict guidelines for posture before and during blood sampling in an anti-doping context.

1. Introduction

Nearly 30 000 blood samples are collected yearly for the Athlete Biological Passport (ABP) (Aikin, 2018). The ABP tracks blood markers longitudinally and based on changes in these variables helps identifying patterns of doping (Sottas, Robinson, Rabin, & Saugy, 2011; Vernec, 2014). However, it is now certain that athletes adapted their doping protocols using rather first generation of rhEPO and frequent microdosing in the evening to maintain a supraphysiological level of hemoglobin while reducing the chances of being tested positive (Hamilton, 2012). Therefore, accurate and precise measurement of blood variables with low bias is paramount to ensure the indirect detection and targeting potential of the ABP. The blood matrix as a suspension of living cells in plasma transports oxygen to the working muscles. Variations in the fluid balance and thus plasma volume may inevitably alter variables for which concentration values are reported (e.g. hemoglobin concentration ([Hb]) or hematocrit (HCT)) while absolute measures (e.g. reticulocytes percentage (Ret%)) remain stable (Ahlgrim et al., 2010; Fahraeus, 1929). Significant shifts in plasma volume were hence reported in connection with many situations of an athletes' daily life such as acute physical exercise, heat exposure, psychological stress, and/or postural changes (Collins, Hill, Cureton, & DeMello, 1986; Imelik & Mustimets, 1992). Acute or chronic plasmatic volume variations may also occur with endurance training periods (Sawka, Convertino, Eichner, Schnieder, & Young, 2000), repeated sauna bathing (Stanley, Halliday, D'Auria, Buchheit, & Leicht, 2015), or hypoxic exposure with a high individual variability (Young et al., 2019). Overall, this underlines the numerous confounding factors affecting concentration based on blood markers and the need for robust procedures to limit pre-analytical variations when analyzed for the ABP. For instance, the World Anti-Doping Agency (WADA) enacted specific blood collection guidelines (WADA, 2016a), in addition to precise Blood Analytical Requirements for the ABP (WADA, 2009). Currently, the guidelines specify that 2 hours waiting is necessary after any physical exercise

and require the athletes to be seated for 10 minutes before sample collection to allow the vascular volumes to equilibrate, based on the results from a prior study (Ahlgrim et al., 2010). The world health organizations' current recommendation to draw blood from the patient in a supine position (if possible) is however not the standard in an anti-doping context but might be more comfortable for certain athletes (World Health Organisation, 2010). The aim of this study was thus to investigate the influence of body position during phlebotomy (ie, seated vs supine) on [Hb] and HCT potentially altered by plasma volume shifts. This study also assessed if a short position change (e.g. walking a short distance) influences hematological biomarkers (e.g. [Hb] and HCT) used in the ABP and may thus be acceptable in the context of the normal anti-doping blood sample collection sequence.

2. Material and methods

2.1 Study subjects

Thirty-eight nonsmoking healthy Caucasian subjects were included in this study in three groups: 10 elite cyclists (Cyc, 10 males), 12 trained apnea divers (Apn, 7 males, 5 females), and 16 moderately trained control subjects (Con, sport sciences students, 9 males, 7 females). The Cyc group included International Elite licensed cyclists successful at an international level (eg, UCI World Tour races or UCI World Cup participations) in road, track, and mountain-bike events. The Apn group included apnea divers with a regular practice of apnea training (ie, at least two weekly training sessions), competing at a national or international level (breath-holding experience 8 ± 2.3 years). Inclusion criteria for the control group were a total weekly volume of aerobic sport activities (e.g. running, triathlon, cross-country skiing) not exceeding 4 hours. Participants living permanently at an altitude above 800 m were also excluded. Procedure and risks were fully explained to the subjects, and all of them gave their written consents to participate in this study. This study was approved by the local ethics committee

(CCER-VD, Lausanne, Switzerland, Agreement 2018-01019) and conducted in respect of the Declaration of Helsinki.

2.2 Study design, pre-analytical conditions and hematological analyses

To mimic as closely as possible an ABP blood sample collection, the current WADA guidelines on analytical procedures were strictly followed.[15] A total of 10 successive blood samples were collected from each subject over a single visit of 70 minutes between 09:00 and 15:00 in the exact same sequence in order to limit putative circadian variations (Sennels, Jorgensen, Hansen, Goetze, & Fahrenkrug, 2011). The study design is illustrated in Figure 1. At least 2 hours after any physical activity or exercise training, subjects reported to the laboratory as part of their daily activity. Room temperature was kept constant at approx. 21°C. A 10-minutes period of normalized activity was imposed upon arrival to the laboratory: walking 1 minute, sitting down to read a newspaper (4 minutes), walking down the stairs one floor and up again (1 minute), and waiting in a standing position (2 minutes) before walking to the phlebotomy laboratory (2 minutes). Subjects were then asked to sit down, and a first blood sample was taken within 1 minute (B1). Subsequently, subjects remained seated for 10 minute and were requested to fill in a food and exercise training diary for the last 24 hours prior to the laboratory visit to control for their activity and hydration status. A second blood sample was taken after 10 minutes seated (B2). In an anti-doping context, this sample typically corresponds to a reference sample that could have been collected for an ABP analysis. Subjects were then asked to stand up and walk 50 m before a third sample was drawn in order to mimic a situation where an athlete in a waiting room would have to change seat for the blood collection (B3). After 5 and 10 minutes in the seated position, two other samples were drawn (B4 and B5). Immediately after, subjects were requested to replicate the initial 10-minutes period of standardized activity before lying down on an examination table and remaining in a supine position with the head slightly above the rest

of the body (back of the examination table angle of 30°). Blood samples were then taken exactly after 5, 10, 15, 20, and 30 minutes in the supine position (B6-B10) (Figure 1). “Butterfly” 21G needles with a short manifold were inserted in one of the antecubital veins (Sarstedt Safety-Multifly®, Sarstedt AG) after proper disinfection of the site. Intravenous access using a butterfly was preferred to reduce hemolysis. A tourniquet was used as standard to facilitate puncture and removed once the butterfly was inserted in the antecubital vein and fixed with medical tape. Tourniquet time was approx. 25 seconds and never exceeded 60 seconds as required by the WADA guidelines.[12] Blood was collected in two EDTA-coated tubes (Sarstedt S-Monovette 2.6 mL, Sarstedt AG). The first tube only served to purge the butterfly manifold by collecting ~0.3 mL of blood. In order to minimize any possible impact of the amount of withdrawn blood, the second tube was approximately filled to the half since ~1 mL of blood was amply sufficient to perform the subsequent analyses (<100 µL required by the flow cytometer). Consequently, the total blood collected for each subject over the time course of the study was thus <20 mL, representing only a potential difference of ~0.002 g/dL in [Hb] for example (based on the calculated blood volumes). Tubes were inverted 10 times directly after blood collection in order to ensure proper mixing with the anticoagulant. All blood samples were collected by the same experienced phlebotomist throughout the study. Five samples were collected in the seated position from one arm and the subsequent 5 blood samples in the supine position from the other arm. The first arm was chosen randomly. Blood samples were drawn from one single venous access maintained in one arm during the seated position and from the other arm during the supine position. In (the rare, n = 3) case of clotting, another butterfly was immediately inserted above the precedent puncture site. Blood variables were determined via flow cytometry using a fully automated cell counter (Sysmex XN1000, Sysmex Europe GmbH). Internal quality controls provided by the manufacturer were run before each analytical batch as described thoroughly elsewhere (Robinson et al., 2018). Once collected, blood samples

were stored in a fridge at 4°C for a time lasting between 30 minutes and 12 hours. All samples from one single subject were subsequently analyzed at the same time as part of the same batch after being rolled during 15 minutes at room temperature for homogenization and temperature stabilization purposes. All samples were analyzed at least twice in order to ensure valid recording of [Hb] and Ret% according to the WADA guideline in force (WADA, 2019). At the end of the blood sampling procedure, circulating volumes (eg, blood volume, plasma volume, and total hemoglobin mass (Hbmass)) were measured with a carbon monoxide (CO) rebreathing technique with a fully automated system (OpCo: Detalo Instruments) as described elsewhere (Siebenmann, Keiser, Robach, & Lundby, 2017). Plasma volume changes (ΔPV) were calculated using the formula introduced by Dill and Costill (Costill & Fink, 1974):

$$\text{DPV}\ (\%) = 100.\ \frac{Hb(B2)}{Hb(t)}.\ \frac{100 - \text{HCT}\ (\text{t})}{100 - HCT\ (B2)} - 1$$

[Hb] and HCT measured at B2 (i.e. after 10 min seated) were used as reference baseline values; t indicates the different collection timepoints (from B1 to B10). Blood pressure was additionally monitored with an automated wrist manometer (Beurer BC85, Beurer Gmbh, Ulm, Germany) at B1 and B6 to verify that the normalized activity imposed for the protocol only produced a low sympathetic stimulus.

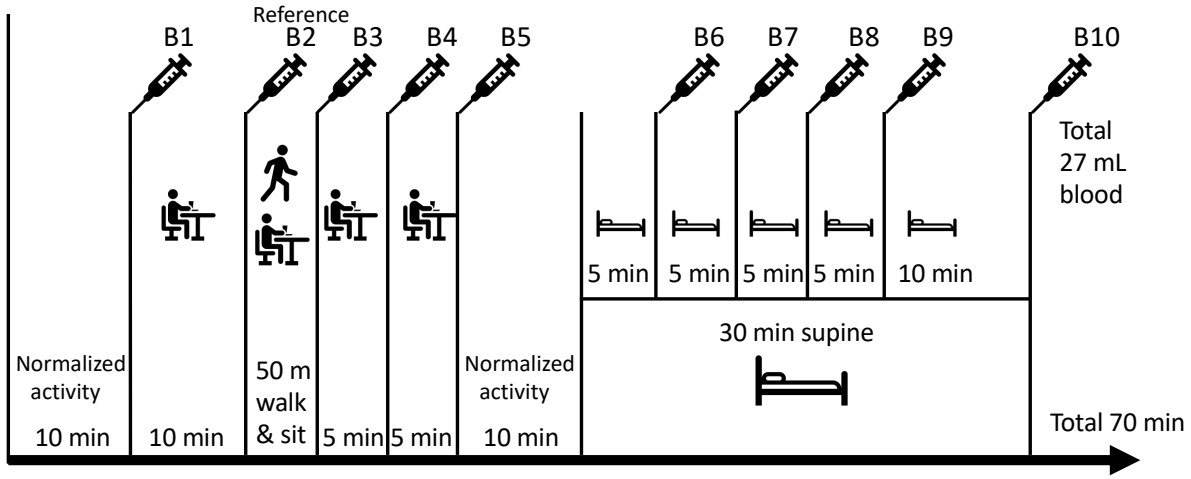

Figure 1. Study design and blood collection times seated or supine

2.3 Statistical analyses

Descriptive values are reported as means ± SD. Variability of [Hb] and HCT over time was calculated as a coefficient of variation (CV, %) from the mean of each individual's CV over the 10 measurements.

Normality of the distributions was successfully tested with the Shapiro-Wilk test. Sphericity was not assumed and the Geisser-Greenhouse correction was used. Baseline variables were compared between groups with Student's t test. Differences in [Hb], HCT and Ret% at the different time points were thus assessed with a linear mixed-model procedure with fixed and random effects to explain target variables where subjects represented random effects; time, sex and group were the fixed effects. The F statistic was used to test for significant fixed effects.

The repeated measures were analyzed by comparing each time point with the reference value (at B2) with correction for multiple comparisons using statistical hypothesis testing (Dunnett's test). The null hypothesis was rejected for $P<0.05$. All statistical analyses were performed using the Jamovi open-source dedicated statistical software (Jamovi, 2019).

3. Results

Relevant baseline variables (such as [Hb] and HCT) were similar in the three study groups (ie, Cyc, Apn and Con) with an average [Hb] of 13.8 ± 1 g/dL and an average HCT of 39.7% ± 2.3%. However, absolute total Hbmass was significantly higher in the Cyclists group when. compared to Apnea divers (+14.5%, P < .01) and Controls (+20.5%, P < .01). Similarly, circulating blood volume was significantly higher in the Cyclists group when compared to Apnea divers (+18.2%, P < .01) and Controls (+24.9%, P < .01) (Table 1). Anthropometrical data, variables measured at baseline (or after all blood samples for total hemoglobin mass and circulating volumes), for the study subjects are reported in Table 1. In females compared to males, total Hbmass (636 ± 84 g) and blood volume (4864 ± 646 mL) were significantly lower than in males (957 ± 121 g and 6748 ± 1054 mL, respectively, P < .001). Similarly, [Hb] (12.7 ± 0.7 g/dL) and HCT (37 ± 2.1%) were significantly lower (P < .01) than in males (14.3 ± 0.9 g/dL and 40.8 ± 2.2%, for [Hb] and HCT, respectively).

	Cyclists (*n*=10)	Apnea divers (*n*=12)	Controls (*n*=16)
Males/Females	10/0	7/5	9/7
Age (y)	28.4 ± 6	37.0 ± 9*##	24.8 ± 3
Height (cm)	180 ± 6	176 ± 9	173 ± 7**
Body mass (kg)	72.2 ± 7.0	71.9 ± 11.2	68.3± 10.0
Total haemoglobin mass (g)	988 ± 94	863 ± 189*	820 ± 208*
Total haemoglobin mass (g/kg)	14.0 ± 0.4	11.9 ± 1.9*	11.5 ± 1.8**
Plasma volume (ml)	4338 ± 598	3638 ± 727*	3888 ± 598*
Blood volume (ml)	7201 ± 789	6093 ± 1197*	5764 ± 1348*
Haemoglobin concentration (g/dL)	13.8 ± 0.6	13.9 ± 1	13.7 ± 1.3
Hematocrit (%)	39.9 ± 1.9	39.8 ± 2.1	39.5 ± 3.0
Systolic Blood pressure at B1 (mmHg)	124 ± 10	118 ± 9	118 ± 8
Diastolic Blood pressure at B1 (mmHg)	70 ± 8	79.3 ± 6*	74.5 ± 10

Systolic Blood pressure at B6 (mmHg)	128 ± 13	127 ± 4	126 ± 16
Diastolic Blood pressure at B6 (mmHg)	76 ± 7	82.8 ± 8	75 ± 7

** $P<0.01$, * $P<0.05$ for difference with Cyclists
$P<0.01$ for difference with Controls.

Table 1. Anthropometrical data and baseline variables (at B2), otherwise specified. Mean values ± SD.

Box-and-whisker plots for [Hb], HCT, and Ret% over the 10 timepoints are presented in Figure 2. Average coefficients of variation for all subjects over all time points for [Hb] and HCT were of 1.4% ± 0.2% for [Hb] and 1.6% ± 0.4% for HCT. When compared to the B2 reference value, significant differences ($P < .01$) were observed after the initial normalized activity (B1) (+0.4 gr/dL for [Hb] and +1.5% for HCT), after 50 m of walking (B3) (+0.1 gr/dL for [Hb] and +0.4% for HCT) and after at least 10 minutes in supine for [Hb] (−0.2, −0.2, −0.3, −0.2 gr/dL at B7, B8, B9, and B10, respectively) and HCT (−0.9%, −1.2%, −1.3%, −1.1% at B7, B8, B9, and B10, respectively) (Figure 3). No significant difference was observed in the reticulocytes percentage (Ret %) over the 10 timepoints ($F (9, 324) = 1.13$, $P = .339$). Plasma volume changes (ΔPv) compared to B2 ranged from –5.3% to 5.1% with a significant decrease of −1.4% in average ($P < .05$) at B3, and with a systematically significant ($P < .01$) mean increase in supine position of 2.3% at B7, 3.3% at B8, 3.7% at B9, and 3.0% at B10. There was a group effect on [Hb] ($P < .05$) indicating that the cyclists had higher values when compared to the apnea divers and controls; similarly, an interaction of sex on [Hb] and HCT ($P < .001$) indicating the lower values measured in female subjects. However, no interaction (group x timepoints or sex x timepoints) was identified for the posture-related changes of [Hb], HCT, or ΔPV over the 10 timepoints in our study (NS) (Table 2).

	[Hb]		Hct		ΔPV	
Effect	F	P	F	P	F	P
Measure	42.083	<0.001	38.073	<0.001	43.624	<0.001
Sex	39.216	<0.001	32.703	<0.001	1.17	0.28
Group	3.508	0.042	2.296	0.117	1.57	0.22
Measure * Group	0.426	0.982	0.516	0.95	0.41	0.98
Measure * Sex	0.951	0.481	1.403	0.186	1.31	0.22

Table 2. Statistical results for the influence of the various fixed effects (measure, group, gender) and interactions on hemoglobin concentration ([Hb]), hematocrit (Hct) and calculated plasma volume changes (ΔPV).

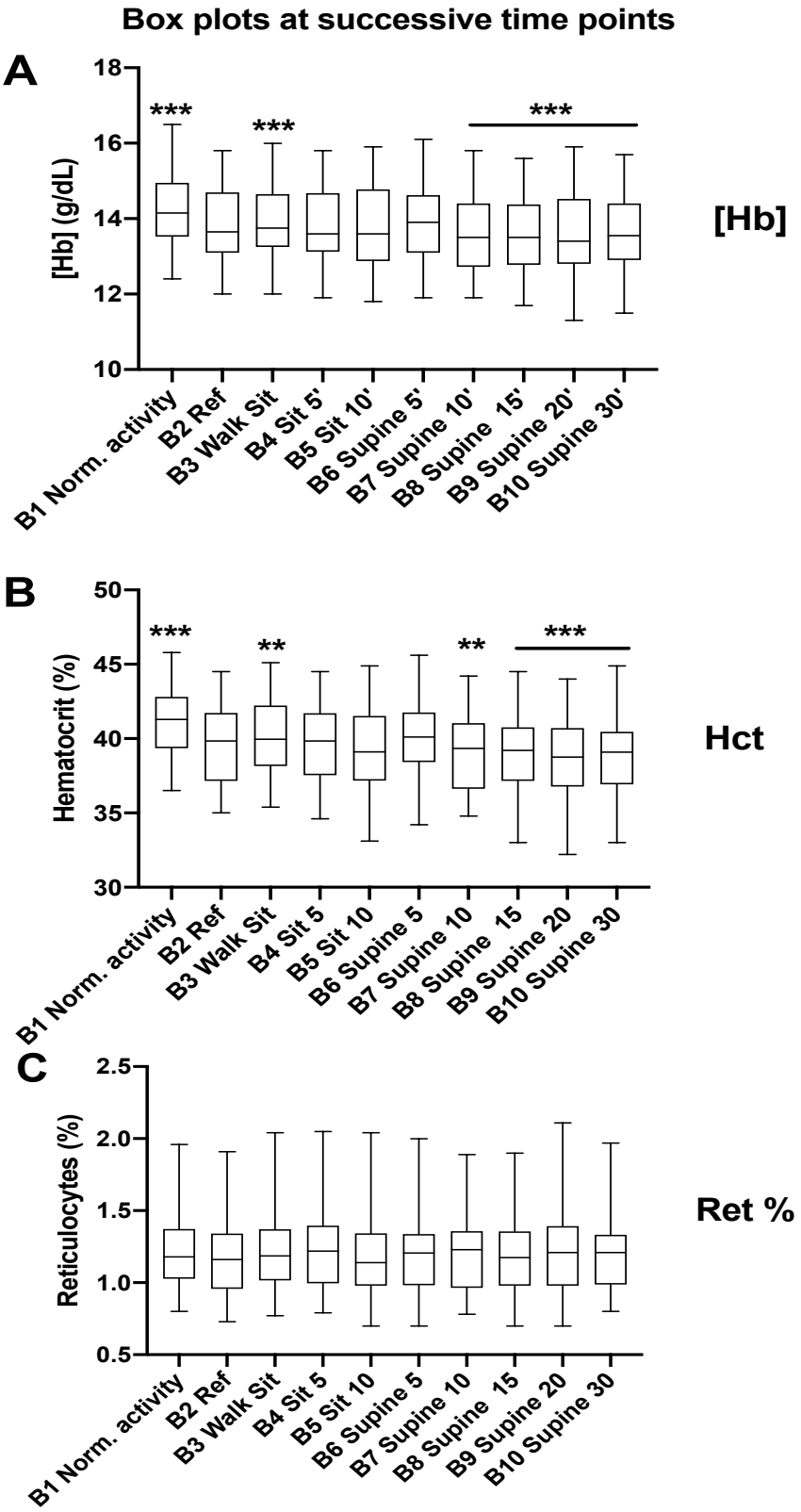

Figure 2. Box plots at successive timepoints (B1 to B10) for hemoglobin concentration (A), hematocrit (B), and reticulocytes % (C) for all subjects (n=38).

*** $P<0.001$, ** $P<0.01$ for difference with baseline (B2 Ref)

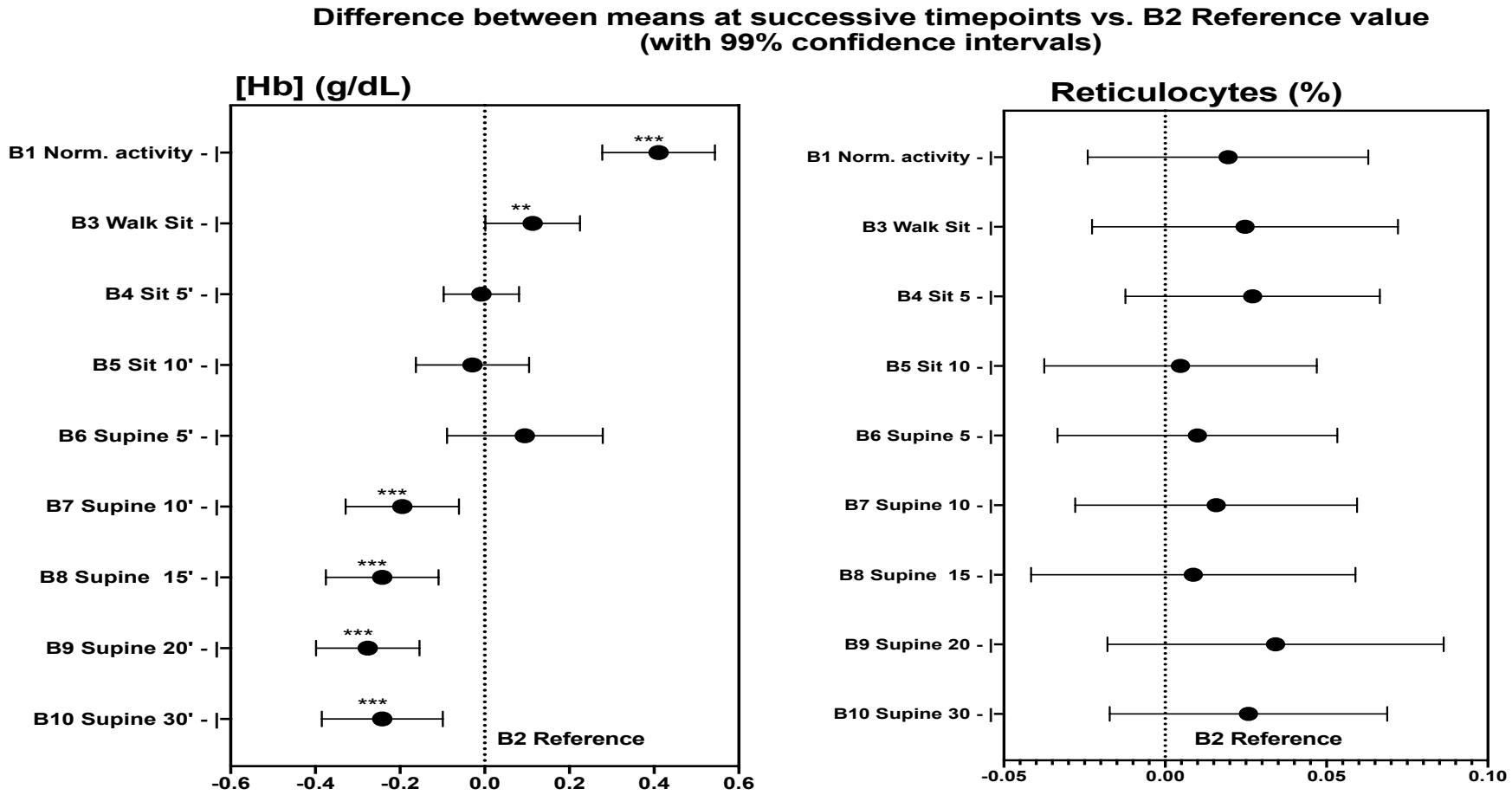

Figure 3. Difference between different timepoints and B2 reference values for hemoglobin concentration and reticulocytes percentage with 99% confidence intervals. *** $P<$ 0.001, ** P <0.01 for difference with baseline (B2 reference).

4. Discussion

The major finding of the present study is that standing up and walking 50 m after being seated for 10 minutes increased [Hb] and HCT, while values were not different from baseline after 5 minutes in a seated position again. The present study also underlined that blood samples collected after 10-30 minutes in supine position resulted in lower [Hb] and HCT values (−0.2 g/dL and −1.1% in average, respectively) values compared to a sample taken after 10 minutes seated. In an anti-doping context, our results indicate that digressing from current guidelines imposing 10 minutes seated before sampling may result in altered results for variables measured as concentrations, due to plasma volume shifts. Practically, however, if an athlete has to change seats (e.g. walking a few meters), only 5 minutes may be required seated again before collecting a sample acceptable for the ABP. Our results are in line with the initial study by Ahlgrim et al showing that posture significantly influences blood volume responses in its volumes distributions (Ahlgrim et al., 2010). It may be hypothesized that a rapid pooling of blood to the lower extremities is due to the law of gravity (Jacob et al., 2005). This phenomenon has been largely documented with an increase in vascular transmural hydrostatic force (Lundvall & Bjerkhoel, 1994). Our results are also in accordance with an older study where a drop in blood volume up to 8% from the seated to the standing position and an increase up to 12% in supine position were reported (Hinghofer-Szalkay & Greenleaf, 1987). An increased capillary pressure in the feet (that can exceed 12 kPa) causing an outward filtration was mentioned as a possible explanation. The smaller change observed at B3 in the present study could be explained by the very short standing (and walking) time of 50 seconds. Our result however indicates that values rapidly stabilize again (i.e. after 5 minutes) when returning to the seated position after the brief walk. In comparison with a seated position, a supine position induces a decrease in vascular resistance and a shift of approximately 500-1000 mL of blood, representing approximately 12%

of blood volume (Robertson, 2008). This important shift may explain the hemodilution observed in our results in the supine position. Moreover, our study results also highlight the time course of hemodilution from the standing to the supine position, in line with research realized on patients undergoing hemodialysis (Inagaki, Kuroda, Watanabe, & Hamazaki, 2001): A hemodilution was apparent after 10 minutes in supine position as highlighted mainly by the lowered [Hb] and an increase in plasma volume. In a medical context, a study showed that a 4% increase in HCT values from supine to standing position with a PV decrease between 6% and 25% (Jacob et al., 2005). The magnitude of changes in PV observed in our study compared to the seated phase is in line with previous research with variations ranging from −5% to +5%, showing interindividual variability (Ahlgrim et al., 2010). In other research, taller subjects had a higher PV volume decrease when standing up that the authors explained by a greater orthostatic load (Lundvall & Bjerkhoel, 1994). However, our results with the significant hemoconcentration observed at B3 in our (significantly taller) Cyc group at B3 did not confirm the latter. The similar variations in [Hb] and HCT indicate that the initial circulating volume and total hemoglobin content does not influence variations due to posture changes (ie, no group x timepoints variation). Our study included almost one-third of female subjects and our results indicate that sex had an effect on [Hb] and HCT ($P < .001$) with lower values at baseline that are well documented (Murphy, 2014). However, the effect of sex on the changes in blood variables is not significant indicating that our conclusions may apply to both male and female subjects or independently of the initial [Hb] or HCT. From a clinical point of view, the observed differences due to posture (e.g. with for example a 0.3 g/dL lower [Hb] value in supine position) are of minimal importance even though statistically significant. However, in an anti-doping context, the robustness of the analysis for each sample collected for the ABP is crucial. Since the variations in [Hb] and HCT are being used in the longitudinal analysis of the athlete's hematological monitoring, it is very important to minimize any confounding factor leading to

additional variation of the true and actual value for the subject at the moment of sampling. Since the variables of the ABP are monitored with a Bayesian approach to tackle the use of blood doping, very slight variations of [Hb] resulting from plasma volume changes due for example to voluntary salt loading (Mora-Rodriguez & Hamouti, 2012) or heat exposure (Sawka et al., 2000) may eventually cause atypical passport findings. The latter underlines the need for stable variables as a result of adequate pre-analytical conditions (i.e. stable seated posture as in the current WADA requirements). In addition, in the supine position, variables were not different in comparison to the reference value after 5 minutes. However, [Hb] and HCT then stabilize at a significantly lower level indicating that the posture-induced plasma volume shift is occurring. It is thus difficult to support the 5 minutes supine position for blood collection as warrant of the most valid or robust condition in an anti-doping context. Beyond postural changes, other factors have to be considered to ensure the quality of the measurements. For instance, tourniquet time was less than 20 seconds on average and the collection of the blood sample was completed in 45-60 seconds. Prolonged tourniquet use (>180 seconds) was thus avoided since it may lead to alterations in [Hb] and HCT values due to the increase in venous pressure causing a fluid shift into the extravascular space (Kuipers et al., 2005). The stable Ret% values obtained over the 10 successive timepoints can be interpreted as a positive quality indicator for both sample collection and analytical measurements with the automated analyzer (Sysmex XN1000) (Lombardi, Lanteri, Colombini, Lippi, & Banfi, 2011). The Ret% values in our study are in line with references values in the general population varying between 0.5% and 2.5% (Banfi, 2008). Ret% are not modified by plasma volume change as they are measured as an absolute value. In conclusion, our study indicates that standing up shortly during an anti-doping blood collection process (and walking up to 50 m to change seats for example) significantly alters the [Hb] and HCT values in athletes and healthy subjects. Values however stabilized after 5 minutes upon returning in a seated position. Blood sampled in a supine position may result in lower [Hb] and

HCT values that do not result in clinically significant differences while it can however affect an ABP profile. Blood samples for anti-doping purposes in the context of the ABP should therefore not be collected in a supine position. If a subject has to stand up shortly after having. waited for 10 minutes (e.g. to change seat from a waiting room to the phlebotomy location), acceptable samples could be obtained after 5 minutes in a seated position. These findings can complement the current WADA guidelines for blood sampling in the context of the ABP.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

RF, YOS, MS, and TA conceived the project. RF and MS obtained the project funding. TA contributed to the collection of data. TA, RF, and FCvR statistically analyzed the data. TA, RF, YOS, and MS drafted the final version of the manuscript. All authors contributed to revising the manuscript and expressed their approval of the final submitted version.

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CHAPITRE 4

ARTICLE II

The influence of training load on hematological athlete biological passport variables in elite cyclists[8]

[8] Tables, figures and references follow their own numbering system within this chapter.

4. Article II. The influence of training load on hematological athlete biological passport variables in elite cyclists

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Abstract

Introduction: The hematological module of the Athlete Biological Passport (ABP) is used in elite sport for antidoping purposes. Its aim is to better target athletes for testing and to indirectly detect blood doping. The ABP allows to monitor hematological variations in athletes using selected primary blood biomarkers (hemoglobin concentration ([Hb]) and reticulocyte percentage (Ret%)) with an adaptive Bayesian model to set individual upper and lower limits. If values fall without the individual limits, an athlete may be further targeted and ultimately sanctioned. Since [Hb] vary with plasma volume (PV) fluctuations, possibly caused by training load changes, we investigated the putative influence of acute and chronic training load changes on the ABP variables.

Methods: Monthly blood samples were collected over one year in 10 male elite cyclists (25.6 ± 3.4 yrs, 181 ± 4 cm, 71.3 ± 4.9 kg, 6.7 ± 0.8 $W \cdot kg^{-1}$ 5-min maximal power output) to calculate individual ABP profiles and monitor hematological variables. Total hemoglobin mass (Hbmass) and PV were additionally measured by carbon monoxide rebreathing. Acute and chronic training loads – respectively 5 and 42 days before sampling – were calculated considering duration and intensity (training stress score, TSS™).

Results: [Hb] averaged 14.2 ± 0.0 (mean ± SD) $g \cdot dL^{-1}$ (range: 13.3 to 15.5 $g \cdot dl^{-1}$) over the study with significant changes over time ($P = 0.004$). Hbmass was 1030 ± 87 g (range: 842 to 1116 g) with no significant variations over time ($P = 0.118$), whereas PV was 4309 ± 350 mL (range: 3688 to 4751 mL) with a time-effect observed over the study time ($P = 0.014$). Higher acute – but not chronic – training loads were associated with significantly decreased [Hb] ($P < 0.001$).

Conclusion: Although individual hematological variations were observed, all ABP variables remained within the individually calculated limits. Our results support that acute training load variations significantly affect [Hb], likely due to short-term PV fluctuations, underlining the importance of considering training load when interpreting individual ABP variations for anti-doping purposes.

1. Introduction

To prevent blood doping in elite cycling, in 2008 the Union Cycliste Internationale (UCI) spearheaded the introduction of the Athlete Biological Passport (ABP) (Zorzoli, 2010). The World Anti-Doping Agency (WADA) then progressively implemented the ABP more widely and currently more than 30'000 blood samples are collected yearly to longitudinally track various blood markers of athletes (WADA, 2019). Starting with average population levels as initial reference, biomarkers in successive samples from a given athlete allow an individually expected range to be predicted within which the series of marker values should fall assuming physiological conditions (WADA, 2019). This range is calculated with an adaptive Bayesian statistical model using levels of probability (i.e. specificity) chosen to estimate the limits of normal physiological variation (Sottas, 2011). The premise is that repeated sampling allows for a progressive narrowing of the range of values considered as physiological for a given individual. The adaptive model uses hemoglobin concentration ([Hb]) and a stimulation index, the OFF-score (combining reticulocyte percentage (Ret%) and [Hb] in $g{\cdot}L^{-1}$ with the formula: OFF-Score = [Hb] - 60 x √Ret%, to generate an Atypical Passport Finding (ATPF) if a marker falls outside the expected range with a 99% specificity (i.e. 1:100 chance or less that this result is due to normal physiological variation) (WADA, 2019).

The individualized ranges for the ABP variables need to be sufficiently large and robustly defined to avoid an ATPF caused by fluctuations related to factors independent of blood doping

(Sottas, 2011). The strict WADA guidelines for blood collection, transportation and storage prevent misinterpretation of variations due to such confounders (WADA, 2019; WADA, 2019; WADA, 2019) while other physiological confounding factors shall be considered.

Some markers of the ABP may be influenced by plasma volume (PV) variations altering their concentration in whole blood (e.g. [Hb] or hematocrit (HCT)), even though they may not be sufficient to prove doping (Schmidt, 2000). Both [Hb] or hematocrit HCT, when considered too high, have been used to apply "No-Start" rules by international federations such as the International Ski Federation (FIS) and the UCI (Saugy, 2020). Exposure to extreme environments (e.g. hot or hypoxic) may also alter PV and [Hb] (Sawka, 2000; Stanley, 2015; Young, 2019; Coffman, 2020; Lobigs, 2018). Further, in competitions over several days (e.g. elite cycling stages races), variations observed in ABP profiles were shown to relate to the repeated strenuous exercise and altitude exposure (Schumacher, 2015). Even though Hbmass remained stable in cyclists over a 6-day cycling race, unlike HCT and [Hb] (Garvican, 2010), stage racing at altitude was also reported to induce a hemodilution surpassing any altitude-induced increase in Hbmass (Garvican-Lewis, 2014).

Since such variations alter the ABP profiles (Gough, 2013) these confounders should be mentioned on the blood control forms (WADA, 2016; WADA, 2016) to allow for an informed evaluation of the ABP. In response to a call for inclusion of "all other relevant information also comprising training and competition results" (Vernec, 2014), monitoring athletic performance (and hence training content) has been proposed (Faiss, 2019), to further strengthen the ABP and its interpretation. Hematological biomarkers vary during a competitive season in athletes among disciplines (Banfi, 2006; Banfi, 2011; Andelkovic, 2015; Diaz, 2011). This could be due to plasma volume variations induced by effort in competition vs. out of competition (Morkeberg, 2009), or strength and endurance training periods (Collins, 1986; Imelik, 1992; Sawka, 2000). Little is known about any direct influence of training load variation on

hematological variables (Varamenti, 2018; Guglielmini, 1989). There are no studies investigating the influence of training load over a prolonged period on the ABP variables in elite cyclists.

The purpose of this study was therefore to monitor training load in elite cyclists over one year and analyze if and how individual ABP profiles constructed from monthly blood samples vary with training load. To further address the within-subject variance of [Hb] (Lobigs, 2016; Garvican-Lewis, 2020), we also determined PV and Hbmass indirectly to assess whether changes due to environmental conditions (seasonal effect) or prolonged periods of high vs. low training loads alter PV and Hbmass to an extent affecting ABP profiles. We hypothesized that acute (5 days) and chronic (42 days) training before an ABP sample would notably change the profile readings without exceeding the individual ranges of the ABP adaptive model.

2. Materials and methods

2.1 Study participants

Ten male elite cyclists (25.6 ± 3.4 yrs, 181 ± 4 cm, 71.3 ± 4.9 kg, 6.7 ± 0.8 $W \cdot kg^{-1}$ 5-min maximal power output) volunteered to participate in the study. All were members of the Swiss national cycling team or an elite cycling team registered at Swiss Cycling and competing in road, track and mountain-bike cycling events at an international level (e.g. UCI World & Europe Tour races or UCI World Cups). Initially, 12 subjects were recruited. One subject withdrew due to personal reasons. Another subject was excluded because of a medical condition during the study affecting his hematological variables, precluding the training load from being considered as the major factor of any variation in the hematological variables. Subjects all lived < 800 m and were healthy. No iron supplementation was used for the duration of the study. Prolonged exposures to hypoxic environments (>6 h at an inspired O2 pressure <120 mmHg) were entered into the training diaries. Among the 10 cyclists two were exposed to prolonged

hypoxic stimuli with a possible effect on erythropoiesis. Cyclist 6 spent 27 days above 3000 m during a vacation in Peru during which training load was drastically reduced (see Figure 3). This resulted in a large increase in Ret% with no influence of training load for the highest value in this specific case. Cyclist 7 slept in a hypoxic tent for 36 nights with an average daily exposure of 9 hours at an inspired oxygen pressure of 14.8 % (simulating 2500 m) before measurement 11 (see Figure 4) with a subsequent rapid increase in Ret% and delayed increase in Hbmass.

All participants provided a fully informed written consent to participate after the procedures and risks were explained. The study protocol was approved by the regional research ethics committee (CER-VD, Lausanne, Switzerland, #2018-01019) and conducted in respect of the Declaration of Helsinki.

2.2 Blood sampling and analysis

Venous blood samples were collected once monthly from every participant by the same experienced phlebotomist. Due to competition schedules and training camps, the samples were separated by 32 ± 12 days. Venipuncture was realized with a 21G short manifold butterfly needle inserted into an antecubital vein (Sarstedt Safety-Multifly®, Sarstedt AG, Nümbrecht, Germany). WADA blood collection guidelines were strictly followed with no physical exercise allowed in the 120 min preceding sampling and blood collection done after 10 min in a seated position, with the exception that instead of the recommended BD Vacutainer® tubes (K2EDTA (K2) CE cat no 368856/ref US 367856) we collected blood in Sarstedt S-Monovette tubes (EDTA-K 2.7 mL, Sarstedt AG, Nümbrecht, Germany), which we considered equivalent. (WADA, 2016;WADA, 2019). VSamples were stored at 4° C for 30 min to 12 h after collection before analysis, depending on instrument and technician availability. Samples were homogenized at room temperature (21° C) on a roller system for 15-45 min before analysis with

a fully automated flow cytometer (Sysmex XN1000, Sysmex Europe GmbH, Norderstedt, Germany). Internal quality controls provided by the manufacturer (Sysmex XN-Checks, levels 1, 2, and 3) were run three times before each batch of samples. The analysis was repeated to produce two successive analyses with differences equal or less than 0.1 $g{\cdot}dL^{-1}$ for [Hb], and 0.15% or 0.25% for Ret% (depending whether Ret% was inferior or superior to 1%) conforming to the applicable WADA guidelines (Wada, 2019). The first valid test result was then recorded. The stimulation index OFF-score was calculated as ([Hb] x 10) - 60 x √RET% and the Abnormal Blood Profile Score (ABPS) was calculated combining RET%, [Hb], hematocrit (HCT), red blood cell number (RBC#), mean red cell volume (MCV), mean red cell Hb and mean cell Hb concentration (MCHC) using the mathematical algorithms in WADA's Anti-Doping Administration and Management System (ADAMS) (WADA, 2019).

2.3 Individual ABP profiles

For each participant an individual longitudinal ABP profile was constructed with the values obtained from the collected blood samples using the official ABP-module in WADA's ADAMS Training Software entering data from each sample individually. The system calculates ABPS and OFF-score for each sample and then generates individual ABP profiles with [Hb] and OFF-score as primary markers and Ret% and the ABPS as secondary ones. Population-based upper and lower limits are used for the first blood sample after which an adaptive model generates individually varying limits for each subsequent blood sample considering previous individual analytical results. An Atypical Passport Finding (ATPF) is generated when a) a [Hb] and/or OFF-score value of the last entered sample falls outside the lower and upper intra-individual limits or b) when the last 2 to 5 [Hb] and/or OFF-score values deviate from the expected range (a so-called “sequence ATPF”). For the first case, the applied specificity is 99% (i.e. 1:100 chance or less that the deviation is due to normal physiological variation). For the latter, the

applied specificity is 99.9% (i.e. 1:1000 chance or less that the sequence deviation is due to normal physiological variation). An ATPF results in a notification to the Athlete Passport Management Unit (APMU) handling the administration of the individual passport on behalf of a passport custodian. The APMU may request expert opinions and declare an adverse passport finding (APF) after 3 independent experts with all available information unanimously deemed the profile likely to result from doping. An APMU may also request an expert opinion in the absence of an ATPF when abnormal variations (e.g. compatible with artificial hemodilution) is observed with ABP profiles remaining within individual limits.

To complement the analysis of variations in ABP variables, for [Hb] and the OFF-score, we also calculated the shortest absolute distance to the closest individual limit (i.e. from the upper or the lower limit).

2.4 Total hemoglobin mass and plasma volume

Hbmass was determined monthly over the last eight months of the study with a fully automated blood volume analyzer (OpCo: Detalo Instruments, Birkerod, Denmark) based on a carbon monoxide (CO) rebreathing technique, as described elsewhere (Siebenmann, 2017). Briefly, participants were comfortably installed in supine position with a nose clip and a mouthpiece connected to a closed rebreathing circuit. They then breathed 100% oxygen (O_2) for 4 min to flush the airways of nitrogen. Subsequently, a bolus of 1.5 mL/kg of 99.997% chemically pure CO (Carbagas, Liebefeld, Switzerland) was introduced into the circuit after which the participants rebreathed the O_2-CO mixture for 9 minutes. Rebreathing was done in supine condition to improve CO mixing (Keiser, 2013); nine minutes were reported sufficient to observe a peak in venous carboxyhemoglobin content (HbCO%). Venous blood was drawn from an antecubital vein and immediately analyzed in triplicate for HbCO% with a calibrated gasometer (ABL80-Co-Ox, Radiometer, Copenhagen, Denmark). Initial duplicate

measurements in our laboratory yielded a typical error (TE) of 1.8% for Hbmass, in line with previously reported values (Siebenmann, 2017; Rønnestad, et al., 2020). The CO remaining in the system was measured with a CO meter (Monoxor Plus, Bacharach, New Kensington, USA) and subtracted from the initial amount introduced to define the exact CO bolus received with a 0.1 mL typical error. Hbmass was calculated from the difference in HbCO% before and after CO-rebreathing with the formulas detailed by Siebenmann *et al*. (Siebenmann, 2017). Total red blood cell volume (RBCV), plasma volume (PV) and blood volume (BV) were derived from [Hb], hematocrit (HCT) and Hbmass as explained in the same reference.

2.5 Training load quantification

Participants were instructed to follow their habitual training and competition schedules as planned with their personal trainer and to report all their training and competition activities in a commercially available online training monitoring interface (Training Peaks™ (TP), PeaksWare, Lafayette, CO, USA). Since all participants were already using TP to monitor their training, we could collect training data for the 42 days prior to the first blood sampling in addition to the twelve months of the monitoring of their hematological variables. All used a crank-based power meter (SRM, Schoberer Rad Messtechnik, Juelich, Germany) for their cycling-based training sessions allowing their training load to be accurately quantified as a function of the duration and intensity of each training session. They were instructed to proceed to regular static calibration and zero-offset calibrations of their power meter according to the manufacturers' recommendations.

The Training Stress Score (TSS™, arbitrary units) was selected in this study to quantify training load because it was reported to be very reliable in competitive cyclists providing a strong dose-response relationship for the changes in aerobic fitness (Sanders, 2017). TSS was automatically calculated for each training session in TP using the following formula: TSS (a.u.)= [(*t* x *NP* x

IF) / (*FTP* x 3600)] x 100 where *t* is the duration in seconds, FTP represents the functional threshold power calculated as 95% of the average power from a recent 20-minute steady-state all-out time trial or maximal effort, *NP* is the normalized power, representing a calculation of the power that could have been maintained for the same physiological "cost" if the power had been perfectly constant, and *IF* is the intensity factor indicating the relative intensity of the session calculated as the ratio of NP to FTP (Coggan, 2019). Individual FTP values were determined at the beginning of the study based on the results of a maximal 20-min field or laboratory test realized under supervision of their personal trainer. The initially calculated FTP value was not modified during the study to allow for an adequate comparison of training loads and their variation throughout the study.

Acute training load (ATL) was calculated as the load during the 5 days preceding each monthly blood sample as a score cumulating the TSS over 5 days. Chronic training load (CTL) was defined as the load for the 42 days (6 weeks) preceding blood sampling and calculated again as a cumulated TSS over the period.

Training loads for High vs. Low training load periods for each cyclist were obtained by identifying the periods with the highest and lowest 12-week cumulative TSS. Seasonal training variation (Winter vs. Summer) was quantified calculating the cumulated TSS during three winter months (December, January and February) and three summer months (June, July, August), respectively.

2.6 Statistical analyses

Values are reported as means and standard deviations. The range reports the maximum and minimum from the individual values to describe data dispersion. Using data from 12 monthly blood samples for each participant (cluster variables, random factor), repeated measures analyses were conducted for each of the four primary variables of the ABP (i.e. [Hb], Ret%,

OFF-score and ABPS) with a mixed model to determine whether changes in the dependent variables (ABP variables) differed over time (fixed factor). This technique was preferred to a repeated measures ANOVA because it also allows to handle dynamic predictors. The effect on the ABP variables, Hbmass, and plasma volume of acute or chronic training load, seasonal variations in training load as well as periods with High vs. Low training load were assessed with mixed models using training load as time-dependent covariates. To indicate the effect of an independent variable, the value of the statistic with its degrees of freedom and the *P* value of the test are presented in parenthesis. In case of a quantitative independent variable (a covariate), the estimate of the parameter associated to it is presented in addition. Visual inspection of residual plots allowed excluding any obvious deviations from homoscedasticity or normality. Polynomial contrasts were used for time in mixed models, employing the Bonferroni method. The Pearson correlation coefficient was calculated for the relationship between time-trial performance (s) and Hbmass. The level of significance was set at $P < 0.05$. All statistical analyses were conducted with an open source dedicated statistical software (Jamovi, Jamovi Project Software, retrieved from (Ortiz-Prado et al., 2019)).

3. Results

3.1 Hematological variations over 12 months

[Hb] averaged 14.2 ± 0.1 $g \cdot dl^{-1}$ (range: 13.3 to 15.5 $g \cdot dL^{-1}$) over the study, while the ABPS averaged -1.32 ± 0.41 a.u. (range: -1.68 to -0.40 a.u.), both with significant variations over the 12 months ([Hb] ($F(11, 99) = 2.76$, $P = 0.004$) and ABPS ($F(11, 99) = 3.54$, $P < .001$). Ret% averaged 1.2 ± 0.31 % (range: 0.76 to 1.81 %) and the OFF-score 76.6 ± 10.0 a.u. (range: 64.0 to 91.7 a.u.) with no significant variation over the study time (Ret% ($F(11, 99) = 1.85$, $P = 0.056$) and OFF-score ($F(11, 99) = 1.77$, $P = 0.069$). Average hematological values over the 12 months for each cyclist are presented in Table 1.

Hbmass averaged 1030 ± 87 g (range: 842 to 1116 g) with no significant variation over the study time (F (7, 52) = 1.75, $P = 0.118$). Conversely, a significant time effect was observed for PV (F (7, 52) = 2.83, $P = 0.014$) which averaged 4309 ± 350 mL (range 3688 to 4751 mL).

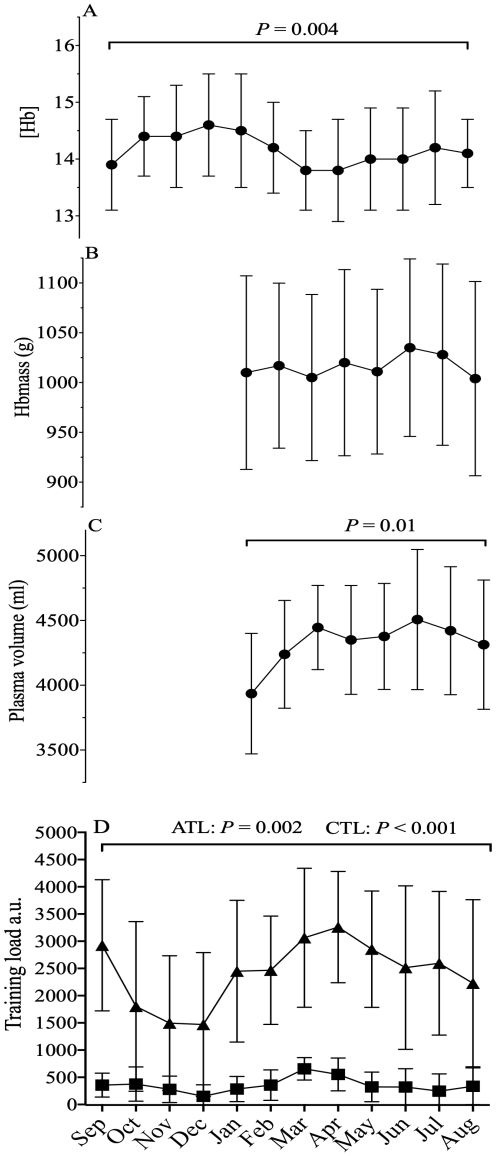

Figure 1. Average 12 months representation of **(A)** [Hb]: hemoglobin concentration; **(B)** Hbmass: total hemoglobin mass; **(C)** PV: plasma volume; along with **(D)** training load (a.u.) (cumulated daily TSS) with acute training load (5 days, ATL) and chronic training load (42 days, CTL) before sampling; a.u.: arbitrary units. *P* – values for the statistical difference over the 12 months.

	[Hb] g/dl	OFF-score a.u.	Ret %	ABPS a.u.	Hbmass* g	PV* mL	ATL a.u.	CTL a.u.	Total TSS a.u.
Cyclist 1	14.4 ± 0.4	91.7 ± 4.7	0.76 ± 0.1	-1.47 ± 0.2	1021 ± 45	4222 ± 225	317 ± 276	1572 ± 986	18871
Cyclist 2	13.5 ± 0.8	72.9 ± 8.3	1.07 ± 0.2	-1.68 ± 0.3	1021 ± 51	4703 ± 491	243 ± 252	2234 ± 1122	26814
Cyclist 3	13.8 ± 0.5	65.3 ± 6.5	1.49 ± 0.2	-1.59 ± 0.3	1045 ± 28	4751 ± 185	256 ± 276	1872 ± 1244	22466
Cyclist 4	15.5 ± 0.6	86.9 ± 5.8	1.31 ± 0.2	-0.40 ± 0.5	1157 ± 24	3998 ± 641	110 ± 193	1395 ± 1146	16751
Cyclist 5	14.8 ± 0.4	85.2 ± 4.4	1.08 ± 0.2	-0.91 ± 0.5	1014 ± 33	3944 ± 374	524 ± 319	2579 ± 1314	30957
Cyclist 6	13.3 ± 0.6	64.4 ± 11.0	1.32 ± 0.4	-1.59 ± 0.2	950 ± 35	4318 ± 377	583 ± 273	4004 ± 1079	48050
Cyclist 7	13.4 ± 0.4	80.3 ± 5.7	0.8 ± 0.2	-1.58 ± 0.1	842 ± 17	3688 ± 208	237 ± 179	1619 ± 876	19439
Cyclist 8	14.3 ± 0.6	74.1 ± 7.0	1.32 ± 0.2	-1.55 ± 0.3	1116 ± 21	4637 ± 338	357 ± 285	3174 ± 1584	38090
Cyclist 9	14.2 ± 0.5	80.9 ± 5.5	1.05 ± 0.1	-1.52 ± 0.4	1079 ± 18	4417 ± 286	517 ± 236	2768 ± 829	33223
Cyclist 10	14.4 ± 0.6	64.0 ± 5.7	1.81 ± 0.3	-1.04 ± 0.5	1056 ± 33	4421 ± 309	387 ± 324	3051 ± 968	36620
Mean	14.2 ± 0.01	76.6 ± 10.0	1.2 ± 0.31	-1.32 ± 0.41	1030 ± 87	4309 ± 350	307 ± 118	2427 ± 840	29128 ± 10091

Table 1. Average individual hematological variables and training load over 12 months. Values reported as means ± SD. [Hb]: hemoglobin concentration; OFF-score; Ret%: reticulocytes percentage; ABPS: abnormal blood profile score; Hbmass: total hemoglobin mass; PV: plasma volume; along with acute training load (5 days, ATL), chronic training load (42 days, CTL) before blood sampling and total training stress score (TSS) over one year; a.u.: arbitrary units. *: Hbmass and PV were monitored during the last eight months of the study.

3.2 Within-subject variations

The measured variables in the ABP profiles remained within the individualized limits; and no ATPFs were outlined. The distance to the individual limits for [Hb] and OFF-score values yielded an average mean lowest distance to the limits of 1.0 ± 0.4 g·dL$^{-1,}$ and 18.5 ± 3.9 (a.u.), respectively. Over the 120 measurements (10 subjects x 12 blood samples), 10 [Hb] values (8.3%) fell within a distance to the (upper or lower) individual limit < 0.5 g·dl^{-1} and 4 within a < 0.1 g·dl^{-1} (3.3%) distance. Four individually calculated OFF-score values were closer than 5 points (a.u.) to an individual limit (3.3%). Three illustrative examples of individual ABP profiles are presented in Figures 2, 3 and 4; and the hematological profiles of the remaining cyclists are available as Supplementary files.

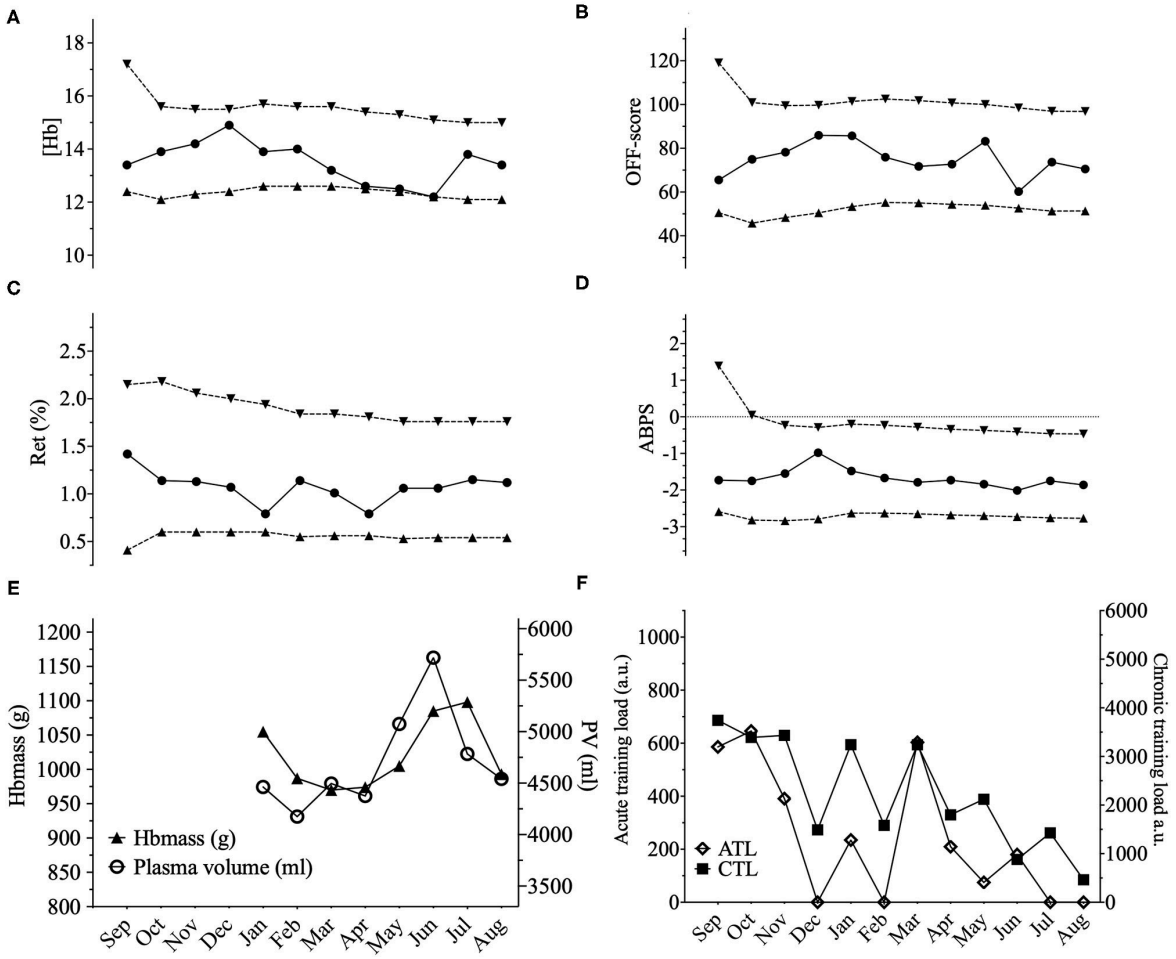

Figure 2. Representations of the Athlete Biological Passport (ABP) hematological profile for cyclist 2 with **(A)** (Hb): hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid lines represent the athlete's values, dotted lines represent the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details). **(E)** Triangles figure total hemoglobin mass (g) and circles represent plasma volume (mL) over the last eight months of the study; **(F)** Acute training load (ATL) and chronic training load (CTL) represent the load respectively 5 and 42 days before sampling. Vertical grey bars represent hypoxic exposure. ABP profile for cyclist 2 showing a prolonged state near the individual limits calculated for (Hb). (Hb) decreased from December to June by 17.6% however, an increase in Hbmass of 3% between January and June was observed with an increase of 28% of PV (4,461–5,719 mL) despite a decrease in chronic training load of −65% from January to August.

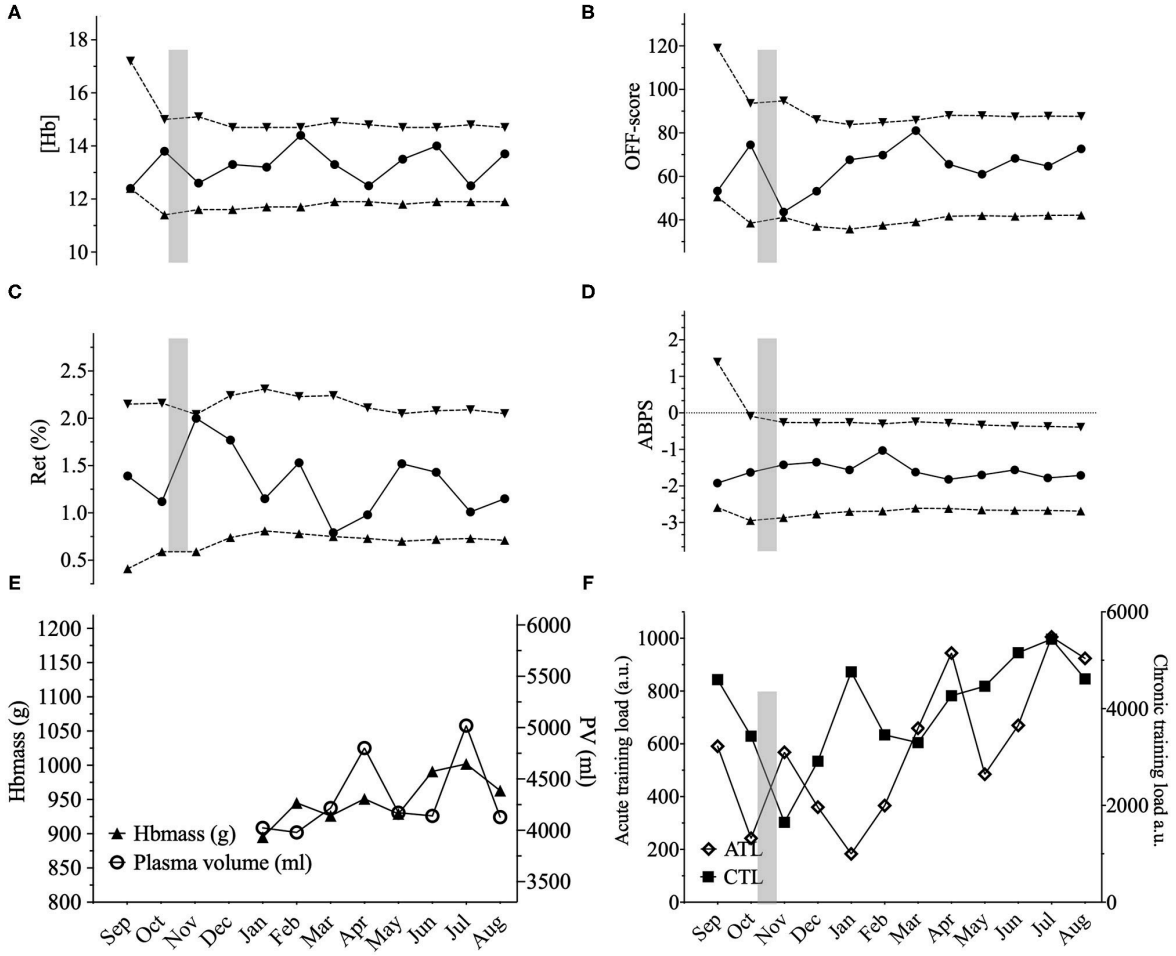

Figure 3. Representations of the Athlete Biological Passport (ABP) hematological profile for cyclist 6 with **(A)** (Hb): hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid lines represent the athlete's values, dotted lines represent the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details). **(E)** Triangles figure total hemoglobin mass (g) and circles represent plasma volume (mL) over the last eight months of the study; **(F)** Acute training load (ATL) and chronic training load (CTL) represent the load respectively 5 and 42 days before sampling. Vertical grey bars represent hypoxic exposure.

ABP profile for cyclist 6 showing a high variability in hematological parameters, especially for (Hb). Mean (Hb) was 13.5 ± 0.77 g·dL^{-1} for this cyclist with the highest SD among all the cyclists with values ranging from 12.2 to 14.4 g·dL^{-1} t (variation of 16%). Values close to the limits are also encountered for the OFF-score and Ret%. This cyclist went on holidays for 4 weeks at an average altitude of 2,750 m in Peru before sample 3 (October). The hypoxic dose amounted to 1,782 km· h^{-1} calculated according to (Garvican-Lewis et al., 2016) explaining the increased Ret% value. Besides, Hbmass varied only by 2% between February until August (945–963 g) and only 3% variations in PV for the same period (3,979–4,129 mL). Despite high variations in the ABP biomarkers, chronic training load did not significantly vary for this cyclist from January to August (+15%).

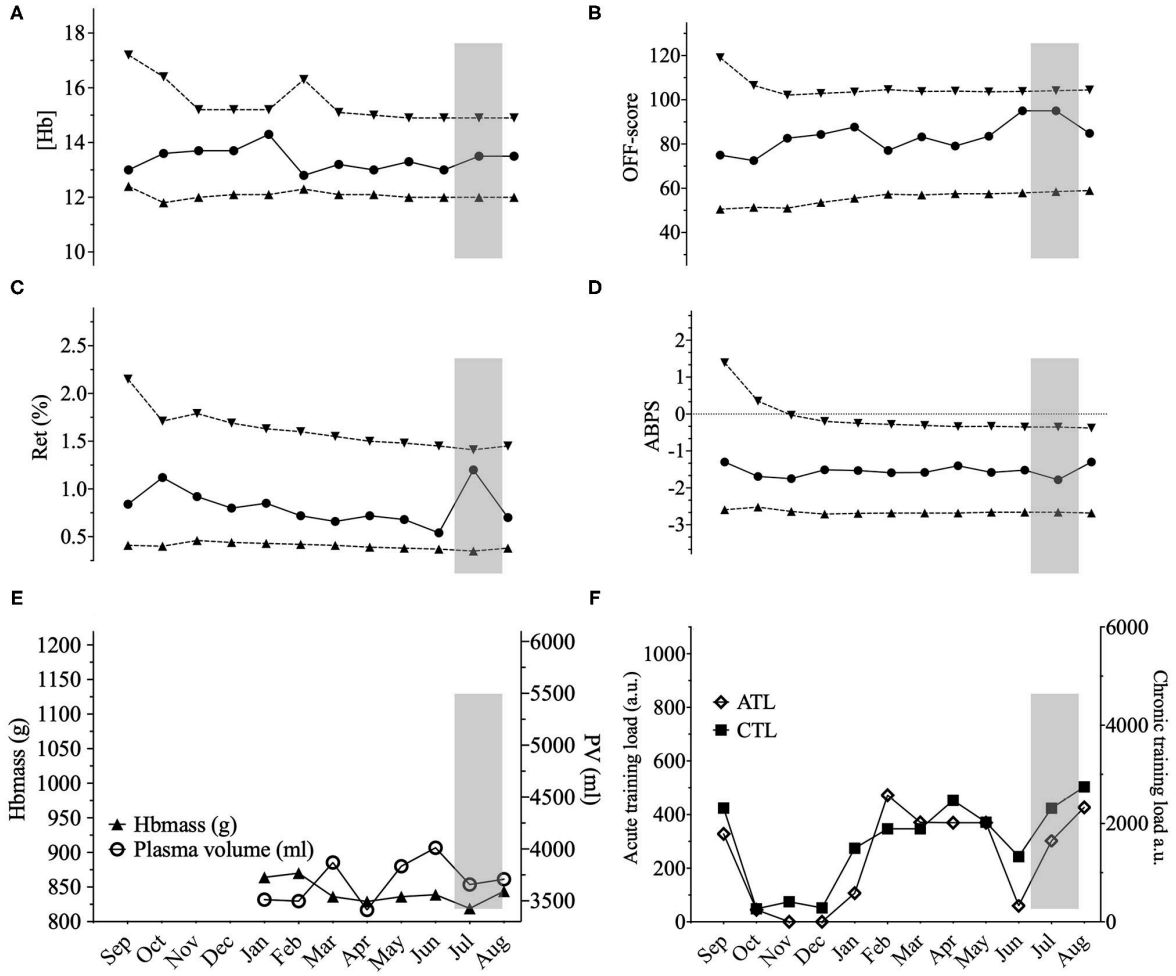

Figure 4. Representations of the Athlete Biological Passport (ABP) hematological profile for cyclist 7 with **(A)** (Hb): hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid lines represent the athlete's values, dotted lines represent the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details). **(E)** Triangles figure total hemoglobin mass (g) and circles represent plasma volume (mL) over the last eight months of the study; **(F)** Acute training load (ATL) and chronic training load (CTL) represent the load respectively 5 and 42 days before sampling. Vertical grey bars represent hypoxic exposure.

ABP profile for cyclist 7 showing few variations. (Hb) varied from 12.8 to 14.4 (10%) with a 6% Hbmass variation (819–870 g) despite a 18% change in PV (3,413–4,012 mL) and a chronic training load increase of 83% from January to August. Cyclist 7 slept in a hypoxic tent for 36 nights with an average daily exposure of 9 h at an inspired oxygen pressure of 14.8% (simulating 2,500 m). The hypoxic dose amounted to 972 km·h–1 calculated according to Garvican-Lewis et al. (2014) with 459 km·h–1 and 513 km·h–1 before after and measurement 11, respectively, explaining the sustained high Ret% with a delayed 3% Hbmass increase between measurement 11 and 12.

3.3 Training load analysis

The average cumulated TSS over 12 months amounted to 29128 ± 10091 a.u. For the High load period average cumulated TSS amounted to 10389 ± 2933 a.u. vs. 3440 ± 2544 a.u. during the Low load period. Cumulated TSS during the Low load period represented on average 31 ± 18% of the High Load TSS. The average cumulated Winter TSS was 7396 ± 2817 a.u., representing on average to 89 ± 50% of the Summer TSS (7609 ± 3658 a.u.) P = 0.75) (Table 2). Cumulated TSS was 353 ± 292 a.u., and 3310 ± 1466 a.u over the 5 (ATL) and 42 days (CTL) preceding blood sampling, respectively. Both ATL and CTL varied significantly with time (P = 0.002 for ATL and P < 0.002 for CTL) (Figure 1 and Table 1).

3.3.1 Seasonal and training load period influence on the ABP

Training load period (High load vs. Low load) did not significantly affect ABP variables (i.e., [Hb] (P = 0.50), OFF-score (P = 0.49), Ret% (P = 0.16), ABPS (P = 0.29)) or Hbmass (P = 0.36) and PV (P = 0.86). There was no significant effect of season on the ABP variables (i.e., [Hb] (P = 0.87), OFF-score (P = 0.96), Ret% (P = 0.87), ABPS (P = 0.20) and on Hbmass (P = 0.45) and PV (P = 0.06). Hematological variations with respect to High vs. Low training load periods and Winter vs. Summer are summarized in Table 2.

	Summer	Winter	Change (%)	High load	Low load	Change (%)
[Hb] (g/dl)	14.1 ± 0.8	14.2 ± 0.7	0.7	14.0 ± 0.6	14.4 ± 1.0	2.8
OFF-score (a.u.)	76.8 ± 10.0	78.8 ± 11.1	2.6	77.1 ± 9.3	77.6 ± 11.9	0.6
Ret%	1.18 ± 0.3	1.15 ± 0.3	-2.5	1.1 ± 0.3	1.3 ± 0.3	18.2
ABPS (a.u.)	-1.42 ± 0.5	-1.25 ± 0.4	12.0	-1.44 ± 0.4	-1.23 ± 0.6	14.5
Hbmass (g)*	1032 ± 84	1023 ± 86	0.9	1048 ± 99	1079 ± 41	3.0
PV (mL)*	4442 ± 403	4148 ± 393	-6.6	4399 ± 354	4342 ± 502	-1.3
TSS (a.u.)	7609 ±3658	7396 ± 2817	-2.8	10389 ± 2933**	3440 ± 2544	-67

Table 2. Average hematological variables according to season and to training load. Data are presented as means ± SD. [Hb]: hemoglobin concentration, OFF-score, Ret%: reticulocytes percentage; ABPS: abnormal blood profile score; Hbmass: total hemoglobin mass; PV: plasma volume; a.u.: arbitrary units. Summer vs. winter were calculated as the cumulated TSS over three months, high vs. low training loads are calculated as the maximal and minimal cumulated TSS per months over three successive months. * Hbmass and PV were monitored during the last eight months of the study. ** P< 0.001 for the difference with Low load (TSS

3.3.2 Influence of acute and chronic training load on the ABP variables

There was a significant effect of cumulated TSS over the five days preceding blood sampling (ATL), on [Hb] (F (1,102) = 12,8, $P < .001$), b = - 0.0036, and PV (F (1,55) = 7.76, $P = 0.007$, b= 2.2, (indicating that a higher ATL was associated with a lower [Hb]), but not ABPS ((F (1,102) = 3.35, $P = 0.07$), OFF-score ((F (1, 102) = 3.77, $P = 0.055$), Ret % ((F (1, 102) = 1.18, $P = 0.28$ or Hbmass ((1, 52) = 2.05, $P = 0.16$). No significant effect of CTL was observed neither on the ABP variables nor Hbmass and PV ([Hb] (F (1,106) = 1.86, $P = 0.176$), Ret% (F (1,106) = 1.62, $P = 0.2$), OFF-score (F (1, 106) = 0.016, $P = 0.89$), ABPS (F (1, 106) = 2.34, $P = 0.129$), Hbmass (F (1,55)= 1.72, $P = 0.195$), PV (F (1, 57)= 0.85, $P = 0.36$). For each cyclist, ATL, CTL and total training load over the 12 months are reported in Table 1.

Cumulated TSS over the 42 days preceding blood sampling had no effect on [Hb] (F (18,82) = 1.19, *P* = 0.35), OFF-score (F (18, 82) = 1.17, *P* = 0.36, Ret% (F (17.3,82) = 1.87, *P* = 0.071), ABPS (F (16.7,82) = 2.02, *P* = 0.053), Hbmass (F (2,54) = 1.29, *P* = 0.53), PV (F (2,54) = 1.99, *P* = 0.39), Cumulated TSS over the 5 days preceding blood sampling had a significant effect on [Hb] (F (1,101) = 12.91, *P* < .001), b = -7.35e-4 , OFF-score (F (1, 100) = 4.32, *P* = 0.04), b = - 0.00545 and PV (F (1,56) = 7.76, *P* = 0.007), b = 0.44 There was no significant effect on Ret% (F (1,100) = 0.79, *P* = 0.38), ABPS (F (1,101) = 3.20, *P* = 0.08), PV (F (2,56) = 18.5, *P* = 0.053) and Hbmass (F (1,52) = 2.05, *P* = 0.16). For each cyclist, ATL, CTL and total training load over the 12 months are reported in Table 1.

4. Discussion

By collecting and analyzing monthly blood samples together with training stress score in a cohort of elite cyclists over a one-year period, we could test the hypothesis that variations in training load over time lead to relevant changes in ABP parameters. The main finding of this study was that acute changes in training load (5 days) prior to blood sampling influenced ABP parameters (e.g. [Hb]), via changes in plasma volume. Chronic changes in training load (42 days) did not influence the ABP parameters. We observed significant variations in PV (but not Hbmass) over time. Despite the highlighted variations ABP variables remained within the individual limits at all times.

Endurance athletes reportedly have greater PV in comparison with team sports athletes, power endurance athletes, and disabled or untrained subjects (Fellmann, 1992). Endurance athletes also have fluctuations in PV, potentially inducing variations in biological markers that are concentration sensitive such as [Hb] (Lobigs, 2016). In agreement we found [Hb] and PV to significantly vary over a one-year period, while Hbmass did not. Monitoring PV would hence allow to interpret [Hb] alterations adequately. Variations in environmental

temperature may also affect blood variables and circulating volumes (Sawka, 1987; Doupe, 1957). We found for instance that a training period in warm summer months was associated with a 4.7 % increase in PV, although not significant, (when compared to winter) even though ABP variables or Hbmass were not influenced. The seasonal discrepancies in PV were not clearly associated with training in our study, although they were in line with the above-mentioned literature considering winter vs. summer temperature in Switzerland where the study was conducted (Table 2).

Our study allowed to contrast the influence of acute (5 days, ATL) variations of training load with the load considered over a longer time (42 days, CTL) before each blood sample. We found that higher ATL was accompanied by lower [Hb] and increased PV. This strongly suggests a hemodilution associated with short-term ATL fluctuations as reported in the literature with PV increase after an acute increase in training load (at the start of an exercise training program) (Sawka, 2000).

An acute hemodilution was recently observed in professional floorball players immediately after a game (>3% decrease in [Hb]) while values returned to baseline after 2-h (Wedin et al., 2020). In an anti-doping context, the pre-analytical bias possibly due to the acute effect of one single strenuous effort is avoided with the compulsory 2-h waiting time after the exercise before blood sampling is allowed (WADA, 2019). The latter rule does however not apply to repeated exercises (i.e. training load) on the days before a blood sample. Our results therefore suggest that monitoring or at least reporting the training load for several days before blood sampling would reasonably allow a better interpretation of ABP variables in an anti-doping setting.

Phases with possibly lower training loads (e.g. holidays or off-season periods, taper period) are expected to have an influence on blood variables (Mujika, 2000).

In our cohort of elite cyclist, we identified prolonged three-months periods with significantly higher training loads, but these did not have a significant effect on the variation of ABP profiles (Table 2). Conversely, [Hb] was shown to decrease in a workload dependent manner while red blood cell count remained constant in 19 elite competitive soccer players over half a competitive season of three months with a controlled training program (Andelkovic, 2015). This underlines the prime relevance of within-subject variation in the ABP that needs to be considered on an individual basis, despite the scientific relevance of cohort results.

The ABP was hence designed to allow "switching the focus from comparison with a population to the determination of individual values" (Sottas, 2010). Bayesian networks were used for the ABP, because they allow to represent the causal relationship between blood doping and its effect on hematological biomarkers (Koski, 2009;Kruschke, 2011). For instance, if blood doping (e.g. recombinant human erythropoietin (rhEPO) use) leads to an increased [Hb], rhEPO is the cause and a higher [Hb] the effect. Monitoring hematological biomarkers in a longitudinal profile is thus challenging because it goes against the causal direction. The way the ABP was designed allows however to analyse the probability that hematological variations may be due to doping rather than natural fluctuation based on existing data showing reference ranges and within-subject variability of either doped or non-doped populations (Malcovati, 2003). More precisely, the model relies i) on a dichotomic variable with two states: doped or non-doped, and ii) a continuous variable represented by hematological biomarkers. Bayes' theorem then expresses the probability of being in a doped state as a function of the measured biomarker. Individual limits of each biomarker of the ABP are set with a high specificity (e.g. 99%). This means that there is less than 1:100 chance that a value outside of the limits is due to a normal physiological condition. The advantage of Bayesian Networks is that they allow to include heterogeneous and

confounding factors (e.g. age, sex, ethnic origin, type of sport, altitude exposure) (Sottas, 2010). Already when it was launched, the potential of the ABP in integrating new potential confounding factors (i.e. training load) to the Bayesian adaptive model was acknowledged (Sottas, 2011). Now the inclusion of performance models has also been proposed (Faiss, 2019).

In our study, to complement the ABP approach, we addressed within-subject variance, and the influence of PV variations by looking at the lowest distance to the individual limits calculated by the Bayesian model for each successive sample. For example, we observed 3 successive [Hb] values within 0.1 $g \cdot dL^{-}$ to the individual limit with a concomitant increase in PV of 1344 mL in one cyclist (Figure 2). However, when considering all 10 elite cyclists (120 ABP points over one year) and despite noticeable differences in PV or [Hb], no ATPFs were observed, with the lowest distance to the individualized upper and lower limits for [Hb] falling only 10 times < 0.5 $g \cdot dL^{-1}$. The inspection of individual ABPs however revealed not a single pattern in the cyclists population regarding how the training load may potentially alter blood values. The visual inspection of individual variations (Figures 2-4) allows to illustrate some patterns of variation observed in training load, and hematological variables. The variations were not uniform and individual interpretation of within-subject variance in the context of the APB is paramount: a decrease in [Hb] may result from a large increase in PV (over 1200 mL), likely related to increased training load while the concomitant increase in Hbmass is less pronounced (see example in Figure 2). Alternatively, changes in training load may not necessarily result in concomitant fluctuations in [Hb] (see example in Figure 3). Finally, a rather stable ABP profile may appear despite high variations in PV (+ 18%) and Hbmass (+ 6%) (see Figure 4). The latter example would additionally question the usefulness of including training content in the interpretation of a profile with no noticeable [Hb] variation notwithstanding significant changes in training load. To

summarize, despite statistically significant relations obtained on aggregate data, there was no systematic association between PV, Hbmass, and individually interpreted ABP variables. Overall our results suggest that the current individual limits of the ABP seem sufficiently robust to prevent a falsely negative interpretation of an ABP profile even though training load variations are present.

Bearing this in mind, blood doping remains attractive to augment Hbmass and improve convective oxygen transport capacity (Warburton, 2000) even with low-volume transfusions that can have a significant performance enhancing effect (Bejder, 2019). In a laboratory setting, minimal changes in Hbmass, as low as 1 $g \cdot kg^{-1}$, can be accompanied by a significant change in aerobic capacity (Schmidt, 2010). To that extent we confirmed the previously reported link between absolute Hbmass and aerobic capacity in elite cyclists (Garvican, 2011;Hauser, 2017). It could therefore be argued that Hbmass would be a valid marker to complement the ABP analysis. The lack of relative influence of Hbmass (in $g \cdot kg^{-1}$ bodyweight) on time-trial performance may support the effect of a higher PV to reduce peripheral resistance to improve oxygen delivery to the muscle (Mairbäurl, 2013;Warburton, 2000). The relative weight of variations in single biomarkers in influencing the ABP markers should therefore be interpreted with care. This underlines the key role of ABP experts for a qualitative interpretation of suspicious profiles by accounting for and discriminating all possible confounders properly.

Strengths and limitations

The strength of this study is that our cohort was composed exclusively of highly-trained elite cyclists and the first one collecting and interpreting monthly blood samples together with quantification of Hbmass and training load over 12 months. Half of the participating athletes were part of a registered testing pool and subject to anti-doping testing and ABP

profiling. Our findings may thus adequately reflect the situation found in an anti-doping context analyzing ABP profiles of elite athletes. With an informed consent to participate in the project, it can be reasonably assumed (but not fully excluded) that the cyclists did not commit any anti-doping rule violation during the study. We must however acknowledge our small sample size limiting the power of our inferential analyses and Hbmass missing values for the first months of the study due to technical reason with the device. Besides, even with strict measurement procedures for Hbmass yielding an acceptable typical error of measurement, it cannot be excluded that a bias occurred in very few individual measurements for the determination of COHb fraction if a certain degree of hemolysis had occurred in the venous sample collected (Lippi, 2013). Each single measurement was carefully verified, but this may however have resulted in artificially high Hbmass with no other confounder clearly identified (e.g. Figure 2, June). Even though unidentified confounders other than exercise training may have influenced certain variables in single measurements, we feel confident that this does not alter the overall conclusion from our inferential perspective.

In addition, the quantification of training load is notoriously difficult. Arguably our approach using TSS (combining intensity from power output and volume with training duration) was deemed the most pertinent when designing the study, with an interface routinely used by all our cyclists and their trainers. We decided to maintain the same FTP for each athlete during the study time as it is often the case in real setting, however we must admit that this choice might have affect the training load. Characterizing objectively short periods of high acute training load before a blood test definitely remains challenging with the numerous training strategies possible. A simple declaration of high ATL (as a pretended alternative to blood withdrawal) may not be considered ultimately by an ABP expert as a unique pertinent explanation for a drop in [Hb]. Nevertheless, based on our findings we see

a rationale for the inclusion of more complete information on training load on the days preceding an ABP sampling procedure. While athletes would obviously not agree to share training "secrets", adding a simple question to the doping control forms on training volume and intensity during the days preceding a test could represent a first step towards a more transparent and meaningful interpretation of ABP profiles.

In conclusion, we consider the ABP as a powerful tool for targeting anti-doping tests, and indirect detection of doping. Our study suggests that variations of acute training load (i.e. the 5 days before a sample is collected) may influence the ABP readings. Considering specific confounding factors (i.e. training load) is therefore certainly paramount in the qualitative assessment of variations observed in ABP profiles to adequately aim for cost-effective testing plans targeting the right athletes at the right moment.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

RF and MS conceived the project and obtained the project funding. TA and RF contributed to the collection of data. TA, RF and FCvR statistically analyzed the data. RF, TA, BK, FCvR and MS interpreted the data. TA wrote the first draft of the manuscript. All authors contributed to revising the manuscript and expressed their approval of the final submitted version.

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Supplementary files

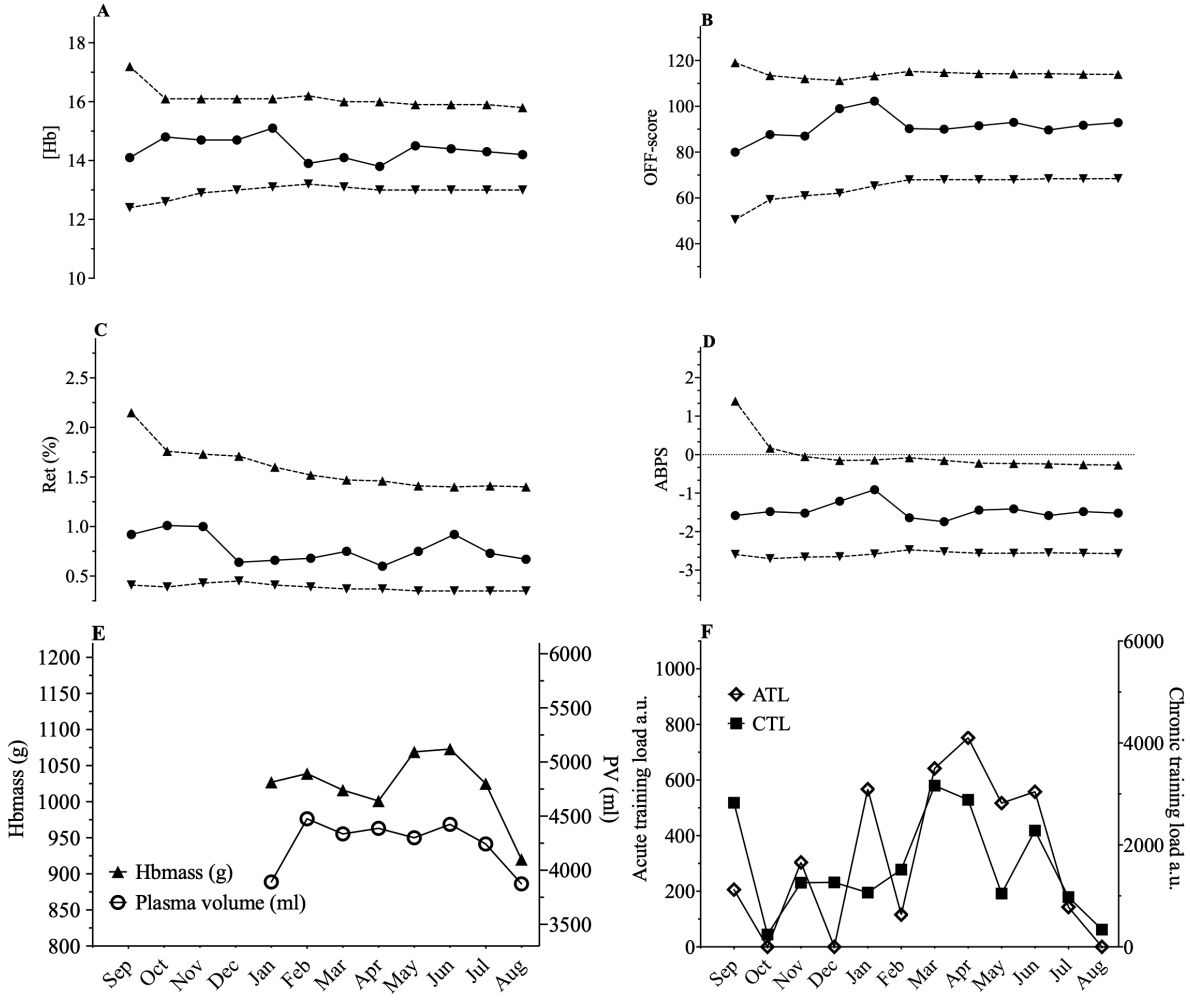


Figure 5. Representation of the Athlete Biological Passport (ABP) hematological profile for cyclist 1, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details). **(E)** Triangles figure total hemoglobin mass (g) and circles represent plasma volume (ml) over the last eight months of the study. **(F)**. Acute training load (ATL) and chronic training load (CTL) represent the load respectively 5 and 42 days before sampling.

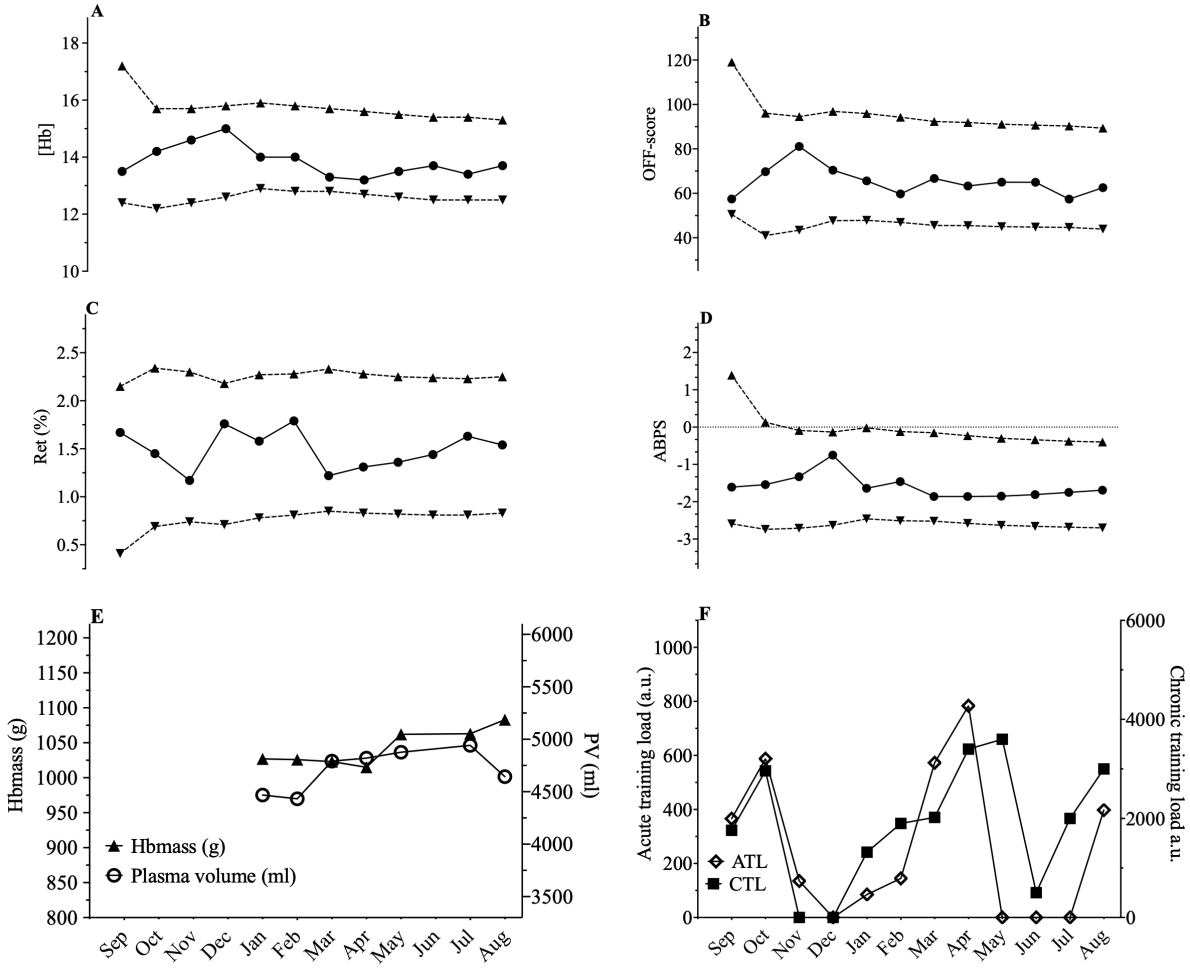


Figure 6. Representation of the Athlete Biological Passport (ABP) hematological profile for cyclist 3, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details). **(E)** Triangles figure total hemoglobin mass (g) and circles represent plasma volume (ml) over the last eight months of the study. **(F)**. Acute training load (ATL) and chronic training load (CTL) represent the load respectively 5 and 42 days before sampling.

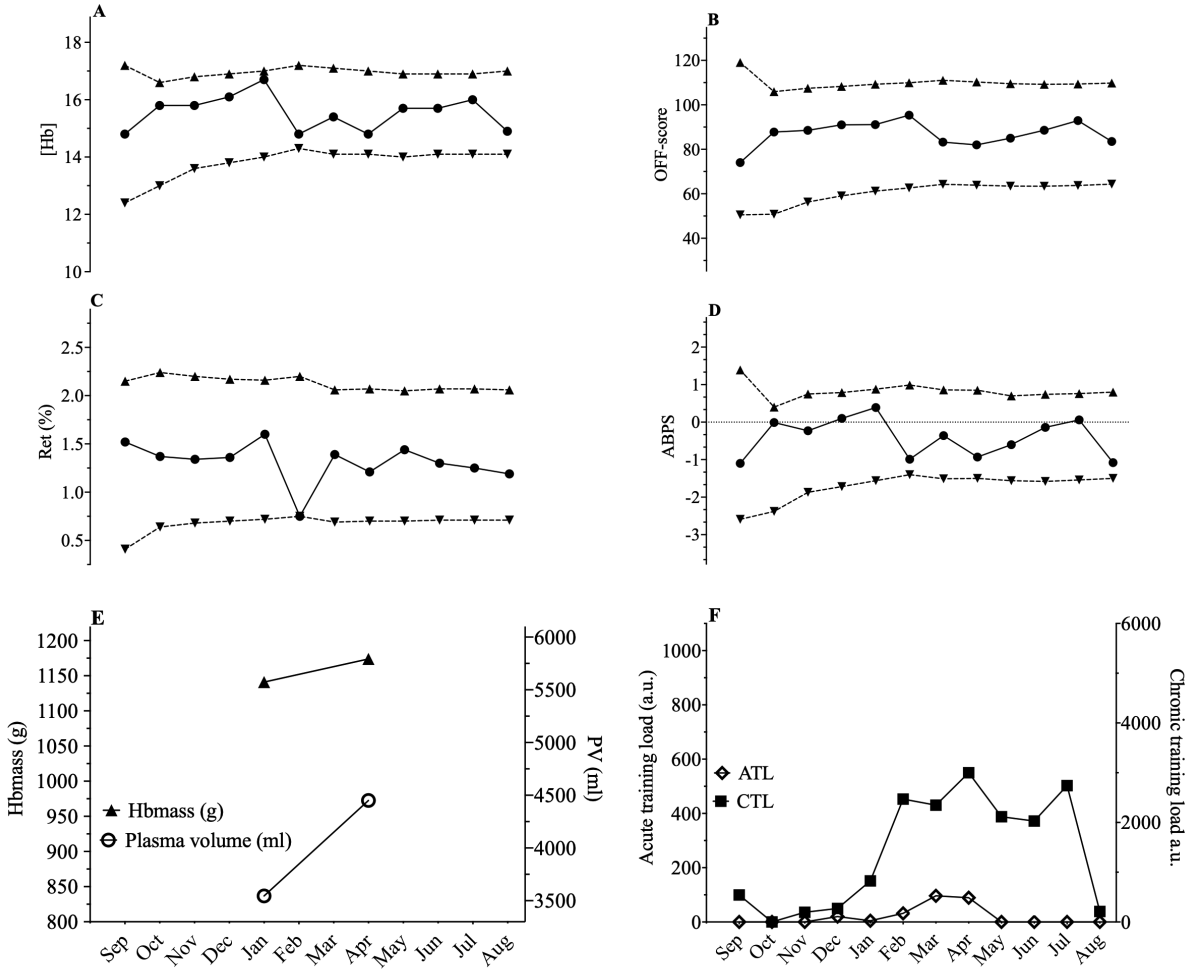


Figure 7. Representation of the Athlete Biological Passport (ABP) hematological profile for cyclist 4, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details). **(E)** Triangles figure total hemoglobin mass (g) and circles represent plasma volume (ml) over the last eight months of the study. **(F)**. Acute training load (ATL) and chronic training load (CTL) represent the load respectively 5 and 42 days before sampling.

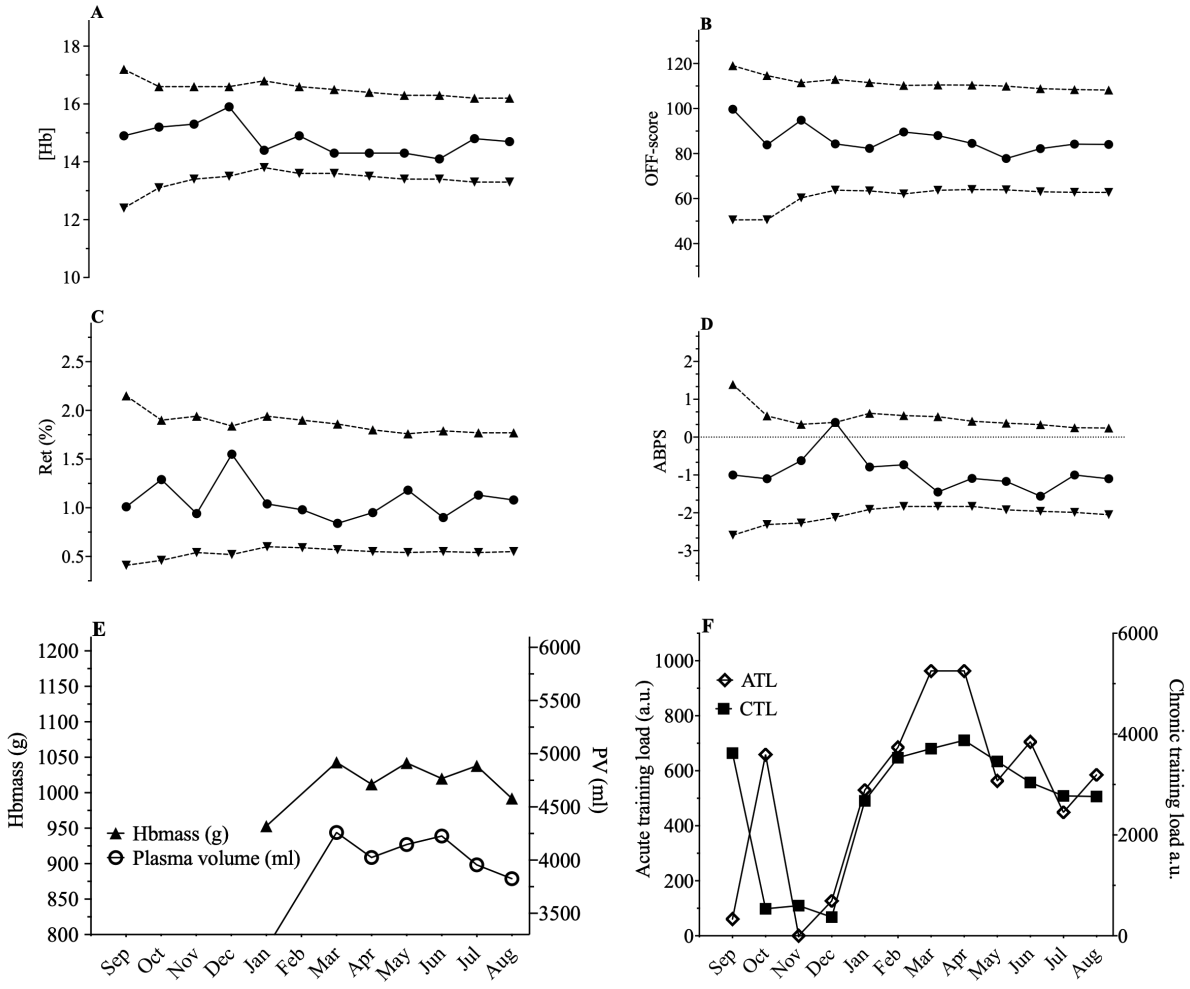
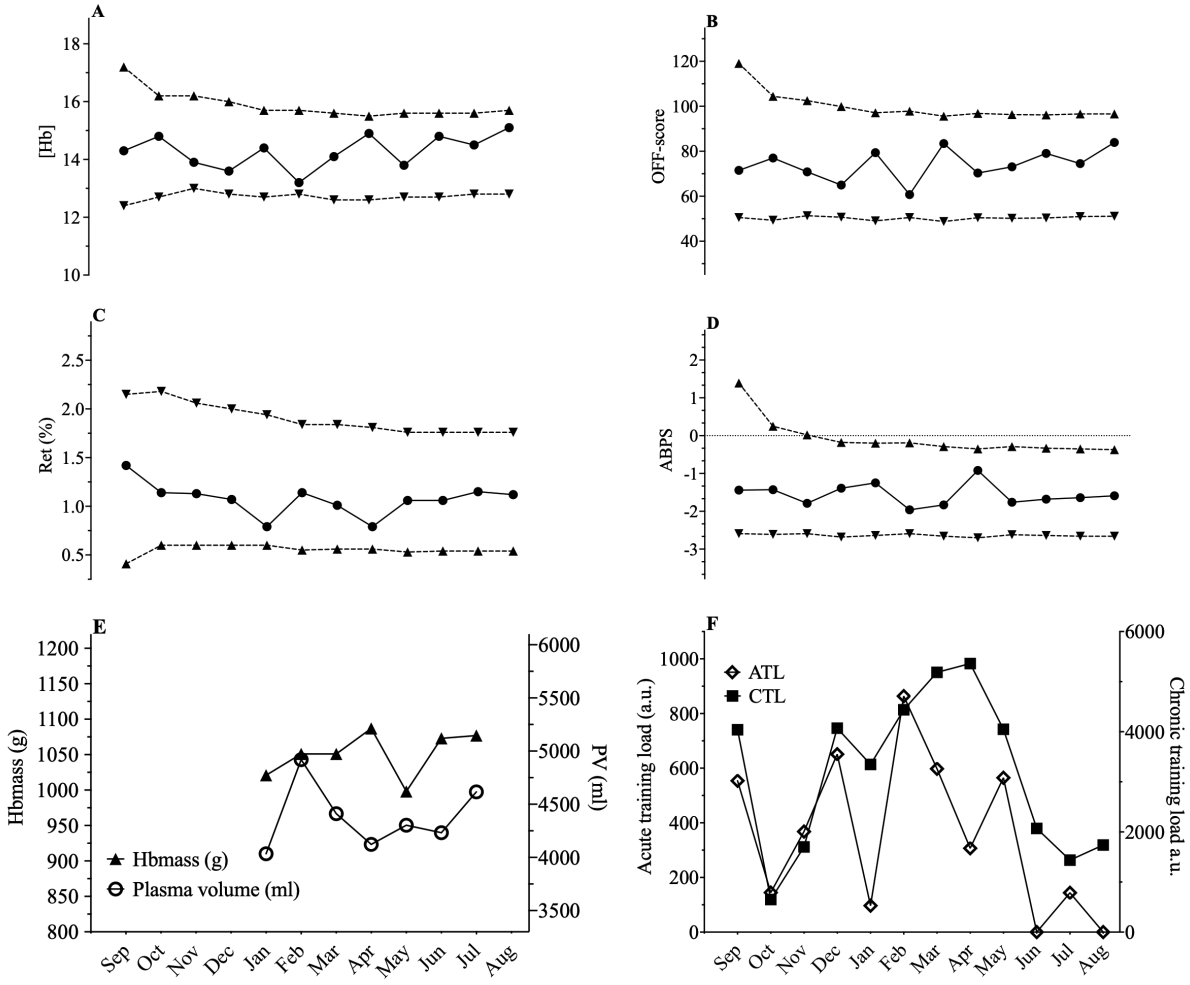


Figure 8. Representation of the Athlete Biological Passport (ABP) hematological profile for cyclist 5, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details). **(E)** Triangles figure total hemoglobin mass (g) and circles represent plasma volume (ml) over the last eight months of the study. **(F)**. Acute training load (ATL) and chronic training load (CTL) represent the load respectively 5 and 42 days before sampling.



Figures 9. Representation of the Athlete Biological Passport (ABP) hematological profile for cyclist 8, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details). **(E)** Triangles figure total hemoglobin mass (g) and circles represent plasma volume (ml) over the last eight months of the study. **(F)**. Acute training load (ATL) and chronic training load (CTL) represent the load respectively 5 and 42 days before sampling.

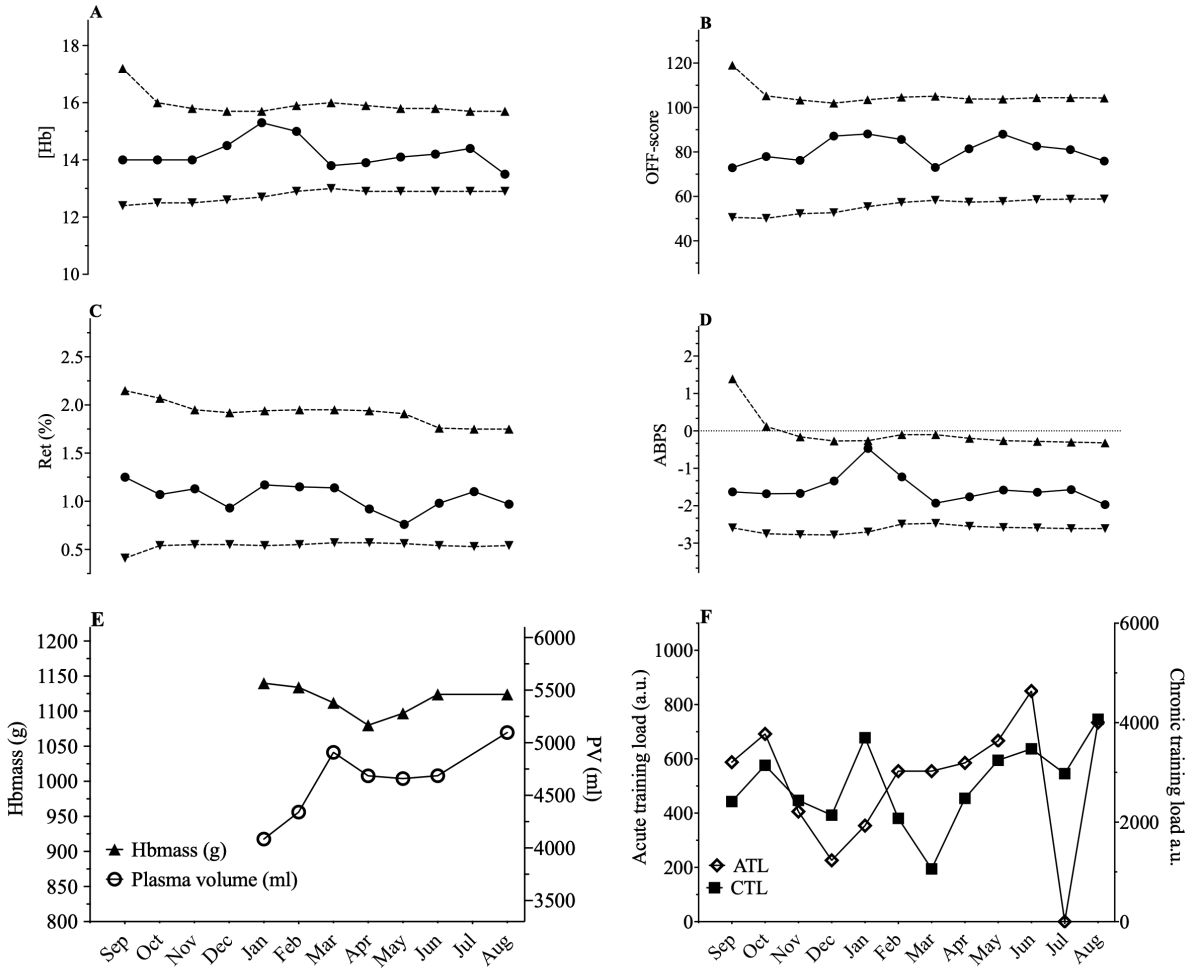


Figure 10. Representation of the Athlete Biological Passport (ABP) hematological profile for cyclist 9, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details). **(E)** Triangles figure total hemoglobin mass (g) and circles represent plasma volume (ml) over the last eight months of the study. **(F)**. Acute training load (ATL) and chronic training load (CTL) represent the load respectively 5 and 42 days before sampling.

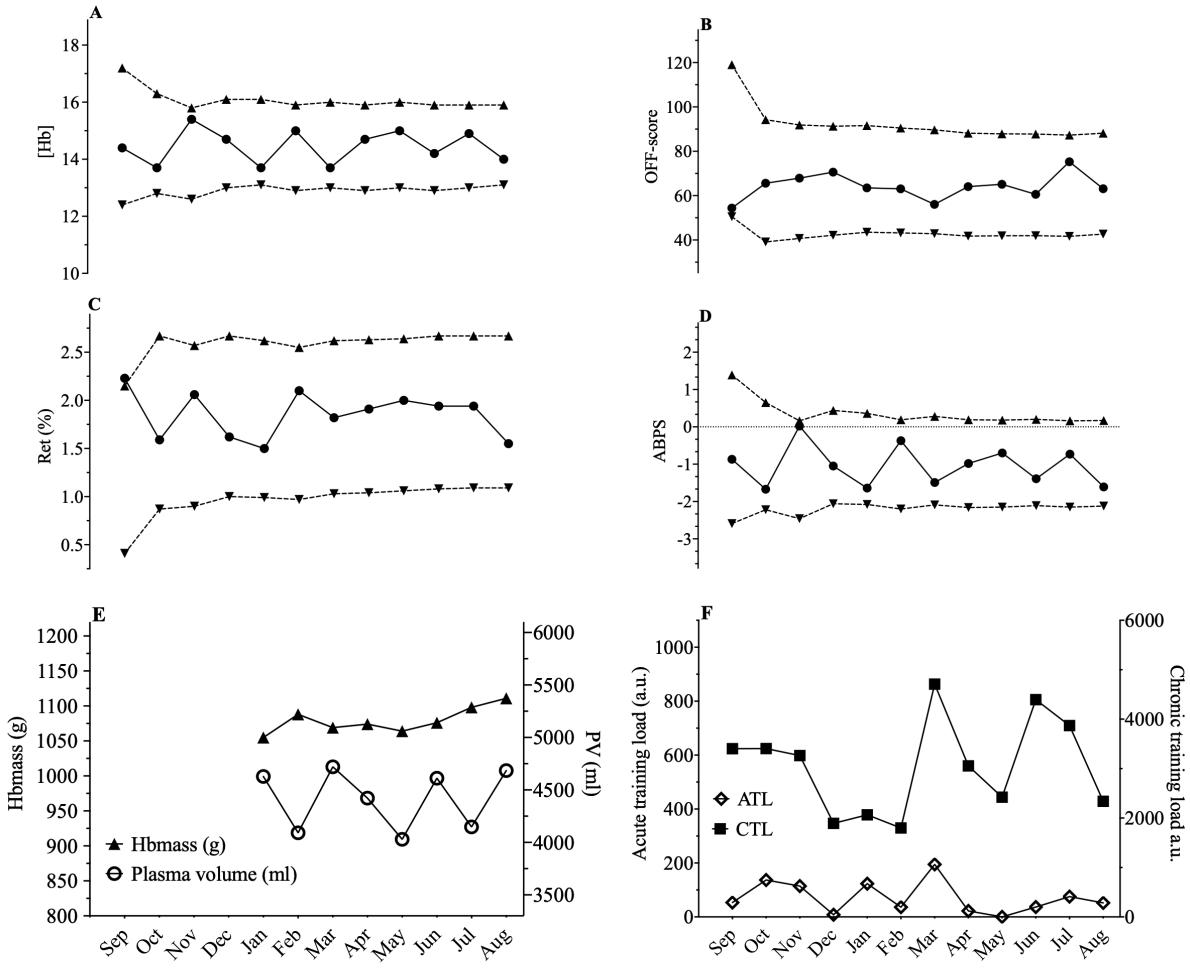


Figure 11. Representation of the Athlete Biological Passport (ABP) hematological profile for cyclist 10, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details). **(E)** Triangles figure total hemoglobin mass (g) and circles represent plasma volume (ml) over the last eight months of the study. **(F)**. Acute training load (ATL) and chronic training load (CTL) represent the load respectively 5 and 42 days before sampling

CHAPITRE 5

ARTICLE III

Hematological variables in recreational breath-hold divers: a longitudinal study[9]

[9] Tables, figures and references follow their own numbering system within this chapter.

5. Article III. Hematological variables in recreational breath-hold divers: a longitudinal study.

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Introduction: The influence of regular breath-hold training on hematological variables is not fully understood. We monitored hematological variables in breath-hold divers' (BHDs) and active controls over a year expecting both breath-hold training and seasonal effects.

Methods: In 11 recreational BHDs (36 ± 9 yrs, 177 ± 8 cm, 72 ± 9 kg) and 12 active controls (22 ± 2 yrs, 174 ± 8 cm, 70 ± 13 kg) monthly blood samples were analyzed with the hematological module of WADA's athlete biological passport. Hemoglobin mass and plasma volume were measured indirectly by the CO-rebreathing method for the last eight months of the study. Breath-hold training sessions were recorded online. Days without breath-hold training, or the number of hours prior to blood sampling when training was realized within the last 24 h, were recorded.

Results: Hematology did not differ significantly between BHDs and controls over the study time ($P > 0.05$). However, hematological values varied significantly over time for both groups suggesting seasonal effects. Blood sampling 19h or more after a breath hold training did not indicate any acute effects of breath holding training.

Conclusions: In comparison with a physically active lifestyle, regular breath-hold training does not induce significant variations over one year for the hematological module of the ABP.

Key words: hematology, chronic apnea training, anti-doping

1. Introduction

Breath-hold diving has emerged as an organized sport with different disciplines, the main ones being dynamic, depth, and static. Breath holding performances are defined by horizontal or vertical under water distance covered, for dynamic and depth respectively, and apnea time for static (Bain, 2018). The common denominator is apnea tolerance. To improve their performance breath-hold divers (BHD) seek to control their urge to breathe, to delay their breakpoint. This is achieved by regular apneas, which increase the tolerance to hypercapnia (Ivancev, 2007; Elia, 2021) and hypoxemia (Henig, 2000). Apart from leading to hypercapnic hypoxia, breath-holding induces the classic diving response with peripheral vasoconstriction and bradycardia. Even though hypercapnia solicits a stronger urge to breathe, it is the progressive hypoxemia which is the main performance limiting factor since it will eventually lead to loss of consciousness. Arterial blood samples obtained at surfacing after a dive to 42 m in six BHDs showed oxygen tensions as low as 31.6 ± 17.0 mmHg (Bosco, 2020).

BHDs are therefore regularly exposed to episodes of hypoxemia. The accompanying diving reflex includes contractions of the spleen expulsing ~100 ml of concentrated red cells into the circulation (Kramer, 1951). This leads to transient increases of blood hemoglobin concentration ([Hb]) and hematocrit (HCT), lasting between 2 and 10 minutes (Schagatay, 2001; Prommer, 2007; Schagatay, 2005). While these acute effects are fairly well described there are few longitudinal studies describing any chronic effects of repeated exposure to hypoxemia during breath-holds in BHDs. During dynamic diving (with locomotion) the hypoxemia was sufficient to stimulate erythropoietin (EPO) release by the kidneys, with blood EPO increasing by 60% up to at least 180 min post apnea (Elia, 2019). Two weeks of breath-hold training in non-BHD increased reticulocyte percentage (Ret%) by 15%, but did not change [Hb] (Engan, 2013). Similarly, six weeks of dynamic breath-hold training

resulted in a 28% increase in Ret%, which returned to baseline values one week after the training, while [Hb] remained unaltered (Elia, 2020). Whether the repeated stimulation of erythropoiesis in those studies was accompanied by an increase in total hemoglobin mass (Hbmass) is unknown since Hbmass nor plasma volume (PV) were quantified. With cross-sectional research, Prommer et al. (Prommer, 2007) found that trained BHDs do not have greater Hbmass compared to controls. By contrast, Zelenkova (Zelenkova, 2019) found that 7 elite BHDs had a somewhat higher Hbmass compared to controls, but lower Hbmass compared to endurance athletes.

The long-term effects of regular breath-holding on erythropoiesis thus appear to be limited, but remain to be better described (Elia, 2021). The Athlete Biological Passport (ABP) was developed for longitudinal monitoring of individual blood parameters in athletes for anti-doping purposes (Zorzoli, 2010; WADA, 2019). Using the ABP we monitored a cohort of BHDs' hematological variations over a year and compared the results to those of an active control group. We tested the hypothesis that regular breath-hold training leads to variations in hematological variables, and more so in BHDs than in controls. We also repeatedly measured total Hbmass and PV. We hypothesized that breath-hold divers would have higher Hbmass and PV and more pronounced seasonal variation compared to controls.

2. Materials and methods

2.2 Participants

Fifteen BHDs (11 men and 4 women) were invited to participate. Four were excluded: two due to medical reasons and two because not reaching the inclusion criterion of an average minimum of one breath-hold training per week. A final sample of 11 healthy non-smoking BHDs (8 men and 3 women; 36 ± 9 yrs, 177 ± 8 cm, 72 ± 9 kg) was studied. They had at least four years of breath-hold training experience and engaged in an average of 1.3 ± 0.5

breath-hold training sessions per week. All were Swiss apnea club members, regularly participated in national and international competitions, and planned to compete in the Swiss championships in May 2019. Eight finally participated in the championships in the various disciplines: static, dynamic with fins and dynamic with no fins. Static time was 273 ± 47 s (258 ± 55 s for the women (n=2) and 278 ± 50 s for the men (n=6)), dynamic distance with fins was 112 ± 30 m (137 ± 53 m for the women (n=2) and 103 ± 19 m for the men (n=6)) and without fins 89 ± 26 m (125 m for the woman (n=1) and 82 ± 22 m for the men (n=6)). Twelve sports science students at the University of Lausanne were invited to participate as a control group (7 men and 5 women; 22 ± 2 yrs, 174 ± 8 cm, 70 ± 13 kg). They participated in-a maximum of 8 h physical training per week. Five were running on a regular basis, four were playing football, two were playing volleyball in local club and one was doing triathlon. They had no prior experience in breath-hold training. The students were somewhat younger than the BHDs ($P < 0.001$), while height ($P = 0.55$) and body mass ($P = 0.82$) were similar.

All participants were healthy, medication free and lived < 800 m. They were informed about the procedure and risks of the study and provided fully informed written consent to participate. The study protocol was approved by the regional research ethics committee (CER-VD, Lausanne, Switzerland, #2018-01019) and conducted in respect of the Declaration of Helsinki.

2.3 Blood analyses

Between September 2018 and November 2019, the participants came monthly to the laboratory to provide a blood sample. World Anti-Doping Agency (WADA) blood collection guidelines were strictly followed (WADA, 2016; WADA, 2019) using the same protocol as described elsewhere (Astolfi, 2020). Briefly, blood samples were collected at

least 120 min after any structured physical activity. Blood was collected from an antecubital vein with a 21G short manifold butterfly needle (Sarstedt Safety-Multifly®, Sarstedt AG, Nuembrecht, Germany) in EDTA-coated tubes (Sarstedt S-Monovette 2.7 ml, Sarstedt AG, Nuembrecht, Germany), the equivalent of the BD Vacutainer®: K2-EDTA (K2) CE cat. no. 368856, as recommended in anti-doping guidelines. Blood samples were stored at 4° C and analyzed, after 15 min of homogenization on a roller system, with a fully automated flow cytometer (Sysmex XN1000, Sysmex Europe GmbH, Norderstedt, Germany) between 30 min-12 h after blood collection. Internal quality controls were run in duplicate before each batch of samples (Sysmex XN-Checks, levels 1, 2, and 3). Test results were validated after two successive measures within a very narrow range for [Hb] (i.e. ± 0.1 $g \cdot dl^{-1}$) and for Ret% (i.e. ± 0.15) (WADA, 2019 #2798). The following blood variables were obtained: hematocrit (HCT), hemoglobin concentration [Hb], red blood cell count (RCB#), reticulocyte percentage (Ret%), reticulocyte count (Ret#), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (standard deviation) (RDW-SD), and immature reticulocyte fraction (IRF%). In addition, Hbmass and volumes (i.e. plasma volume (PV), red blood cell volume (RBCV) and total blood volume (TBV)) were determined with a fully automated blood volume analyzer (OpCo: Detalo Instruments, Birkerod, Denmark) based on the carbon monoxide (CO) rebreathing technique, as described elsewhere (Siebenmann, 2017; Astolfi, 2020). Hbmass and blood volumes measurements could only be performed over the eight last visits to the laboratory due to technical reasons.

2.4 ABP Profiles

An individual longitudinal ABP profile for each participant was created as described elsewhere (Astolfi, 2020). Briefly, blood variables were entered into the ADAMS-training module in order to generate individual ABP profiles (WADA, 2019). The OFF-score (i.e. [Hb] x 10) - 60 x √Ret%) and the Abnormal Blood Profile Score (ABPS) (i.e. a combination of Ret%, [Hb], HCT, RBC, MCV, MCH and MCHC) were obtained via the ADAMS-training algorithm (WADA, 2019). After applying population-based upper and lower limits for the first sample, for the successive samples the reference ranges were adapted to the athlete's own values by use of a Bayesian statistical approach. An Atypical Passport Finding (ATPF) was generated when a) a [Hb] and/or OFF-score value of the last entered sample fell outside the lower and upper intra-individual limits or b) when the last 2 to 5 [Hb] and/or OFF-score values deviated from the expected range (a so-called "sequence ATPF"). For the first case, the applied specificity was 99% (i.e. 1:100 chance or less that the deviation was due to normal physiological variation). For the latter, the applied specificity was 99.9% (i.e. 1:1000 chance or less that the sequence deviation was due to normal physiological variation). In addition, for each participant we calculated the shortest distances to lower and upper personal limits, calculated by the Bayesian model, for each ABP biomarker of each sample (Astolfi, 2020).

2.5 Breath-hold training quantification

BHDs recorded their breath-old training via a training monitoring interface (Training Peaks™ (TP), PeaksWare, Lafayette, Colorado, USA). Breath-hold training type, i.e. static/dynamic, with/without water immersion was recorded. Breath-hold training sessions were categorized into six categories: 1) dynamic breath-holds (i.e. with or without fins in a swimming pool); 2) static apnea training with face immersion; 3) mix of dynamic breath-hold training and static with face immersion in the same session; 4) deep apnea training in

open water; 5) dry dynamic breath-hold training (i.e. on an ergometer, while running or cycling); and 6) dry static breath-holds. Breath-hold training was entered after the first visit so that training activities prior to the first blood sample could not be considered. The number of days/hours without apnea training before a blood sample was quantified.

2.6 Statistical analyses

Descriptive values are given as means and standard deviations. Anthropometric data (i.e. age, height, body mass) and the shortest distances to the individual upper and lower limits for each ABP biomarker (i.e. [Hb], Ret%, OFF-score and ABPS) for both groups were compared with the independent sample t-test, the Welsh test or the Mann Whitney non-parametric test, according to variance homogeneity and deviation from normality. Boxplots representing median, first and third quartiles with whiskers indicating variability outside the first and third quartiles are presented for the ABP biomarkers, Hbmass and PV for each group.

Hematological variables (i.e. HCT, [Hb], RCB#, Ret%, Ret#, MCV, MCH, MCHC, RDW-SD, IRF%), the OFF-score, and the ABPS of the 12 monthly blood samples were analyzed with a mixed model (with participant as random factor) to test the effect of group and time (fixed factors). Visual inspection of residual plots allowed excluding any obvious deviations from homoscedasticity or normality. Breath-hold training influence was investigated in two different ways: 1) using as covariate the number of days without breath-hold training before blood sample; and 2) using as factor a dichotomous variable on the average time between the training and the blood test for the subgroup that had one in the last 24 hours. We performed two series of mixed models (participant as random factor) to test the effect of time and a variable of breath-hold training (as covariate or fixed factors).

Hbmass and PV, monitored for the eight last months of the study, were analyzed with a mixed model (participant as random factor) to test the effect of group and time (fixed factors). Polynomial contrasts were used for time in mixed models, employing the Bonferroni method. The level of significance was set at $P < 0.05$. All statistical analyses were conducted with open source statistical software (Jamovi version 1.0.5.0, Jamovi Project Software, retrieved from https://www.jamovi.org.

3. Results

3.1 BHDs' training

On average 67.6 (± 28.6) breath-hold training sessions were realized over the study period by each BHD. They consisted of: 1) 52% dynamic breath-holds (i.e. with or without fins in a swimming pool); 2) 9% static apnea trainings with face immersion; 3) 8% mix of dynamic breath-hold training and static with face immersion in the same session; 4) 13% of deep apnea training in open water; 5) 9% dry dynamic breath-hold training (i.e. on an ergometer, while running or cycling); and 6) 9% dry static breath-holds.

3.2 Hematological values

All participants had 12 blood samples collected over the study with the exception of one diver who missed one (total of 275 blood samples). Time elapsed between two blood samples was 30.5 ± 3 days for the divers and 31.3 ± 6 days for the control group ($P = 0.77$). Distribution of the values for each ABP biomarkers (i.e. [Hb], OFF-score, Ret% and ABPS) and Hbmass and PV for each group are presented in Figure 1. Individual mean and standard deviation of hematological values for each participant are presented in Table 1.

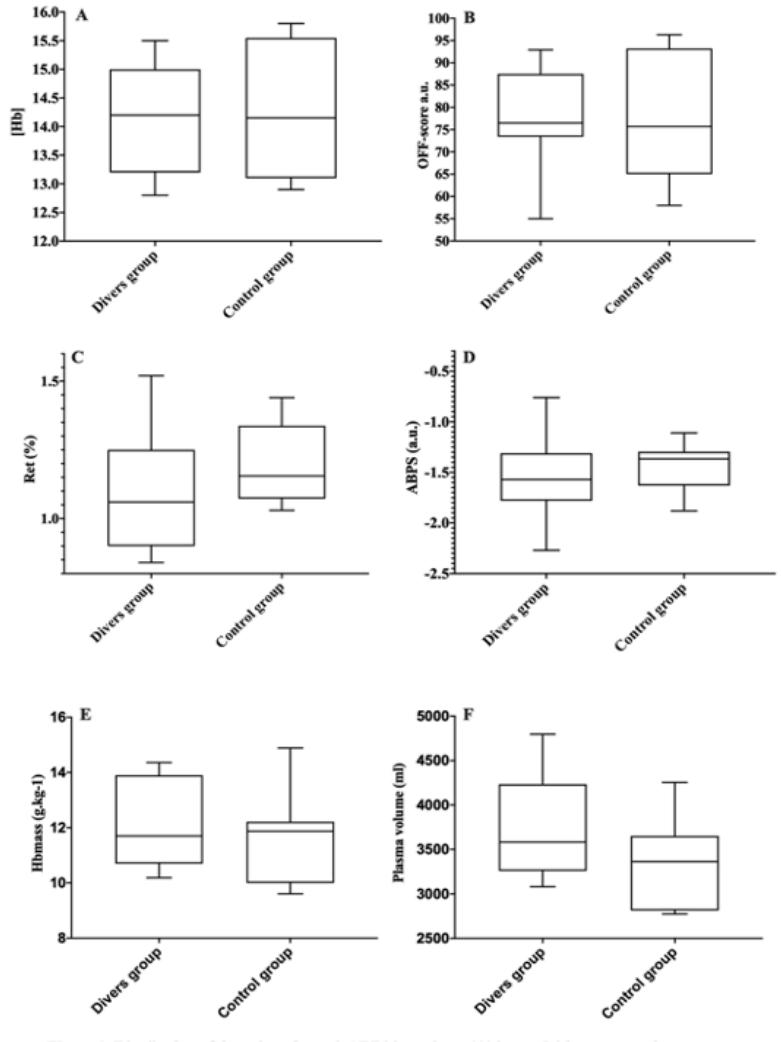

Figure 1. Distribution of the values for each ABP biomarker: (A) hemoglobin concentration ([Hb]), (B) OFF-score, (C) reticulocytes (Ret%) and (D) abnormal blood profile score (ABPS) and (E) hemoglobin mass (Hbmass) and (F) plasma volume (PV) for each group.

	W/M	[Hb] [g/dl]	HCT [%]	RBC [10^6/µl]	Ret [%]	Ret# [10^6/µl]	MCV [fL]	MCH [pg]	MCHC [g/dL]	RDW-SD [fL]	IRF [%]	OFF-score [a.u.]	ABPS [a.u.]	PV* [mL]	Hbmass* [g]	Hbmass* [g.kg^{-1}]
Divers 1	W	13.2 ± 0.3	38.8 ± 1.2	4.3 ± 0.1	0.96 ± 0.13	0.042 ± 0.005	89.5 ± 1.47	30.5 ± 0.3	34.1 ± 0.5	37.5 ± 0.96	2.5 ± 1.7	73.9 ± 6.1	-1.73 ± 0.14	3259 ± 197	710 ± 46	10.7 ± 0.7
Divers 2	M	15.5 ± 0.5	43.3 ± 1.8	4.9 ± 0.2	1.25 ± 0.31	0.061± 0.017	87.7 ± 0.94	31.7 ± 0.7	36.1 ± 0.9	38.8 ± 2.13	4.5 ± 1.6	88.7 ± 6.3	-0.76 ± 0.61	4154 ± 204	1110 ± 36	11.1 ± 0.3
Divers 3	W	13.0 ± 0.5	37.8 ± 1.5	4.4 ± 0.2	0.88 ± 0.16	0.038 ± 0.066	85.8 ± 0.93	29.5 ± 0.2	34.4 ± 0.4	39.0 ± 0.95	4.6 ± 1.4	74.5 ± 7.6	-1.91 ± 0.12	3256 ± 132	683 ± 44	10.5 ± 0.4
Divers 4	M	14.5 ± 0.6	42.0 ± 2.0	4.9 ± 0.2	0.91 ± 0.18	0.045 ± 0.007	85.3 ± 1.13	29.5 ± 0.22	34.5 ± 0.4	41.5 ± 1.00	6.8 ± 1.9	91.1 ± 7.1	-1.49 ± 0.51	3583 ± 342	880 ± 54	13.3 ± 0.9
Divers 5	M	15.5 ± 0.5	43.5 ± 1.6	5.2 ± 0.2	1.06 ± 0.15	0.054 ± 0.007	84.4 ± 0.80	30.0 ± 0.29	35.6 ± 0.4	36.4 ± 0.61	5.2 ± 2.1	95.4 ± 6.0	-1.12 ± 0.47	3361 ± 177	931 ± 40	14.4 ± 0.7
Divers 6	M	13.5 ± 0.5	39.4 ± 1.6	4.5 ± 0.2	1.07 ± 0.11	0.048 ± 0.006	88.1 ± 1.34	30.3 ± 0.26	34.3 ± 0.4	37.0 ± 0.89	6.9 ± 2.2	74.2 ± 5.7	-1.77 ± 0.14	4796 ± 409	1048 ± 43	14.1 ± 0.3
Divers 7	M	13.2 ± 0.2	39.2 ± 0.9	4.4 ± 0.1	0.84 ± 0.17	0.036 ± 0.007	89.6 ± 1.14	30.2 ± 0.28	33.7 ± 0.5	40.0 ± 1.16	3.0 ± 1.1	76.9 ± 6.5	-1.57 ± 0.19	4401 ± 217	981 ± 35	12.1 ± 0.3
Divers 8	M	14.2 ± 0.4	40.6 ± 1.1	4.6 ± 0.1	1.21 ± 0.16	0.055 ± 0.008	88.4 ± 1.03	31.0 ± 0.25	35.1 ± 0.35	37.8 ± 1.12	7.7 ± 1.4	78.1 ± 4.7	-1.57 ± 0.17	3849 ± 179	918 ± 53	11.3 ± 0.5
Divers 9	M	14.3 ± 0.4	42.1 ± 1.3	4.6 ± 0.1	0.90 ± 0.16	0.042 ± 0.007	91.8 ± 1.16	31.1 ± 0.25	33.9 ± 0.97	42.5 ± 0.72	5.3 ± 1.9	87.7 ± 3.8	-1.31 ± 0.23	3328 ± 282	800 ± 16	11.7 ± 0.2
Divers 10	M	15.0 ± 0.7	42.3 ± 1.9	5.3 ± 0.3	1.52 ± 0.15	0.080 ± 0.008	79.7 ± 0.6	28.3 ± 0.32	35.5 ± 0.42	34.4 ± 0.51	4.4 ± 1.5	78.5 ± 9.9	-2.27 ± 0.31	4234 ± 554	1089 ± 51	13.9 ± 0.6
Divers 11	W	12.8 ± 0.5	37.2 ± 1.6	4.3 ± 0.1	1.50 ± 0.21	0.064 ± 0.051	86.6 ± 1.2	29.8 ± 0.27	34.4 ± 0.49	38.7 ± 1.03	7.0 ± 2.2	56.7 ± 8.6	-1.78 ± 0.16	3081 ± 314	623 ± 28	10.2 ± 0.4
Control 1	W	13.0 ± 0.5	38.2 ± 1.8	4.3 ± 0.2	1.44 ± 0.28	0.062 ± 0.001	88.2 ±1.1	29.9 ± 0.27	33.9 ± 0.46	39.8 ± 0.95	4.0 ± 1.9	58.0 ± 9.6	-1.88 ± 0.46	3355 ± 212	719 ± 26	9.3 ± 0.3
Control 2	M	15.0 ± 0.5	44.7 ± 1.6	5.4 ± 0.2	1.30 ± 0.10	0.070 ± 0.006	82.3 ± 0.9	27.7 ± 0.35	33.6 ± 0.50	34.8 ± 0.77	7.1 ± 1.8	81.9 ± 5.9	-1.11 ± 0.67	3469 ± 308	948 ± 45	11.1 ± 0.5
Control 3	W	13.1 ± 0.6	38.6 ± 2.0	4.6 ± 1.9	1.16 ± 0.12	0.052 ± 0.005	84.5 ± 1.4	28.6 ± 0.20	33.8 ± 0.53	37.5 ± 1.44	3.9 ± 1.5	66.0 ± 7.7	-1.11 ± 0.78	2826 ± 188	603 ± 15	9.6 ± 0.3
Control 4	M	15.8 ± 0.3	45.5 ± 1.3	5.4 ± 0.1	1.08 ± 0.19	0.057 ± 0.001	84.9 ± 1.1	29.6 ± 0.2	34.8 ± 0.42	35.8 ± 0.88	3.9 ± 1.7	96.2 ± 7.6	-1.39 ± 0.62	4254 ± 569	1197 ± 51	11.1 ± 0.6
Control 5	W	13.1 ± 0.3	38.4 ± 0.8	4.3 ± 0.1	1.35 ± 0.13	0.057 ± 0.006	90.3 ± 0.7	30.7 ± 0.3	34.0 ± 0.29	41.8 ± 1.12	5.9 ± 2.1	60.9 ± 2.9	-1.31 ± 0.62	2792 ± 104	591 ± 22	9.8 ± 0.3

Control 6	M	15.4 ± 0.4	43.8 ± 1.5	5.2 ± 0.2	1.14 ± 0.17	0.059 ± 0.009	84.0 ± 0.86	29.6 ± 0.33	35.2 ± 0.4	37.5 ± 0.9	4.3 ± 1.5	89.9 ± 3.8	-1.34 ± 0.88	2774 ± 173	749 ± 28	12.3 ± 0.5
Control 7	M	13.6 ± 0.4	39.9 ± 1.3	4.5 ± 0.2	1.17 ± 0.18	0.052 ± 0.007	89.3 ± 1.59	30.4 ± 0.31	34.1 ± 0.4	38.3 ± 1.3	7.6 ± 1.6	71.5 ± 6.6	-1.52 ± 0.50	3995 ± 283	903 ± 18	11.5 ± 0.4
Control 8	W	14.2 ± 0.7	41.3 ± 2.2	4.6 ± 0.2	1.42 ± 0.27	0.065 ± 0.013	90.2 ± 1.07	31.1 ± 0.30	34.5 ± 0.5	39.5 ± 0.8	5.2 ± 1.9	71.2 ± 8.5	-1.29 ± 0.86	2810 ± 300	644 ± 32	10.8 ± 0.6
Control 9	W	12.9 ± 0.4	37.7 ± 1.5	4.5 ± 0.2	1.15 ± 0.26	0.051 ± 0.011	84.1 ± 1.11	28.8 ± 0.35	34.2 ± 0.4	35.2 ± 1.2	4.2 ± 2.1	64.7 ± 9.5	-1.31 ± 0.62	3252 ± 222	665 ± 13	9.6 ± 0.3
Control 10	M	15.8 ± 0.8	43.7 ± 2.5	5.1 ± 0.2	1.07 ± 0.16	0.054 ± 0.008	85.1 ± 1.44	30.8 ± 0.28	36.2 ± 0.6	35.2 ± 1.3	5.6 ± 1.6	96.3 ± 9.2	-1.57 ± 0.59	3374 ± 384	881 ± 25	11.6 ± 0.4
Control 11	M	15.6 ± 0.3	44.7 ± 0.9	5.0 ± 0.1	1.05 ± 0.16	0.052 ± 0.008	90.1 ± 1.19	31.4 ± 0.27	34.8 ± 0.6	40.6 ± 1.0	5.6 ± 1.3	94.3 ± 5.1	-1.65 ± 0.60	3543 ± 205	977 ± 42	13.9 ± 0.7
Control 12	M	14.1 ± 0.4	41.0 ± 1.2	4.6 ± 0.1	1.03 ± 0.14	0.047 ± 0.007	89.2 ± 1.0	30.6 ± 0.38	34.3 ± 0.5	41.3 ± 1.0	6.3 ± 1.8	79.9 ± 3.7	-1.67 ± 0.55	3689 ± 282	878 ± 42	11.8 ± 0.6

Over the 12 assessments, the average values for the hematological variables between groups are presented in Table 2. There was no significant influence of group for any hematological variable ($P > 0.05$). There were significant effects of time for all hematological variables ($P < 0.05$) with the exception of the ABPS ($P = 0.99$). Polynomial contrasts for time were mostly quadratic (Table 2). Interaction group x time was significant for Ret% ($P < 0.001$), Ret# ($P = 0.004$) and IRF% ($P = 0.013$). For those variables, a linear decrease with time appeared for controls, and stability for the BHDs.

	[Hb] [g/dl]	HCT [%]	RBC [10^6/µl]	Ret [%]	Ret# [10^6/µl]	MCV [fL]	MCH [pg]	MCHC [g/dL]	RDW-SD [fL]	IRF [%]	OFF-score [a.u].	ABPS [a.u|.	PV* [mL]	Hbmass* [g]	Hbmass* [g.kg^{-1}]
Divers group	14.1 ± 1.0	40.5 ± 2.5	4.7 ± 0.4	1.10 ± 0.29	0.052 ± 0.015	87.0 ± 3.2	30.2 ± 0.9	34.7 ± 0.9	38.5 ± 2.4	5.3 ± 2.3	78.2 ± 12.0	- 1.57 ± 0.5	3794 ± 616	902 ± 161	12.20 ± 1.6
Control group	14.3 ± 1.2	41.5 ± 3.2	4.8 ± 0.4	1.20 ± 0.23	0.056 ± 0.010	86.9 ± 3.0	29.9 ± 1.1	34.5 ± 0.8	38.1 ± 2.6	5.3 ± 2.1	77.6 ± 15.1	- 1.43 ± 0.7	3372 ± 543	824 ± 176	11.60 ± 1.5
Group effect	0.699	0.428	0.527	0.285	0.269	0.943	0.533	0.347	0.767	0.861	0.888	0.288	0.078	0.311	0.452
Time effect	<.001 Q	<.001 Q, C	<.001 Q	<.001	0.039	<.001 L, C	0.121	<.001 L, C	0.052	<.001 L, H	<.001 Q, C	0.992	<.001	0.001 Q	0.003
Time x Group	0.540	0.507	0.672	<.001 L	0.004 L	0.280	0.619	0.471	0.062	0.013 L, H	0.008	0.090	0.682	0.303	0.137

Table 2. Average divers group and control group hematological variables over 12 months. Values reported as means ± SD. [Hb]: hemoglobin concentration; HCT%: hematocrit; RBC: red blood cell count; Ret%: reticulocytes; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin content; RDW-SD: red cell distribution width (standard deviation); IRF: immature reticulocytes fraction; OFF-score; ABPS: abnormal blood profile score; PV: plasma volume; Hbmass: hemoglobin mass. a.u.: arbitrary units. *: were monitored during the last eight months of the study. P-value for Group, time and Time X Group effect are presented. Polynomial contrasts for Time

3.3 ABP profiles

A typical BHD ABP profile is presented in Figure 2. The hematological profiles of all BHD and control group participants are available as Supplementary Figures I to X for the divers and XI to XXII for the control group. In the full data set, covering a year, only one point ([Hb]) fell outside of the individual ABP limits thus generating an ATPF in the ADAMS-training hematological module. The acute variation in [Hb] for this subject occurred concomitantly with a change in PV between the fourth and the fifth measurement (see supplementary Figure 10 for details).

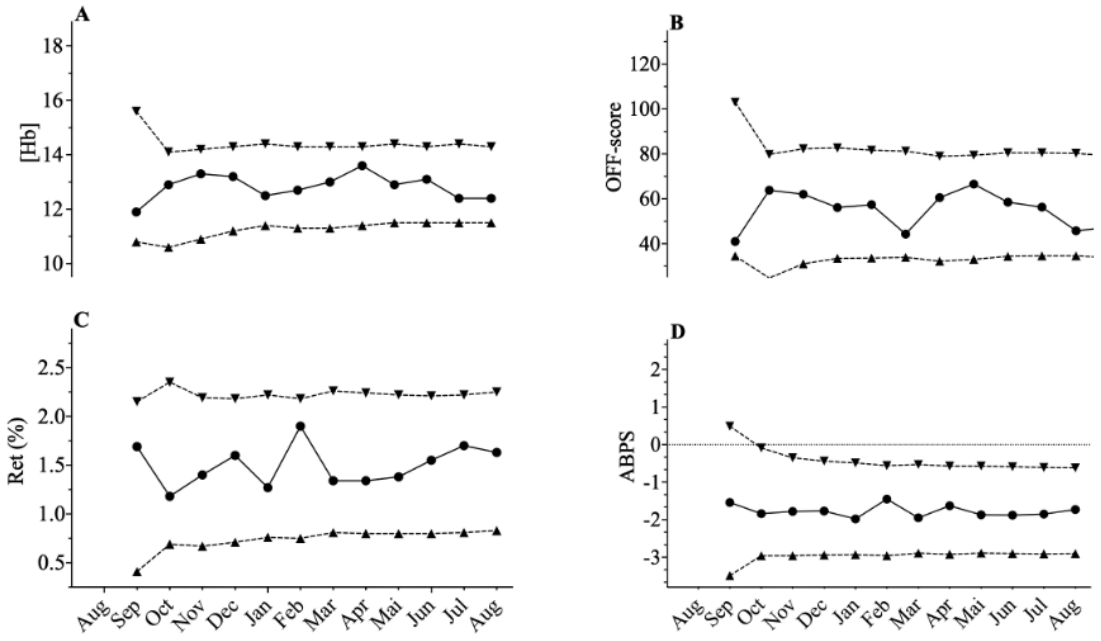

Figure 2. Representation of the Athlete Biological Passport (ABP) hematological profile for diver 11, with (A) [Hb]: hemoglobin concentration; (B) OFF-score, (C) Ret%: reticulocytes percentage; (D) ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).

The average shortest distances to the individual limits of the ABPS variables were not significantly different between groups ([Hb]: 1.2 ± 0.3 vs. 1.2 ± 0.4 $g \cdot dl^{-1}$ ($P = 0.87$), OFF-score: 18.5 ± 4.3 vs. 18.8 ± 5.8 ($P = 0.86$), Ret%: 0.48 ± 0.16 vs. 0.49 ± 0.16 ($P = 0.89$) and ABPS: 0.94 ± 0.21 vs. 0.93 ± 0.31 ($P = 0.93$) for BHD and controls respectively).

3.4 Influence of breath-hold training

The time elapsed between a breath-hold training session and blood sampling averaged 3.5 ± 2 days (range: 0-6 days). This elapsed period of time was not related to variations of the hematological variables (i.e. [Hb], HCT, Ret%, OFF-score and ABPS) ($P > 0.05$) nor to Hbmass or PV ($P > 0.05$). There was a relationship between an increased number of days without apnea training and an increase in the IRF% ($F(1,107) = 5.6$, $P = 0.02$, $b = 0.16$). When considering only the training session that was the closest to blood sampling in all BHDs (average time between training and blood sample: 19.4 ± 2.3 h) there was no influence observed of the latter training session on the hematological variables, Hbmass or PV ($P > 0.05$). For further details, all variables and statistics of the relationships with breath-hold training are presented in supplementary file (Supplementary Table 1).

4. Discussion

This is the first study collecting and analyzing monthly blood samples over one year in recreational BHDs for comparison with samples of an active control group. We tested the hypothesis that breath-hold training induces changes in hematological variables in BHDs over time. The main findings of this study were that: a) over a one-year period, hematological values varied significantly over time for both BHDs and an active control group; b) the time effects were present for all blood variables, with polynomial contrasts

(quadratic effects) on most variables, suggesting seasonal variations; c) Ret% and IRF were stable in BHDs over the study time despite regular apnea training; and d) BHDs do not have different hematological characteristics in comparison to an active control group.

Variations in hematological variables of elite athletes over a competitive season have been reported before, with notable temporary decreases in [Hb], HCT and Ret% after heavy training periods (Banfi, 2011). These hematological variations seem more pronounced for more endurance-type sports like cycling, running and swimming as compared to intermittent-type sports such as football and rugby (Banfi, 2011), even though Diaz et al., (2011) observed a certain stability in Ret% and [Hb] in elite triathletes followed over four consecutive seasons

In addition to the significant effects of time found in our study, for all hematological variables – with the exception of the ABPS – we found that the polynomial contrasts for time were mostly quadratic. These findings are suggestive of a seasonal effect that was not different between BHDs and controls. The ABP analytical module thus seems sufficiently robust to not flag such variations as potentially the result of blood doping practices.

There was variability in the distribution of ABP biomarkers values in BHDs, which were also present in the control group (Figure 1). Regarding the primary (i.e. [Hb], OFF-score) and secondary (i.e. Ret% and ABPS) biomarkers of the ABP, the distances between measured variables to the individual limits of the ABP were quite short and did not differ significantly between groups. We observed one ATPF on a primary biomarker of the ABP (i.e. [Hb]) in a BHD Supplementary Figure 12). This participant had been on vacation in a hot country during which he had refrained from any endurance exercise or breath-holding

training to then return to cold January weather prior to coming to the laboratory for blood sampling. We tentatively explain the drop in PV for this athlete in January compared to his prior and subsequent values to be the result of a combination of detraining and acute cold exposure (Sawka, 2000). Both environmental heat exposure and increased endurance training load are known to increase PV and thereby affect concentration-dependent hematological variables such as [Hb] (Astolfi, 2020). This underlines the relevance of documenting an athlete's location and training, or better, measuring PV, for a more nuanced appreciation of an athlete's hematological values, something particularly relevant in an anti-doping context.

In the case of BHDs one should also make sure that the acute effects of spleen contraction have subsided, since this is known to cause acute temporary changes in [Hb], HCT and Ret%. A study of Richardson et al., (2005) showed that 3 maximal repeated apneas, without immersion and separated by 2 min of recovery, acutely increased [Hb] in divers, skiers and untrained humans (Richardson, 2005). Such effects have been reported when apnea was practiced with (Bakovic, 2003; Bakovic, 2005) and without face immersion (Schagatay, 2001; Schagatay, 2005). The observed increases in HCT and [Hb] are thought to be transient, lasting between 2 and 10 min (Espersen, 2002; Richardson, 2005). However, the exact minimum time needed to allow a complete return to baseline levels is not known. Our study was not designed to determine this, but in our BHDs a minimum of 19 h separating the blood sampling from a prior breath-hold training appeared to exclude any significant variation in the monitored hematological variables. This suggests that for anti-doping purposes leaving a pause of at least 19 hours after a breath-hold training would be sufficient to prevent confounding from the acute effects of splenic contraction. We found that IRF% increased as a function of the number of days without breath-hold training, illustrating a

tenuous link between a variation in IRF% and breath-hold training sessions. Others showed that IRF% was unchanged 30 min after a breath-hold training session to then decrease by 20% from baseline value after 4 h (Dolscheid-Pommerich, 2020). Taken together, this may suggest that breath-hold training sessions activate the release of hemoglobin by splenic contraction (thus altering acutely [Hb], HCT and IRF%) (Schagatay, 2001) instead of generating new precursors of RBC to cope with transient hypoxemia/hypoxia.

Our study design has some limitations. First, we could not record PV or Hbmass during the first four months of the study, due to technical issues. Then, we did not directly monitor iron stores in our participants and therefore cannot exclude that some might have been iron deficient, which could have negatively influenced new RBC production (Brugnara, 2000). However, the hematological variables allowed us to exclude any clear iron-deficiency anemia. While active blood doping by the participants cannot be excluded, its likelihood would seem extremely low given the limited rational to blood dope in our participants and the results of the ABP monitoring (Dimant, 2015). The small number of women in our study and the influence of the menstrual cycle on blood composition (Mullen, 2020) do not allow us to draw any conclusions of sex-specific effects. Finally, the non-elite level of our BHDs precludes generalization to the elite.

From a performance and in an anti-doping perspective, the acute effects of breath-holding on [Hb] and Ret% could be used by athletes to 1) enhance their performance as breath-holding leads to a „natural“ auto-transfusion, as 50% of the 200-250 mL of packed RBC that are stored in the spleen can be released into the circulation upon breath-holding (Bakovic, 2005), increasing blood O_2 transport capacity (Dolscheid-Pommerich, 2020); or 2) use breath-holding right before an anti-doping control to increase their [Hb] and Ret%,

so as to mask any actual blood doping at other times of anti-doping controls where the athlete would refrain from breath-holds prior to sampling. Whether the latter approach is feasible and how the resulting variations of the monitored blood parameters by ABP would play out remains to be investigated directly.

5. Conclusions

In conclusion, we report that regular breath-hold training does not induce significant long term hematological variations in comparison to a being physically active, while seasonal variations occur in blood parameters in both divers and controls. The ABP can be used in breath hold divers by leaving at least 19 h between the last apnea training and blood sampling.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

Raphael Faiss and Martial Saugy have given substantial contributions to the conception or the design of the manuscript, Tiffany Astolfi and Raphael Faiss to data acquisition. All authors participated in the analysis and interpretation of the data. All authors have participated to drafting the manuscript, Tiffany Astolfi revised it critically. All authors read and approved the final version of the manuscript.

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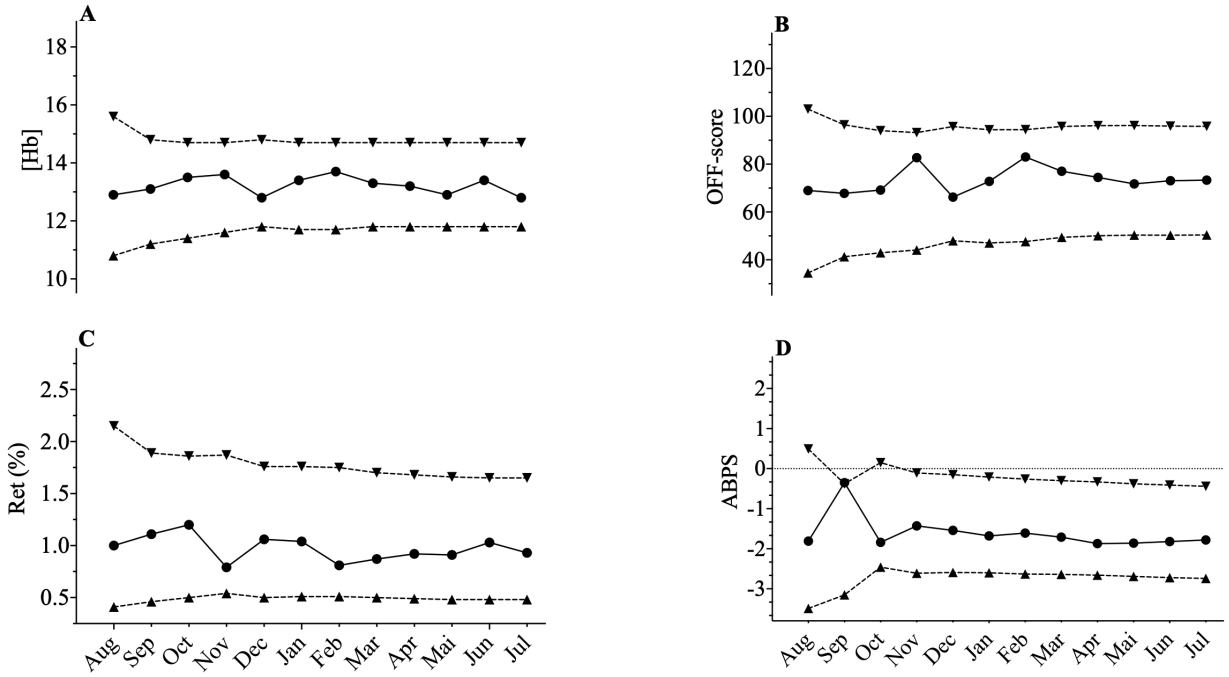
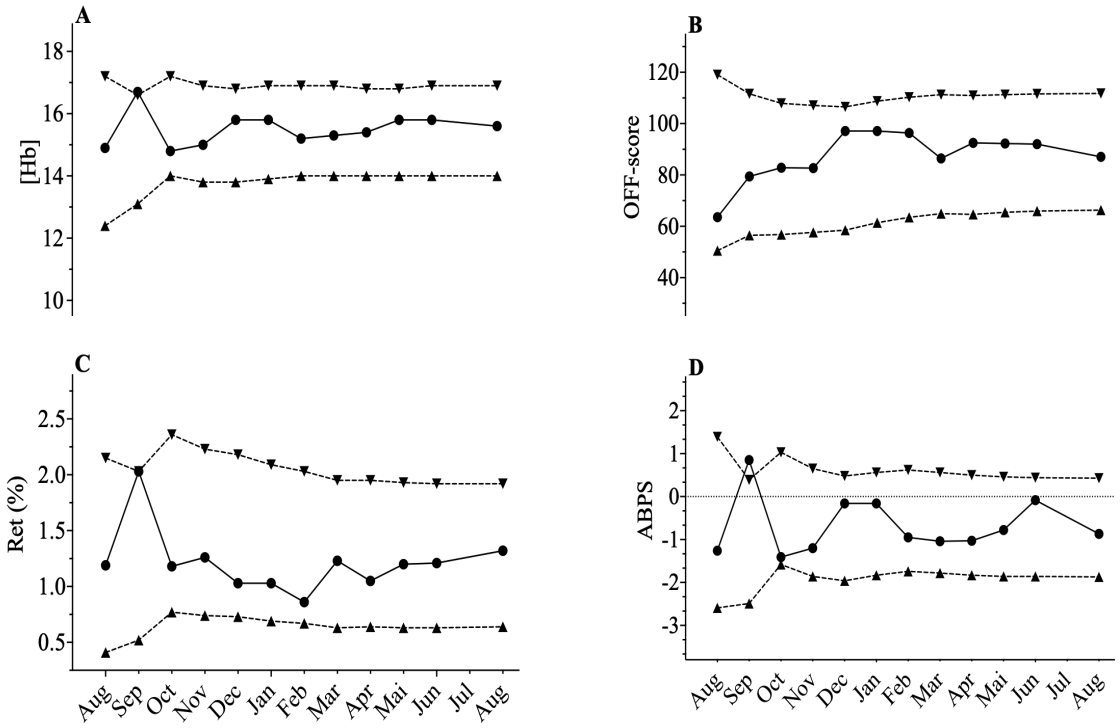
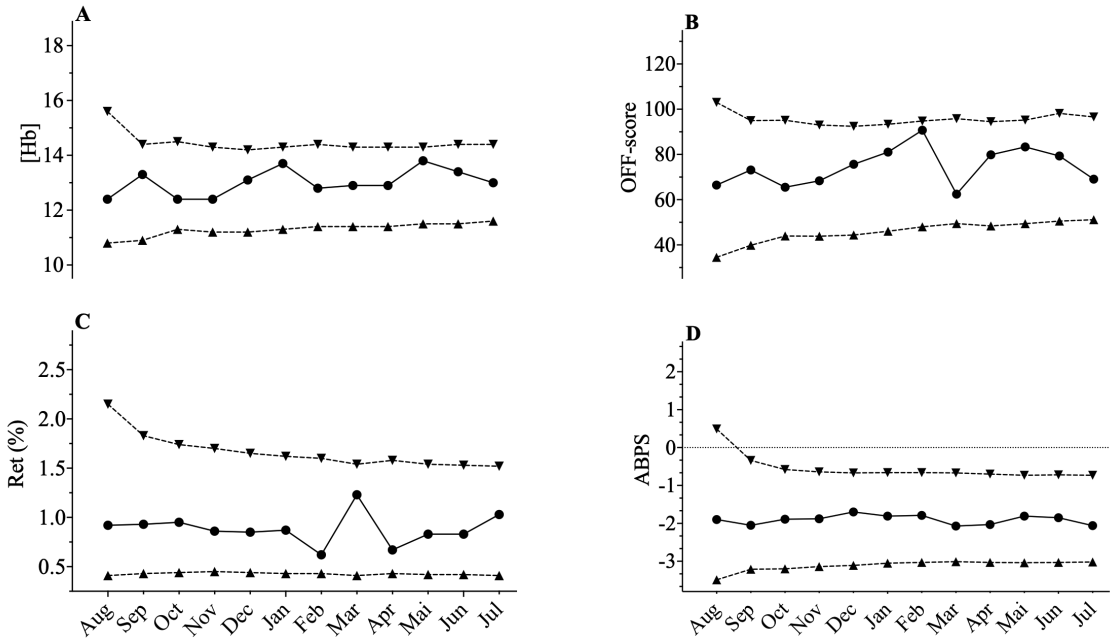
Supplementary figures I – XXII



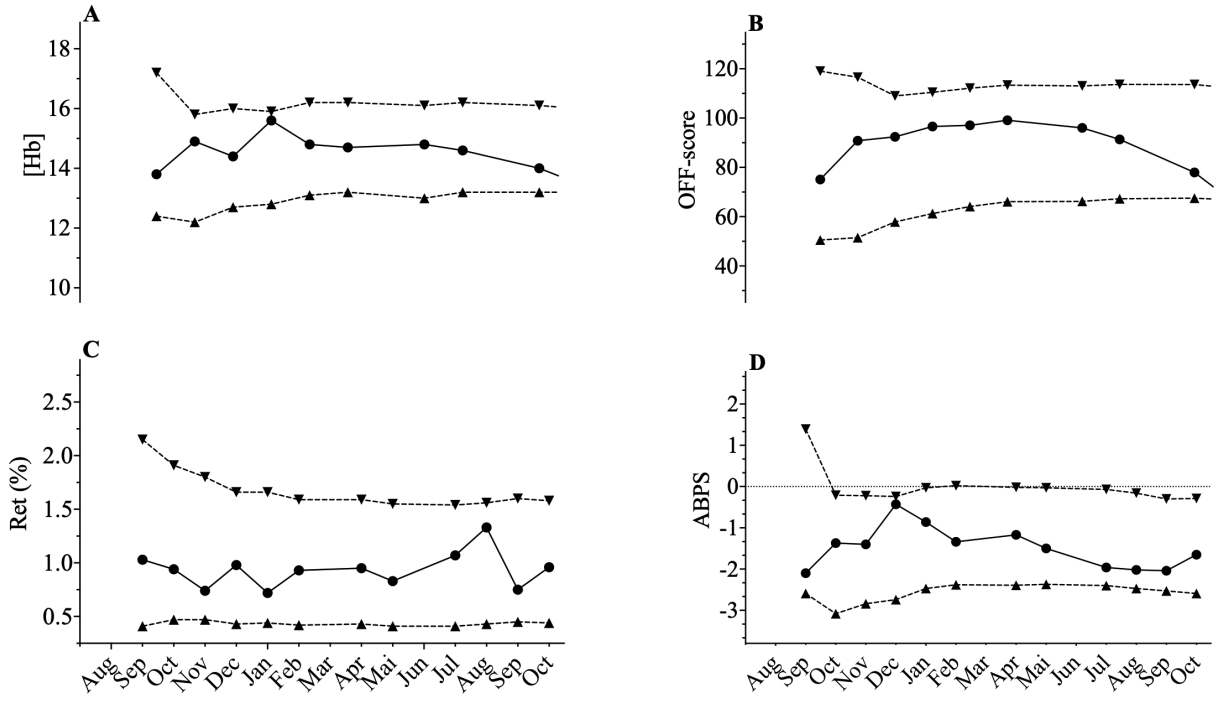
Supplementary figure I. Representation of the Athlete Biological Passport (ABP) hematological profile for diver 1, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



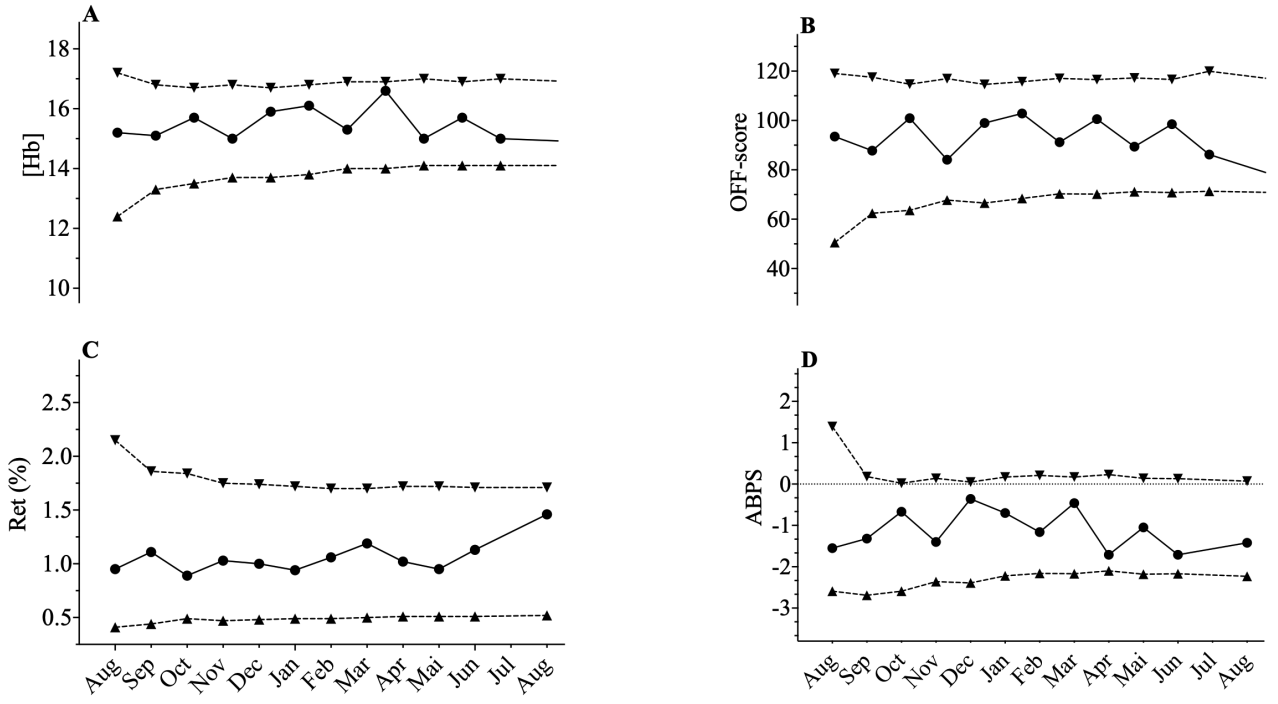
Supplementary figure II. Representation of the Athlete Biological Passport (ABP) hematological profile for diver 2, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



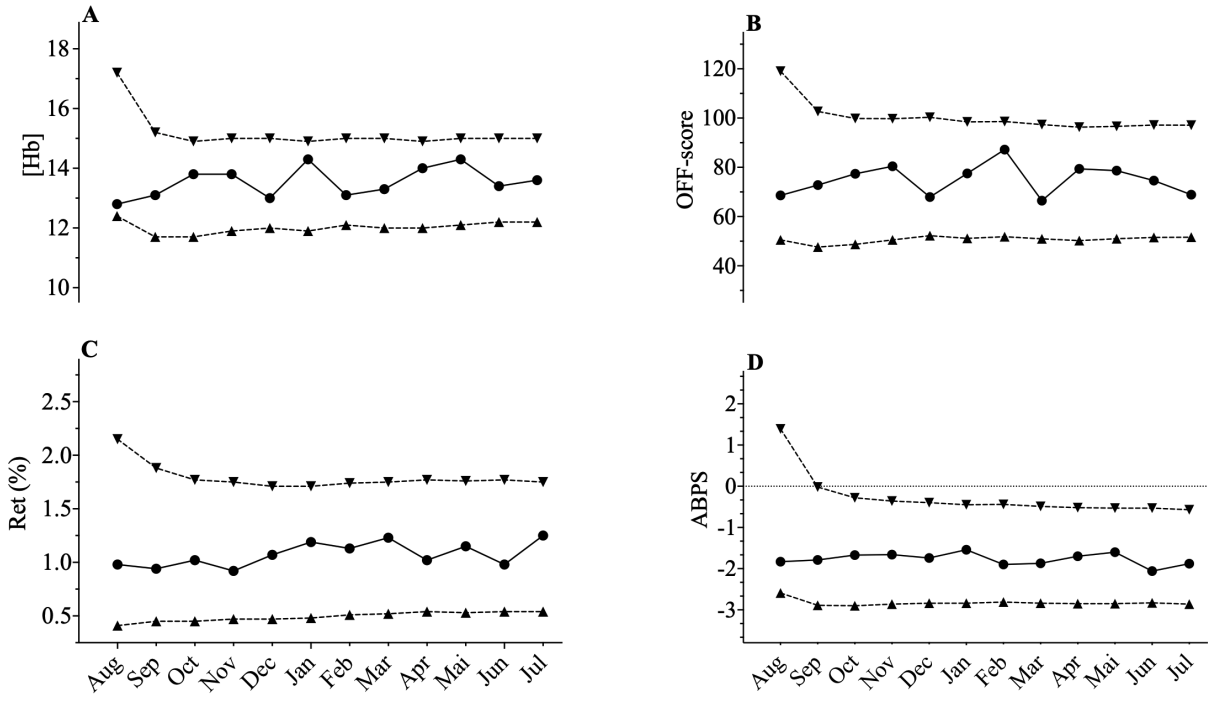
Supplementary figure III. Representation of the Athlete Biological Passport (ABP) hematological profile for diver 3, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



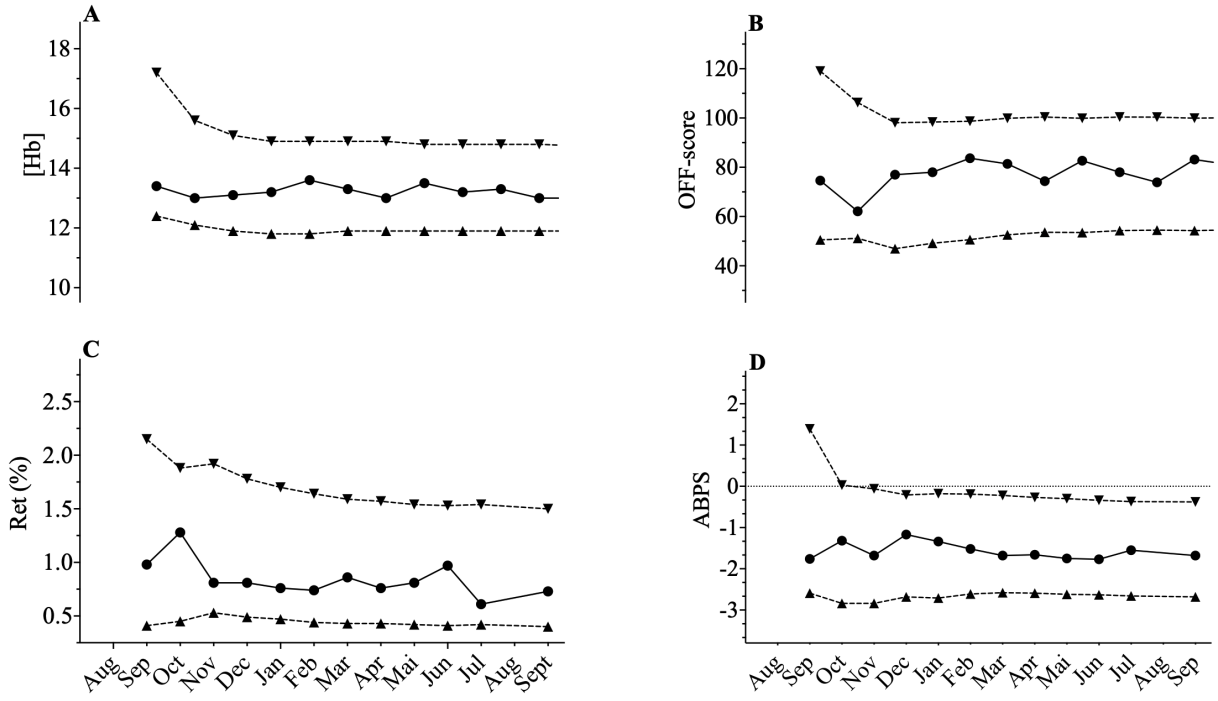
Supplementary figure IV. Representation of the Athlete Biological Passport (ABP) hematological profile for diver 4, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



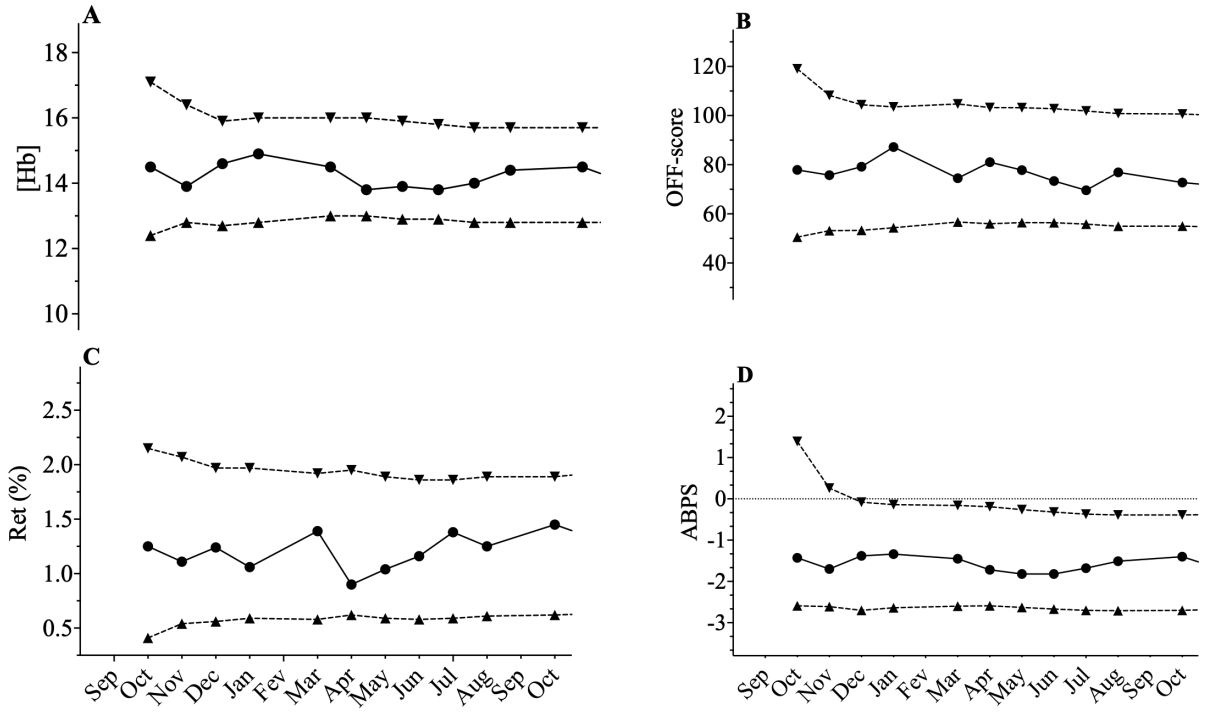
Supplementary figure V. Representation of the Athlete Biological Passport (ABP) hematological profile for diver 5, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



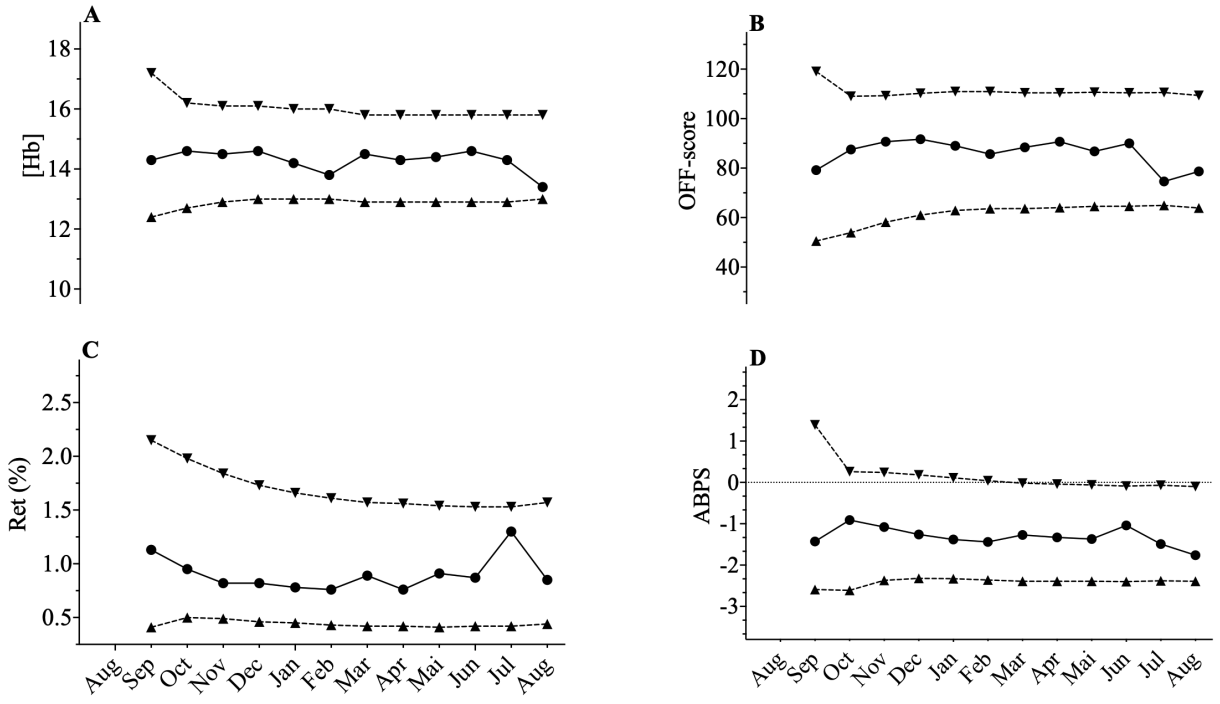
Supplementary figure VI. Representation of the Athlete Biological Passport (ABP) hematological profile for diver 6, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



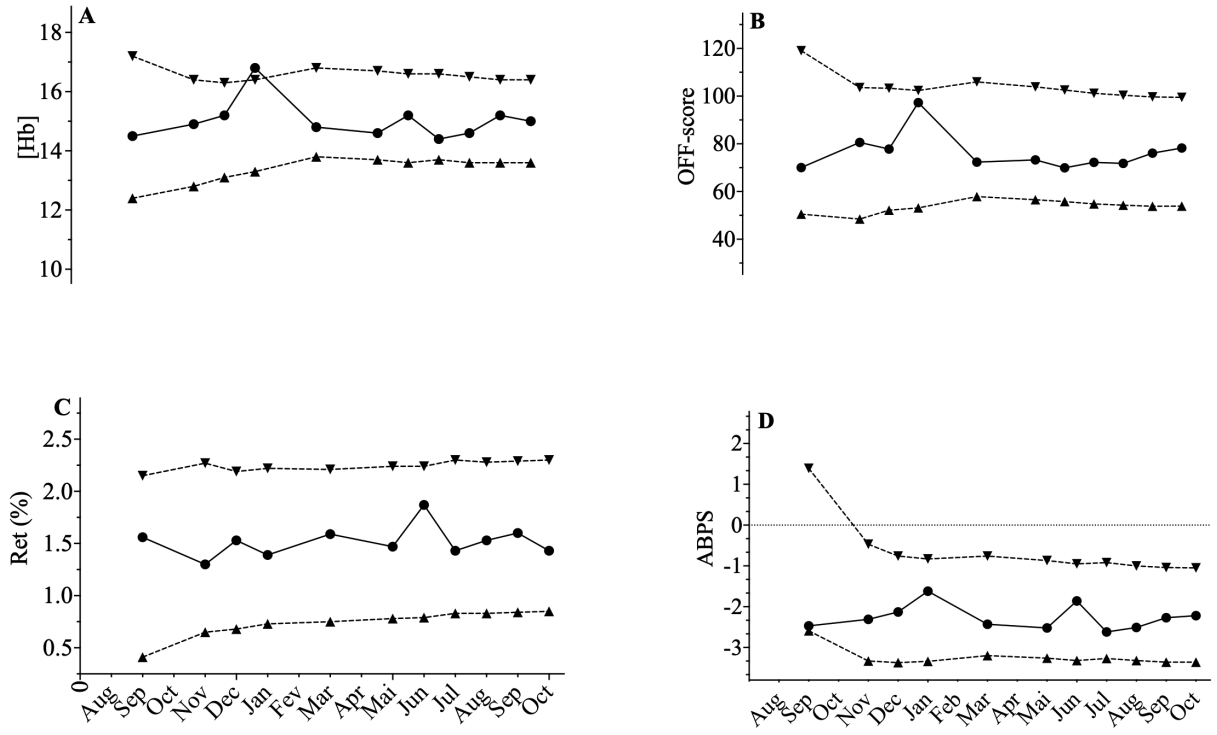
Supplementary figure VII. Representation of the Athlete Biological Passport (ABP) hematological profile for diver 7, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



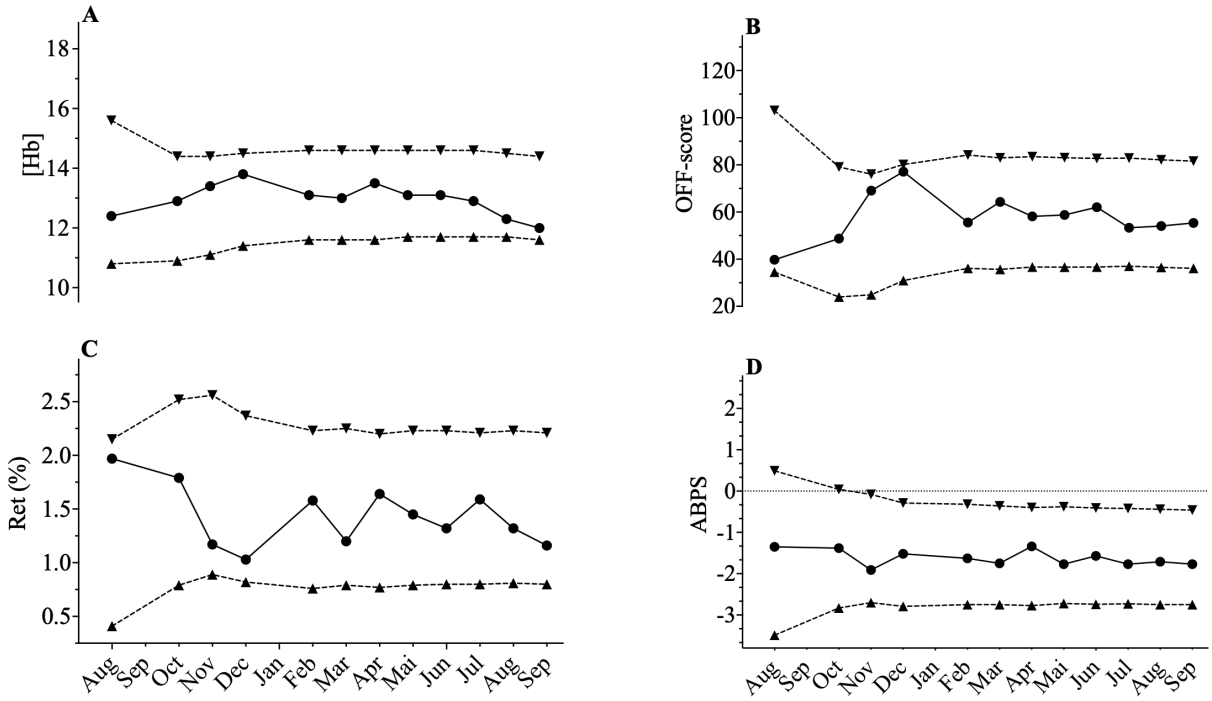
Supplementary figure VIII. Representation of the Athlete Biological Passport (ABP) hematological profile for diver 8, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



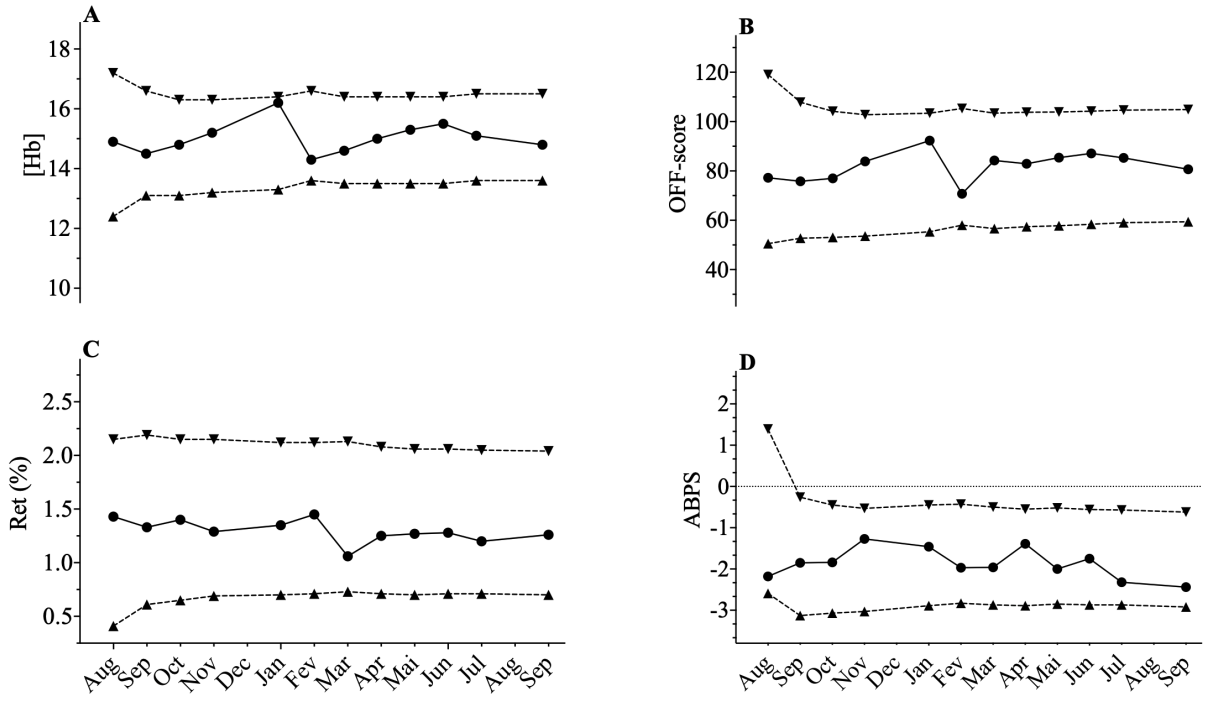
Supplementary figure IX. Representation of the Athlete Biological Passport (ABP) hematological profile for diver 9, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



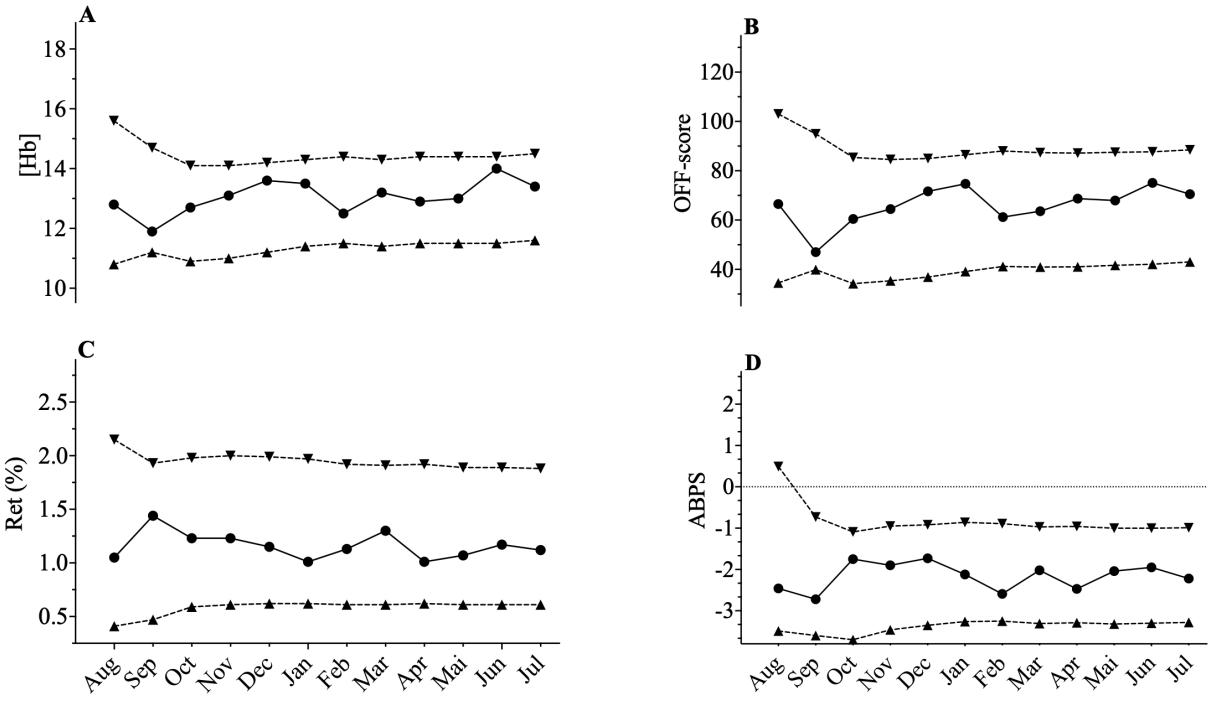
Supplementary figure X. Representation of the Athlete Biological Passport (ABP) hematological profile for diver 10, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



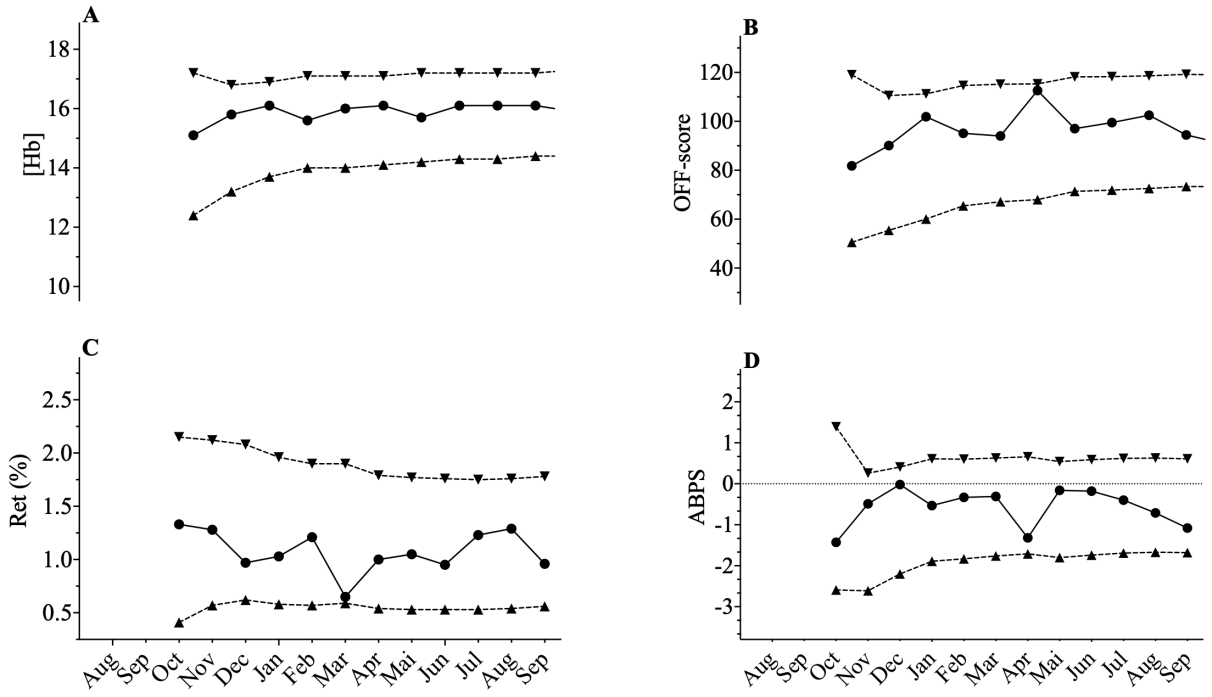
Supplementary figure XI. Representation of the Athlete Biological Passport (ABP) hematological profile for participant (control) 1, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the participant's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



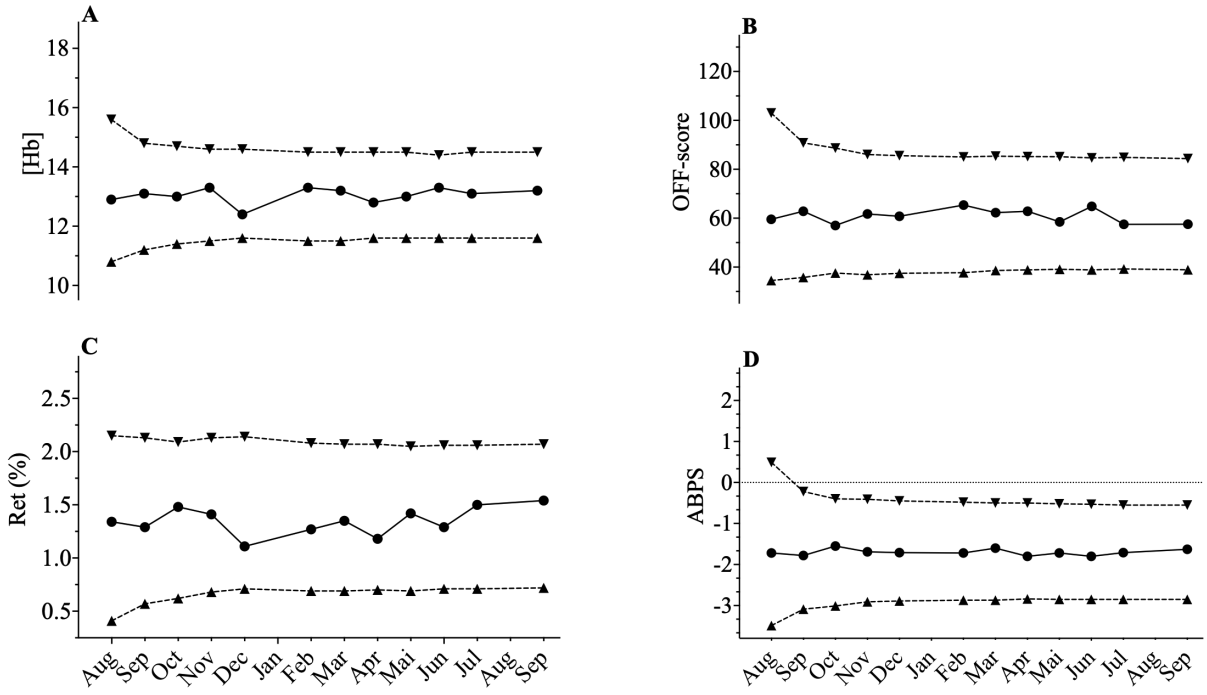
Supplementary figure XII. Representation of the Athlete Biological Passport (ABP) hematological profile for participant (control) 2, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the athlete's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



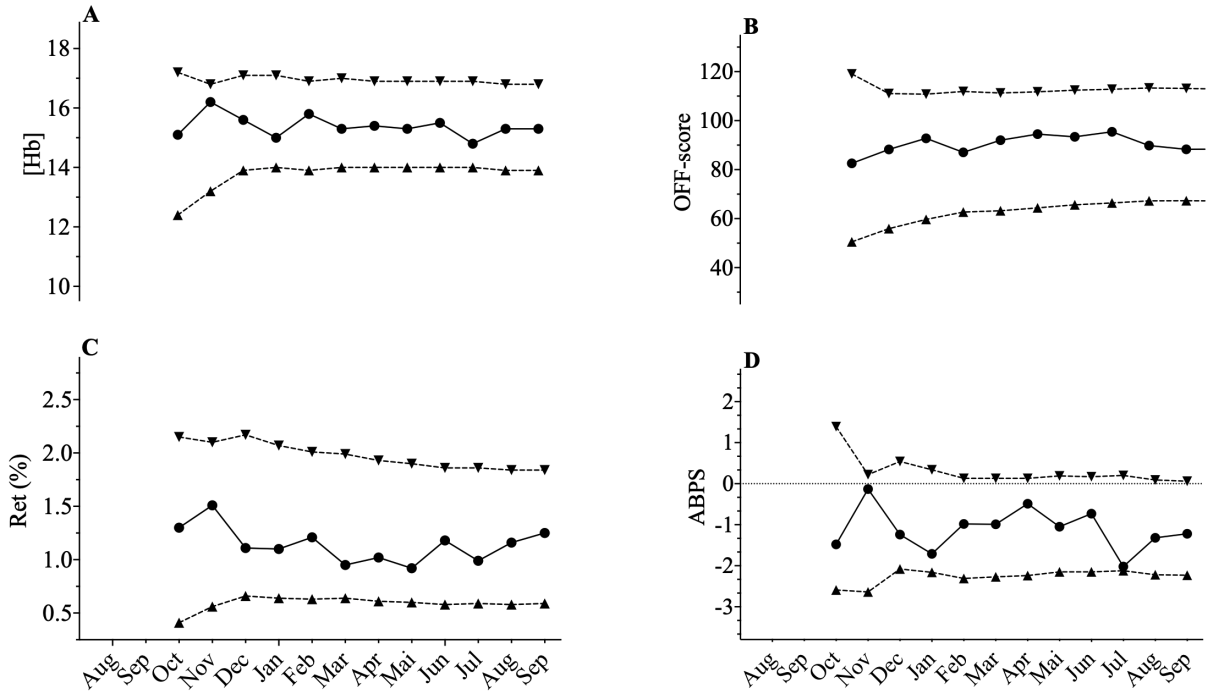
Supplementary file XIII. Representation of the Athlete Biological Passport (ABP) hematological profile for participant (control) 3, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the participant's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



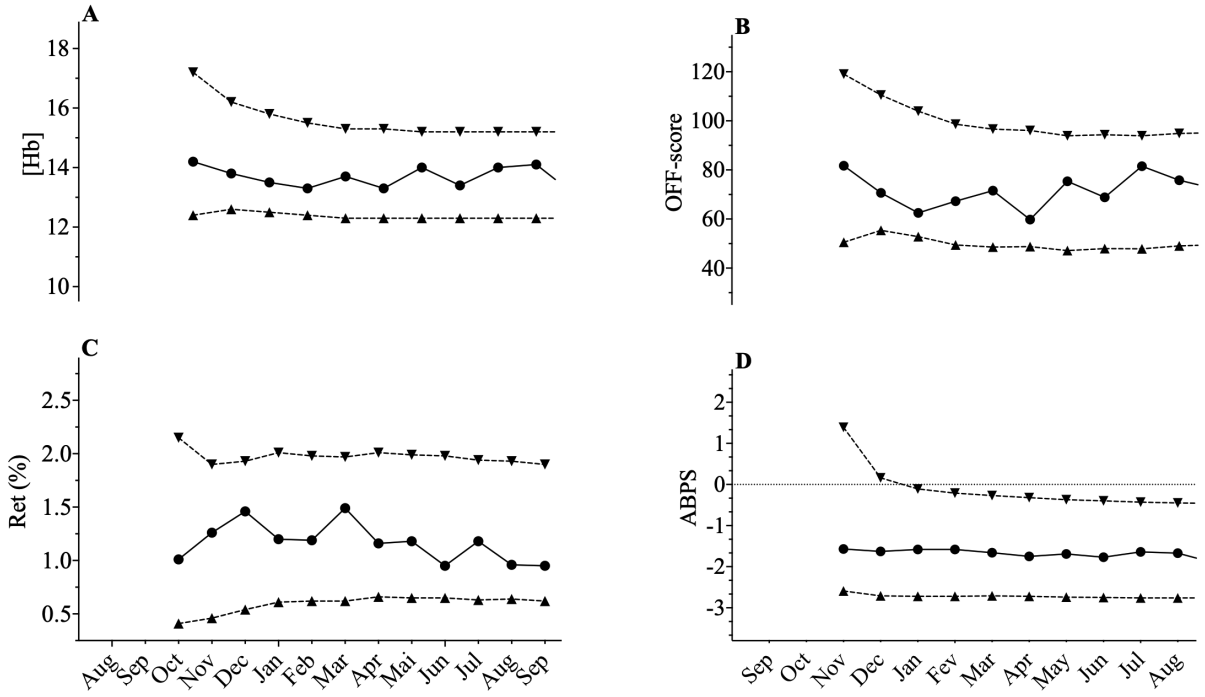
Supplementary figure XIV. Representation of the Athlete Biological Passport (ABP) hematological profile for participant (control) 4, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the participant's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



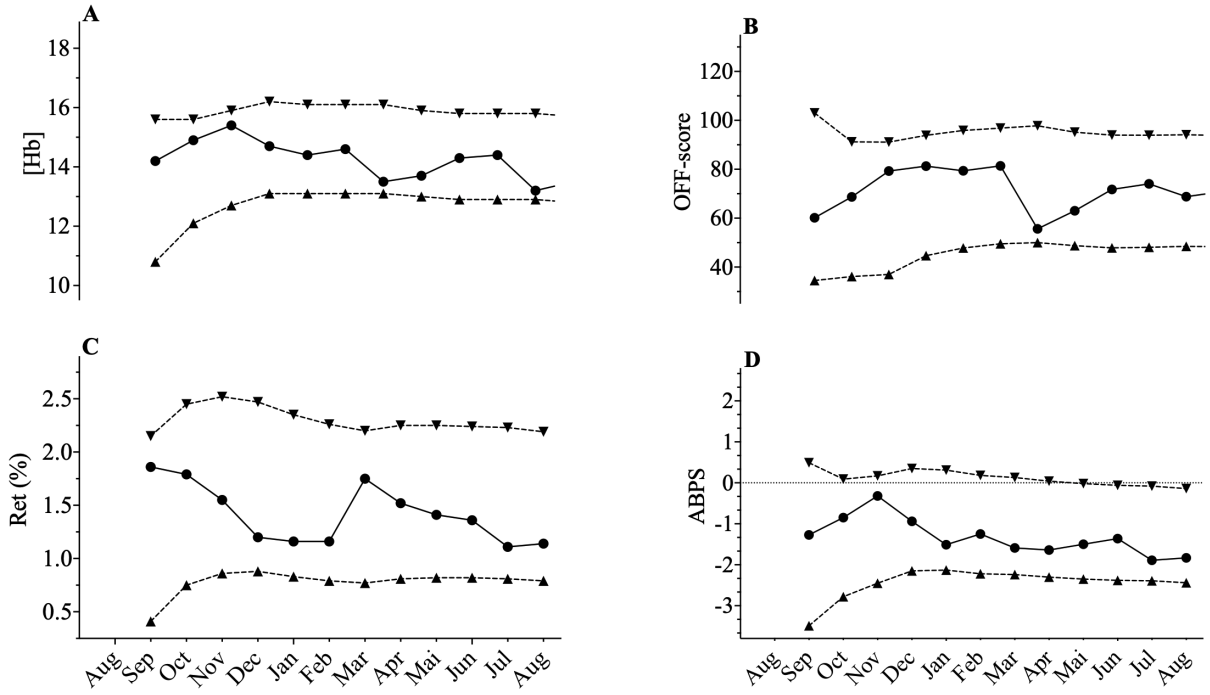
Supplementary figure XV. Representation of the Athlete Biological Passport (ABP) hematological profile for participant (control) 5, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the participant's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



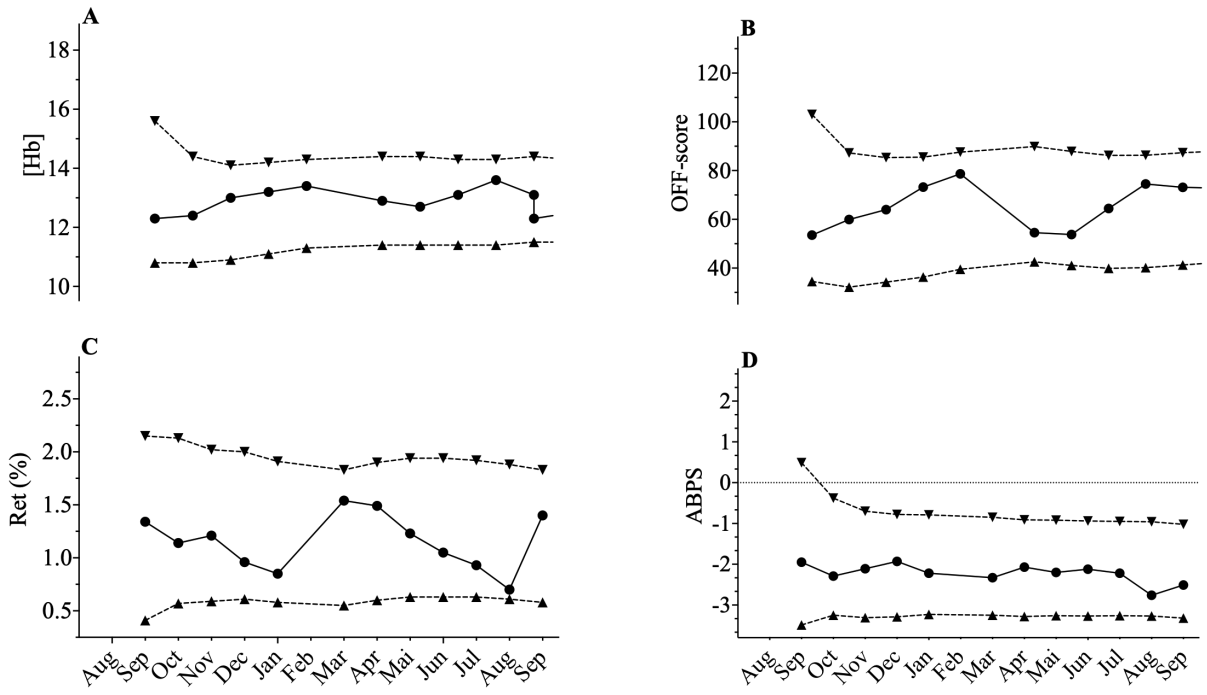
Supplementary figure XVI. Representation of the Athlete Biological Passport (ABP) hematological profile for participant (control) 6, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the participant's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



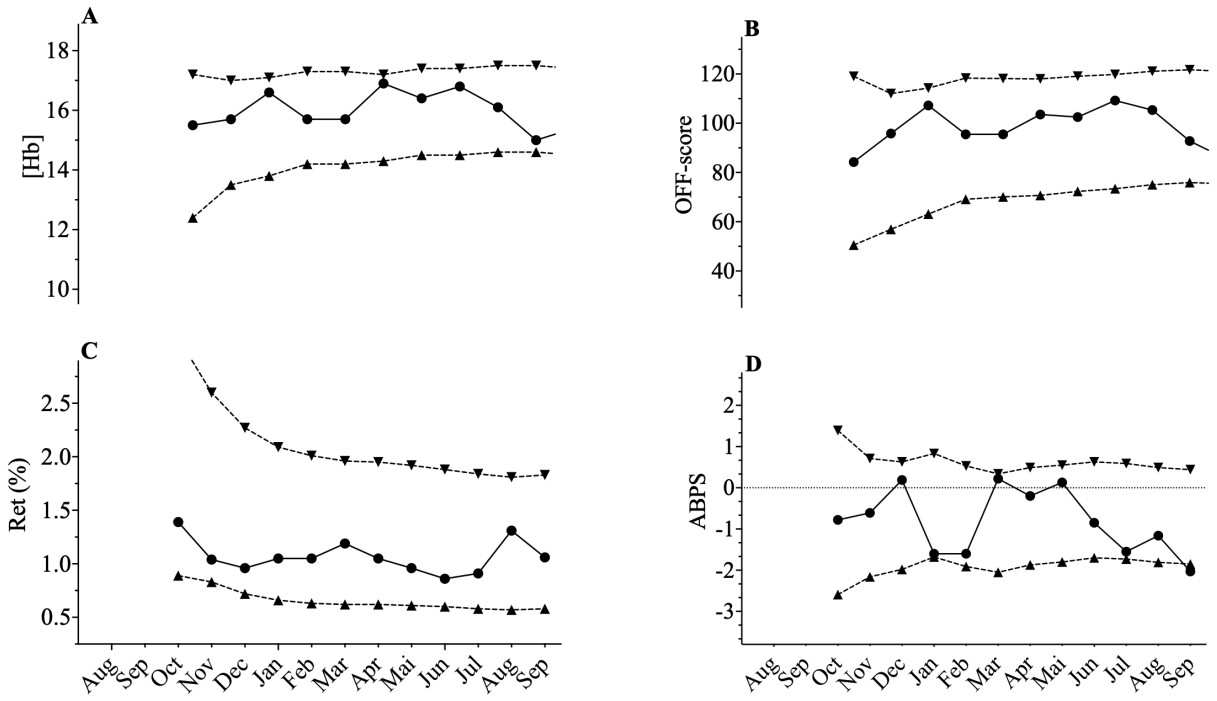
Supplementary figure XVII. Representation of the Athlete Biological Passport (ABP) hematological profile for participant (control) 7, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the participant's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



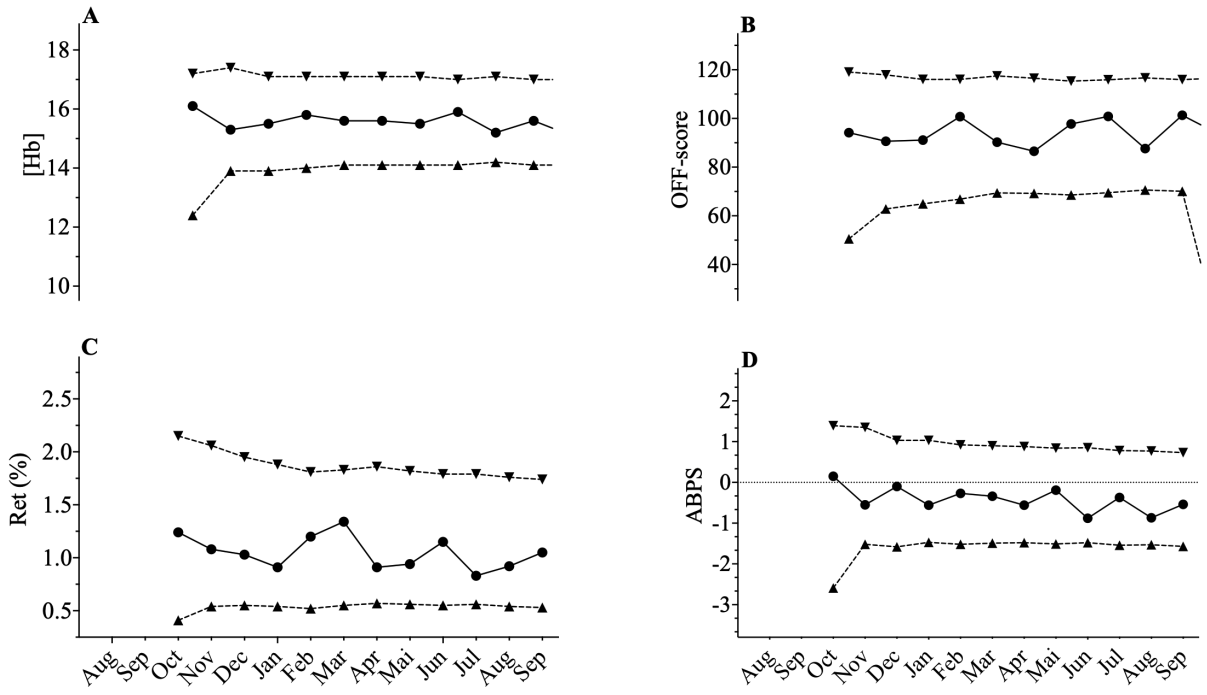
Supplementary figure XVIII. Representation of the Athlete Biological Passport (ABP) hematological profile for participant (control) 8, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the participant's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



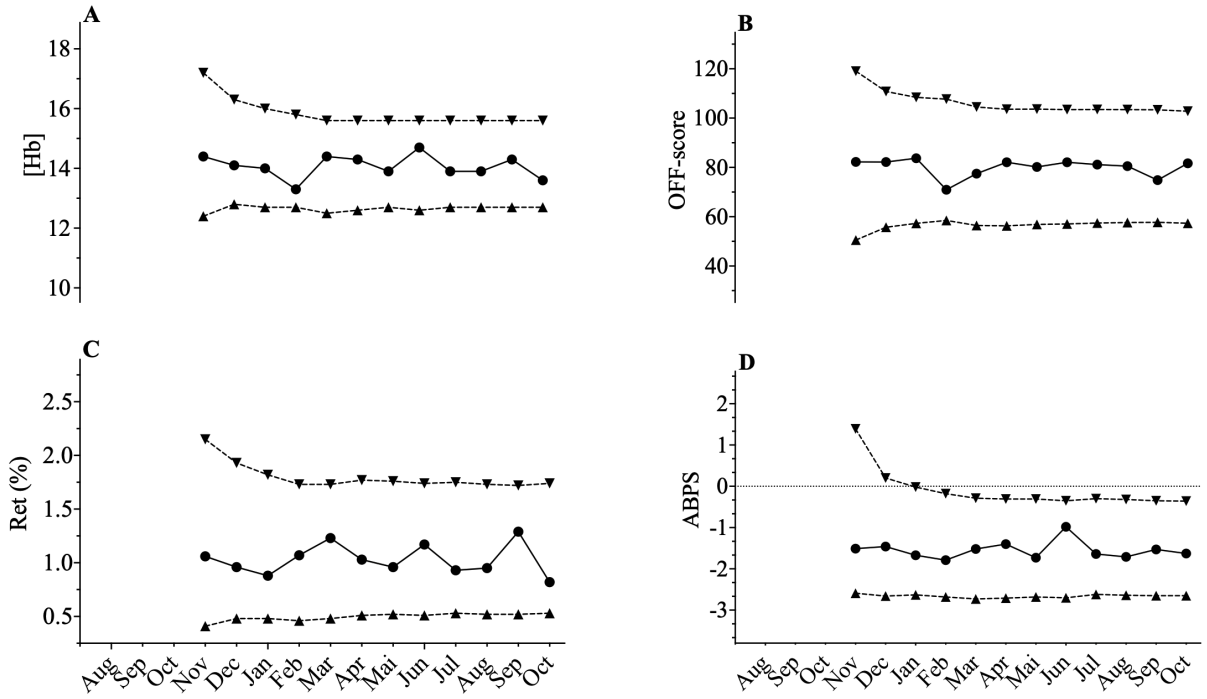
Supplementary figure XIX. Representation of the Athlete Biological Passport (ABP) hematological profile for participant (control) 9, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the participant's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



Supplementary figure XX. Representation of the Athlete Biological Passport (ABP) hematological profile for participant (control) 10, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the participant's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



Supplementary figure XXI. Representation of the Athlete Biological Passport (ABP) hematological profile for participant (control) 11, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the participant's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).



Supplementary figure XXII. Representation of the Athlete Biological Passport (ABP) hematological profile for participant (control) 12, with **(A)** [Hb]: hemoglobin concentration; **(B)** OFF-score, **(C)** Ret%: reticulocytes percentage; **(D)** ABPS: abnormal blood profile score; over the 12 months of the study design. Solid line represents the participant's values, dotted line represents the upper limits and lower limits calculated by an adaptive Bayesian model (see methods section for details).

Supplementary table

	[Hb] [g/dl]		HCT [%]		Ret [%]		IRF [%]		OFF-score [a.u.]		ABPS [a.u.]		PV* [mL]		Hbmass* [g.kg-]	
Effect	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Breath-hold training within the last 24 h before blood sampling	0.01	0.92$	0.01	0.91$	0.02	0.90$	1.24	0.27$	0.004	0.95$	0.09	0.76$	0.74	0.39$	1.16	0.29$
Number of days without breath-h training before blood sampling	0.28	0.59$	0.30	0.58$	1.24	0.27$	5.60	0.02	0.25	0.62$	0.05	0.82$	0.14	0.71$	1.74	0.19$

Supplementary Table 1- Linear mixed model results for the influence of the various fixed effects of breath-hold training (Breath-hold training within the 24hrs before blood sampling; Number of days without breath-hold training before blood sampling) on [Hb]: hemoglobin; HCT%: hematocrit; Ret%: reticulocytes; IRF%: immature reticulocytes fraction; OFF-score a.u.; ABPS a.u.: abnormal blood profile score; PV mL: plasma volume; Hbmass g.kg-^{1}: hemoglobin mass. a.u. arbitrary units. *measurements were only realized over the last eight months of the study.
(F statistics, *P*-value). $ represents time effect $P<0.05$).

CHAPTER 6

DISCUSSION AND PERSPECTIVES

6. Discussion and perspectives

6.1 General discussion

Performance in endurance sports relies heavily on the capacity of the blood to transport O_2 to the working muscles. Athletes try to exploit this to gain an unethical advantage through blood doping, which increases O_2 availability by artificially increasing the number of RBCs. To attempt to counter this, the ABP tracks and monitors blood biomarkers involved in the erythropoiesis cascade. However, the interpretation of solely hematological biomarkers is challenging because some are measured in concentration, which fluctuates with variations in PV. Thus, a multi-dimensional approach, including understanding the influence of potential confounding factors, is paramount for interpreting the ABP.

Overall, this thesis presents new data to assist in the assessment of the influence of three confounding factors related to the ABP: a) body position before and during blood sampling, b) training load in elite cyclists and c) breath-hold training. Our studies mainly show differences in measured values that could be traced directly to changes in PV (i.e. hemoconcentration or hemodilution) that significantly impact some of the ABP biomarkers (i.e. [Hb], HCT). Regardless, our results showed that the ABP was not influenced by these variations, highlighting the robustness of the passport.

Our work focused first on the influence of body position during blood draw on the biomarkers measured for the ABP, hypothesizing that body position (i.e. seated vs. supine) as well as a short position change (i.e. walking 50 m) could alter [Hb] and HCT due to the shift in PV. If these values shift from the “acceptable” range, it will indicate that body position could affect the accuracy of the ABP reading. Firstly, our work confirmed that a short walk (50 meters) is

sufficient to disrupt the ABP blood results. Additionally, the important new finding that we highlighted was that when a short walk occurs with a change in body position from the seated to the standing position (e.g. changing chairs), 5 min is enough to rebalance the volume compartments. In other words, after a period of 10 min in the seated position with feet on the floor as requested using WADA guidelines (WADA, 2019), if the athlete must stand up and walk for less than 50 m, only 5 min in the seated position is required before blood sampling. The protocol chosen was an extreme situation (i.e. walking 50 m) that should never be encountered if the control is performed under anti-doping conditions. Nevertheless, this shows the influence on blood biomarker results.

In addition, we showed that the results from blood drawn while in the supine position are sufficiently changed compared to the WADA standard. This enables us to say that a change in hydrostatic pressure due to body position altered the ABP.

Secondly, we questioned whether training load would affect the ABP reading in elite cyclists. We hypothesized that an acute (representing 5 days) and a chronic (representing 42 days) training load before a blood sampling would influence the ABP variables, exceeding the individual thresholds calculated using the adaptive model. We concluded through our work that only the changes in acute training load (5 days) prior to blood sampling significantly influenced [Hb] and ABPS, but that the chronic training load (42 days) did not affect these values. Here, the important new finding is that Hb_{mass}, contrary to PV, was unaltered over one year of chronic training load in elite cyclists.

The third major aim of our work was to investigate whether regular breath-hold training leads to a variation in hematological variables, again testing the robustness of the ABP against a new potential confounding factor. We hypothesized that breath-hold training would induce more

hematological variations compared to a control group and that we would see a seasonal effect on the markers. Contrary to our hypotheses, we highlighted no significant differences between breath-hold divers and a control group. In fact, most markers remained stable throughout the year also for the breath-hold divers, with only IRF and RET decreasing linearly for the controls. Nevertheless, seasonal effects were suggested by quadratic variations that occurred over the year for each biomarker except the ABPS. With this study, we have been able to examine whether an ABP should be required for BHDs in the near future. We showed that there were seasonal variations, but that these occurred for the controls as well as the divers. Thus, the need for a ABP may not be a priority, as breath-hold training has not been revealed to be a confounding factor in our longitudinal studies.

Although there were not many statistical differences between the confounding factors tested here and the control groups, excepting the acute training load in cyclists, our work still emphasizes the relevance of studying longitudinal hematological data at two different levels: intra- (i.e. within-) and inter-individual level (see Section 6.1.1 for more). Taken together, the results of the studies presented in this thesis provide some clues on the intra-individual variations in hematological biomarkers, indicate the need to include more women in research studies related to the ABP, and highlight the role of Hb_{mass} and PV measurements in an anti-doping context. In addition, we propose the monitoring of training load as a way to better understand these confounding factors for blood biomarkers and performance analyses.

6.1.1 Intra-individual variations

The variations in blood testing data for a single athlete signifies the intra-individual variability, and the variability among a group of different individuals signifies the inter-individual variability (Banfi, 2008). The inter-individual variability refers to what is commonly called

"population references ranges", which are quite insensitive for monitoring individual variations; there is a higher inter-individual variation in biological markers than intra-individual (Sottas et al., 2009). Thanks to the statistical approach of the ABP, which uses a Bayesian-based algorithm, the athlete becomes his or her own reference (Sottas et al., 2009), and this reference is much more accurate than using collective values. The ability to highlight and correctly interpret intra-individual variability afforded by the ABP is of prime relevance in anti-doping. In the past, this would have helped overcome one of the main issues encountered by the previously proposed "no-start" rule (see introduction for further details). Unfortunately, despite advances in the scientific field (Mullen et al., 2020; Moreillon et al., 2022), the universal intra-individual variance from the ABP algorithm is based on males. The need for more research on women, including the influence of the same confounding factors already studied in men, is necessary to propose a universal intra-individual variance tailored for female physiology.

6.1.2 Consideration of Women

Though sex is a major determinant in performance (Tucker & Collins, 2012), women are not prioritized in physiological studies, let alone those examining variations in blood. Considered as the major obstacle, the menstrual cycle causes blood loss, which induces hematological variations (Fraser, 2001; Hallberg et al., 1966; Wyatt et al., 2001). Sometimes, heavy bleeding like menorrhagia, defined as blood loss > 80 mL (Hallberg et al., 1966), are present among women, nevertheless as all the blood loss induce by the menstrual cycle, the exact amount is hard to verify in a scientific context (Wyatt et al., 2001). Although potential sex-induced variations are already included in the adaptive model of ABP as a heterogeneous factor, data regarding the potential influence of menstrual cycles are relevant for ABP readings. Along this line, Mullen et al. (2020) highlighted an increase in Ret# and Ret% during the ovulatory and luteal phases in 17 contraception-free women. No other biomarker variations were noted

(Mullen et al., 2020). Two ATPFs were highlighted in two participants (n = 17). Unfortunately, no data were recorded for PV and Hb_{mass}, which would have improved the understanding of those results. Recently, Moreillon et al. (2022) proposed monitoring menstruation with regard to the ABP in women using oral contraceptive pills (OCP). Interestingly, they highlighted variations in hematological biomarkers related to a PV shift. However, they accounted for the increase in Ret% by the fact that progesterone alters red cell membranes and a reticulocytoses in women using OCP (Devenuto et al., 1969). In their study, Hb_{mass} and PV remained steady throughout the phases (i.e. luteal, ovulatory and follicular). Despite obvious changes in PV, the authors conclude that these were not dependent on menstrual phases. Interestingly, we showed that there was no significant effect of sex on hematological variables (i.e. [Hb], HCT) when studying the effect of body position during phlebotomy, despite changes in PV. However, studies on the effect of blood doping practices in women are still needed. For instance, Solheim et al. (2019) highlighted that sex-specific studies are needed regarding the effect of autologous transfusions. Additionally, there is only one relatively old study that enrolled only women (Robertson et al., 1984).

6.1.3 The role of Hbmass and PV measurement

Hb_{mass} measurement

An individual's baseline Hb_{mass} depends on multiple predispositions, such as genetic factors (Saugy et al., 2014), individual level of health, physical conditions and ferritin level (Garvican-Lewis et al., 2018). As Hb_{mass} is a key parameter for any kind of blood manipulation, an increase Hb_{mass} increase is very attractive for athletes. Thus, the inclusion of Hb_{mass} in the adaptive model may further improve its efficiency (Pottgiesser et al., 2012).

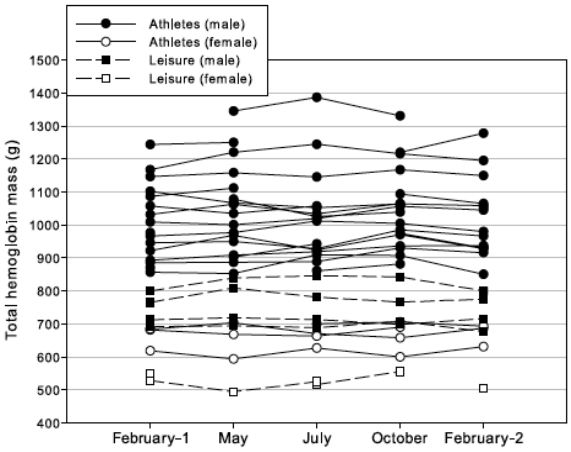


Figure 6. *Individual behavior of Hb_{mass} over one year across various population. Taken from Prommer et al., 2008.*

Hb_{mass} has been found to be stable in an athlete over a period of one year (Figure 6) (Prommer et al., 2008). More importantly, the authors of this study found a typical error of measurement (TEM), indicating both the typical percentage of error (TE) and the real biological changes, of 2.2% for Hb_{mass} which is very small. Considering that the TE in their method was 1.4%, there was very little individual variability in these measurements (Prommer et al., 2008). The TEM for [Hb] was a bit higher, at 3.5%. Eastwood and al. (2008) found the same TEM following a study of athletes over 100 days, with 40 measurements taken per athlete. Put into context, Pottgiesser et al. (2007b) explained that a TE of 1.4% indicates a 95% probability that a change in 3.9% in Hb_{mass}, representing 35 g for an athlete with an Hb_{mass} of 900 g, would fall outside of the noise of the method. Compared to blood transfusions, one unit of blood alters Hb_{mass} by approximately 60 g (Pottgiesser et al., 2007b). Additionally, Parisotto et al., showed that Hb_{mass} can increase between 7 and 12%, (i.e. 12% representing around 107g for an athlete with 890 g Hb_{mass}) with rHuEPO administration (Parisotto et al., 2000), which matched another study (Lundby et al., 2007), nevertheless this amount of rHuEPO is no longer taken by athletes, who now use a more sophisticated regimen. Nevertheless, when microdosing rHuEPO, research shows that the Hb_{mass} can increase by up to 10% (Ashenden et al., 2011).

In our studies, we showed that Hb_{mass} was not affected by training load nor seasonal variations (winter vs. summer) in elite cyclists over the study time (one year). Indeed, Hb_{mass} remained significantly stable over time, averaging 1030 ± 87 g (range: 842 to 1116 g) despite significant variations in PV averaging 4309 ± 350 mL (range 3688 to 4751 mL). Our study group did consist of elite cyclists with higher baseline Hb_{mass} values than the general population. Hauser et al. (2000) showed that even athletes with a high Hb_{mass} should expect a reasonable change in Hb_{mass} following a live-high/train-low camp. They found a moderate inverse correlation between initial Hb_{mass} and Hb_{mass} improvement, though they attributed this to the weight loss following altitude camp.

With that in mind, a large increase in Hb_{mass} would thus be suspicious. Nevertheless, doping regimes have become so sophisticated that large fluctuations can be avoided, which is why a multidimensional approach to fight doping is necessary. Towards this end, we designed studies using training data to help improve the interpretations of hematological variations. The generally stable Hb_{mass} highlighted in our three studies emphasized the consideration, partly debated, of whether or not Hb_{mass} measurements should be implemented in anti-doping efforts (Sanchis-Gomar & Lippi, 2012).

Plasma volume measurement

It is worth noting that the generally stable Hb_{mass} was not always associated with a stable PV. Lobigs et al. (2018) elaborated a PV correction, highlighting that 66% of [Hb] variations are explained by this mean. For instance, in study 1 (Chapter 3), we highlighted a short-term confounding factor involving body position during phlebotomy. Indeed, PV measurement here helps to highlight the hemodilution that occurred when athletes were in the supine position. In study 2 (Chapter 4), we observed a 4.7% increase in PV in the summer compared to winter that

was not associated with a difference in training load. Although the difference was not significant, it helps us to better interpret the significant negative correlation between PV and [Hb] variations. Additionally, the reading of the hemodilution observed with the altered training load 5 days before the blood sample could be improved using the additional PV data, as seen for elite cyclists in Figure 7 or in BHD in Figure 8.

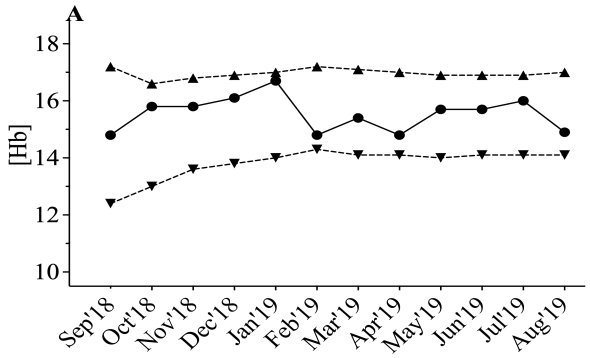


Figure 7. *Representation of hemoglobin concentration ([Hb]) for an elite cyclist as per 12 successive blood samplings over one year. The decrease in [Hb] observed for the 6th blood sample is concomitant with a PV of 3545 mL, whereas the 9th blood sample is concomitant with a PV of 4451 mL. Taken from Astolfi et al., 2021.*

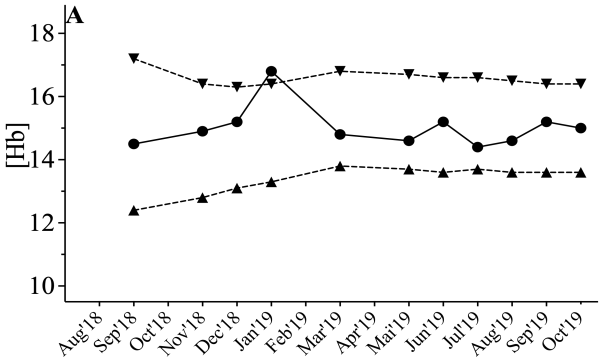


Figure 8. *Representation of [Hb] for a breath-hold diver as per 11 successive blood samplings over one year. A value outside the expected range was calculated by the adaptive model that was concomitant with a change in PV (3138 mL in January compared to 4334 mL in March). Taken from Astolfi et al., 2021.*

For detecting fraudulent practices, the concomitant use of PV and Hb_{mass} would be relevant. For instance, in the case of EPO treatment, it has been shown that the "boosting" effect of RCB

and the increase in [Hb] were due to two mechanisms: an increase in RBCV and a decrease in PV. The authors attributed this to a probable downregulation of the renin-angiotensin-aldosterone axis (Lundby et al., 2007).

Hb_{mass} and PV are not part of the ABP. Though they would likely be useful in the evaluation of the ABP as we have presented above, several remaining issues hinder their implementation. For instance, measuring Hb_{mass} and PV via the CO-rebreathing method reduces the effective VO_2 max by approximately 3% (Schmidt & Prommer, 2005), which makes it inadequate for use shortly before competitions. Among other drawbacks of this method, the CO-rebreathing method is, actually, impossible to implement in an anti-doping control for ethical and logistical reasons. To also ensure a lack of CO and an accurate measurement, which requires proper breathing, the athlete must be completely cooperative. Moreover, research has shown that the CO-rebreathing method, even in a low dose, can also stimulate the production of new RBC (Schmidt et al., 2020). Despite all these points, the measurements of Hb_{mass} and PV in our studies provide another read of the ABP. Nevertheless, to emphasize the applicability of our results, we recommend the use of PV corrections proposed by Lobigs et al. (2017) in addition to the use of the CO-rebreathing method.

6.1.4 Towards an athlete training passport

In anti-doping sciences, the risk of false positives is unacceptable and must be minimized as much as possible, which is why the determination of doping is not only based on thresholds and biomarker values, but also includes expert analysis (Sottas et al., 2008). Confounding factors represent great challenges for the ABP, and many loopholes are still obvious.

In a new attempt to counter doping, an additional approach to the ABP profile has been proposed: the performance profile or performance model (Faiss et al., 2019; Hopker et al., 2020;

Iljukov & Schumacher, 2017). This model relies on training and competition results, as the main goal of doping is to improve performance (Vernec, 2014). To further this model, Iljukov et al. (2018) proposed monitoring performance data in elite runners to highlight a possible gap in performance that did not correspond to a physiological improvement. Faiss et al. (2019) also proposed to monitor performance and included training content as part of the performance monitoring (Faiss et al., 2019). Nevertheless, the APP can be difficult to apply to all sports. For instance, our result regarding the variations of [Hb] with acute training load in cyclists could have been hypothesized regarding previous studies following the Grand Tours[10] (Corsetti et al., 2012; Lombardi et al., 2013). Nevertheless, we also showed that in cycling the use of reliable power data in training may provide relevant values. The main challenge is implementing and using those data in anti-doping due to access issues, data treatment and data reliability.

Regarding data treatment, sophisticated statistical models exist to treat large amounts of data. For instance, the expectation-maximization algorithm (EM), first proposed by Dempster et al. (1977), is an iterative algorithm for estimating the model parameters where the solution cannot be found directly that uses iterations of an expectation (E) step and a maximization (M) step. This relatively old statistical method is useful in many contexts where data are missing. Recently, Roshan et al. (2021) compared statistical methods used in longitudinal biomarker monitoring in the medical field (i.e. cancer biomarkers). They compared three statistical approaches: a) static, b) Bayesian and c) the EM and proposed an alternative method to the EM that would enable fast analyses of a large dataset compared to the static or the Bayesian method. The alternative to the EM algorithm is an interesting solution, though more studies are needed to determine its applicability to the ABP. We are also interested in whether its statistical algorithm would enable the analysis of more training datasets in an anti-doping context. For

[10] Examples of Grand Tours: Giro d'Italia, Vuelta a Espana, Tour de France to name a few.

instance, the intra-individual variation of each athlete in terms of his training would be relevant. As already proposed for the APP, an "athlete training profile" (ATP) could be imagined that would include research on intra-individual variation in various fields of sport sciences, like nutrition and mental preparation, to help better understand the increase that can be achieved for an athlete through various training modalities.

Performance level can also be an indirect marker for blood doping. Along this line, the athlete performance profile (APP) has been proposed to track suspicious performance increases using competition results (Montagna & Hopker 2018). As an example, Kruse et al. found a decrease in performance level in running race speeds in conjunction with improved rHuEPO detection (2014). To strengthen this approach, the application of a APP was suggested that would trigger further testing when a performance exceeds a certain level for a single athlete. This "indirect detection" can also be acceptable under article 2.2 of the WADA Code. To estimate credible or uncredible performances, the data available from competition results can be drawn for the APP for a single athlete using the level of probability as explained previously. Thus, the APP has the potential to help adjust target testing (Iljukov & Schumacher, 2017). However, this longitudinal profile of performance is not currently implemented in practice.

Nevertheless, a drawback of the APP is that performance data, like blood biomarkers, also have confounding factors that make it such that we can never be sure whether an athletic performance reflects an athlete's full capabilities. These confounding factors include pacing strategy, environmental conditions, standardized vs. non-standardized sports conditions, seasonal training and training pattern that can also affect performance. In fact, as the main way to achieve performance is through training, it is relevant to look deeply into alterations induced by training, which is why the proposition to longitudinally follow training data would improve

interpretations of the ABP as well as a future APP. In our second study, we propose a first step for longitudinally monitoring cycling training load, which could be applied to increase the performance sensitivity and specificity for a single athlete. In this way, we expect that an ATP would enable the differentiation between a physiological increase in performance caused by training as opposed to an unphysiological one caused by the use of prohibited substances or/and methods (Iljukov & Schumacher, 2017).

An ATP can be imagined that would state the intra-individual variations expected for a single athlete in terms of training regimens. We could add the training regimen of an athlete into the Bayesian networks, for instance endurance period, intensity blocs, recovery time, etc., first as a way to better understand blood biomarkers and, second, to eliminate potential confounding factors for the APP. In addition, an ATP can help target athletes and refine, for instance, the *Testing Plan* requested in *The International Standard for Testing and Investigations* (WADA, 2021e). Nevertheless, the main, and difficult to surmount, drawback is the availability and reliability of data, even though the emergence of portable data is widespread (Puchowicz et al., 2018).

6.2 Limitations

Although this thesis presents valuable results in the field of anti-doping, our new findings need further development in multiple axes:

- For the third study, our sample size is limited, though representative of the elite population in cyclists. Our participants also highly adhered to the study requirements, furnishing details of their training and coming regularly for blood measurements, which improves the overall statistics. We also regret the non-inclusion of elite women cyclists

and the fact that the tests were all announced, as unannounced testing would have strengthened the transfer of our data to anti-doping settings. Indeed, because our cyclist samples were all in the RTP, we could have used the values from the sampled blood in this context.

- In our third study, the use of EPO serum level would have brought relevant insight into the understanding of the influence of breath-hold training on hematological values.

In addition, the time of the day may influence our results and was not included as a variable in our studies because we emphasized the relevance of participant compliance and reduced the inconvenient of a fixed time as much as possible. Indeed, the circadian rhythm has been shown to influence the PV, with a variability between 10–25% between the morning and evening blood sample of an athlete (Voss et al., 2011). Here, PV was shown to be responsible for the decrease in [Hb] due to a dilutional effect, with a mean decrease in [Hb] of approximately 0.6 g/dL (or 3.7%) from the morning to the evening values. Nevertheless, a prior study highlighted the opposite regarding Ret% and EPO, with both of these values reaching their peak in the evening (McKee et al., 1974). A more recent study showed that the peak of Ret release was around 1 AM, with a 95% confidence interval between 8:00 PM and 4:30 AM. Here, the authors concluded that the circadian rhythm constitutes 37% of the total variability encountered over a 24-hour span (Banfi, 2008). Nevertheless, with these issues withstanding, we kept as close as possible to an anti-doping setting, when a blood sample can be taken at various hours throughout the day.

6.3 Conclusion and overall perspectives

In conclusion, the studies presented in this manuscript shed light on the assessment of three potential confounding factors present in the measurement of hematological variables for the ABP. We first showed that body position influenced the ABP, as blood taken while the athlete was in a supine position showed larger PV variations that cannot meet the current standards. Additionally, we showed that a short walk of 50 m before blood collection required only a 5 min seated waiting time to return to normal values. Secondly, an acute training load (5 days) prior to blood sampling might need to be required additional information for the experts performing the ABP readings, especially for significant variations in [Hb]. Thirdly, we investigated a new potential confounding factor in breath-hold training, showing that it had no influence on hematological variables. With this study we have been able to show the relevance of implementing an ABP for this population.

Here, as we tested only divers training mainly in a swimming pool (13% of the total training was deep diving), further research with deep divers would further inform our results due to the additional physiological responses associated with the pressure change. In addition, evaluating the ABP directly after a dive would have been interesting as well as EPO serum concentration, as there are other physiological responses to a deep dive (i.e. lung volume decreases with increase hydrostatic pressure, risks of nitrogen narcosis) (Patrician et al., 2021).

Additional data

Overall, we emphasize the undeniably valid addition of monitoring Hb_{mass}, PV and training data for interpreting the ABP in elite cyclists. Even though blood doping detection is indirect (Saugy & Leuenberger, 2020), it has already been proven that looking firstly at the effect to understand the cause is relevant. Similarly, we suggest that looking at training data is paramount, as it can bring additional information.

Nevertheless, some obstacles must be overcome, including the use of reliable devices for analyzing multiple sets of training data for each athlete. For instance, the subjectivity of the training related by the athlete cannot be accounted for or has no place in anti-doping sciences. Several high-technology wearable devices are available to accurately track physiological (e.g. heart rate, saturation, temperature) or performance data (e.g. speed, power, frequency) (Seshadri et al., 2017). These analyses would be assisted by one of the foremost developments in sport in this century—big data analysis and the associated artificial intelligence, data analytics and machine learning (Baerg, 2017). In the context of hematological variation analysis, additional training data for a population and a population of athletes (i.e. for population-based reference ranges for training variations and athlete references ranges for the athlete population will help detect inter-individual variations. Additionally, training reference values created for individuals could help to better interpret when hematological values or performances deviates from an expected range.

Consideration of women

Regarding women, we have shown that it is possible to include them using an adequate statistical model. Therefore, we hope that more women will be included in future scientific research regarding blood variations to reduce, little by little, the ever-present caveat seen across the field—"this result should be confirmed in women".

To conclude, future assessments of Hb_{mass}, PV and training data should be emphasized and included for anti-doping measures as well as performance and health concerns. Overall, this thesis brought further support for the robustness of the ABP hematological module and

highlights the need for further work in refining and extending the incorporation of confounding factors in the ABP algorithm.

CHAPTER 7

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