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A Novel Transcriptomic Based Approach to the Detection of Recombinant Human Erythropoietin Doping

by

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A doctoral Thesis

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Abstract:

Administration of recombinant human erythropoietin (rHuEpo) improves endurance performance. Hence rHuEpo is, allegedly, frequently subject to abuse by athletes, although rHuEpo is prohibited by the World Anti-Doping Agency. A transcriptomic-based longitudinal screening approach has the potential to improve further the performance of current detection methods. **AIM:** To assess the effects of rHuEpo on blood gene expression profiles in order to identify a "molecular signature" of rHuEpo doping. **METHODS:** 19 Caucasian trained males at sea-level (Scotland – SCO) and 20 Kenyan endurance runners at moderate altitude (~2,150 m, Kenya – KEN) received rHuEpo injections of 50 $IU \cdot kg^{-1}$ body mass every two days for 4 weeks. Blood was obtained 2 weeks before, during and 4 weeks after administration. 3,000 m time trial performance was measured pre, post administration and at the end of the study. RNA was extracted from blood stabilized in Tempus RNA tubes, amplified, labelled and hybridized to Illumina HumanHT-12v4 Expression BeadChips. Expression data was analyzed using Rank Products with a 5% false discovery rate and an additional 1.5 fold-change threshold. A subset of target and housekeeping genes was further validated using QuantiGene Plex assay. **RESULTS:** Despite markedly different baseline values between SCO and KEN, as exemplified by the haematocrit (41.9 ± 1.8% vs. 45.3 ± 2.6, $p < 0.001$), key blood parameters significantly increased during rHuEpo in both groups ($p < 0.001$) to reach similar levels at the end of administration (49.2 ± 2.0% vs. 49.6 ± 2.6, $p = 0.638$). The relative improvements in running performance post rHuEpo (~5%) and 4 weeks post administration (~3%) compared to baseline were of similar magnitude in both groups ($p > 0.188$). These results confirmed that the perturbation involving rHuEpo worked effectively. Relative to baseline, the expression of hundreds of genes were found to be altered by rHuEpo. In particular, 30 transcripts were already differentially expressed two days after the first injection while 15 transcripts were profoundly up-regulated during and subsequently down-regulated up to 4 weeks post administration in both groups. Importantly, the same pattern was observed in all subjects. The functions of the discovered genes were mainly related to either the functional or structural properties of the erythrocyte or to the cell cycle and its regulation. **CONCLUSION:** This research successfully identified the blood "molecular signature" of rHuEpo administration and provides the strongest evidence to date that gene biomarkers have the potential to substantially improve the performance of current anti-doping methods such as the Athlete Biological Passport for rHuEpo detection.

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Preface

The anemia of chronic renal failure: Pathophysiology and the effects of recombinant erythropoietin

Principal discussant: Joseph W. Eschbach

University of Washington School of Medicine, Seattle, Washington

"Case presentation:

A 40-year-old man has undergone chronic hemodialysis for 7 years. He had nephrotic syndrome from age 2 to 5 years but was apparently well until age 18, when proteinuria was detected again. At age 28, hypertension was noted. Hematocrit was 36%. (...) At age 32, his blood pressure was 145/90 mm Hg. The hematocrit was 20.5%. (...) At age 33, uremic symptoms were evident. The hematocrit was 14%. Two units of red blood cells were transfused and hemodialysis was begun.

He improved and returned to his home in Alaska to continue chronic hemodialysis. Because his hematocrit fell repeatedly to levels of 12%—l4%, he returned to Seattle every 2 to 3 weeks to receive packed red cell transfusions. Five months after starting dialysis, his blood pressure was 152/100mm Hg. (...) Transfusion requirements did not diminish.

At age 34 (15 months after the patient started hemodialysis), he received a cadaveric renal transplant. The hematocrit rose within one month to 27%. (...) Three months following transplantation, the aliograft was rejected, and treatment with chronic hemodialysis was resumed. The hematocrit fell to 11%, and frequent packed red-cell transfusions again were required to stabilize the level at about 19%. (...).

Although he worked 7 days a week running his country store, his energy and "wind" would "give out" by early afternoon and he would rest the remainder of the day. He could no longer unload the supplies delivered to his store.

By age 39, he had received a total of 313 units of red blood cells.

At that time, recombinant human erythropoietin (rHuEpo) was given in an initial dose of 300 units/kg intravenously and was continued three times per week. Within 8 weeks after initiation of rHuEpo therapy, his hematocrit was 38%; no transfusions had been administered over this time

Clinical improvement was dramatic; he was able to play handball and to meet all the physical demands of running his country store. This included working a full day without becoming exhausted, and now being able to unload the heavy boxes of supplies delivered to his store. After one year of rHuEpo therapy (75 units/kg, 3 times/week, intravenously), the hematocrit was 36%. (...)

He continues to enjoy an active life."
(Eschbach 1989)

Following this personal and clinical triumph ...

1997   2002   2006   2012

Acknowledgment:

It would not have been possible to complete this doctoral thesis without the guidance, help and support of several individuals, to whom I would like to express my sincere gratitude.

My supervisors Dr Yannis Pitsiladis and Dr John McClure for their unconditional support, excellent guidance and help in developing my knowledge in molecular biology applied in the field of exercise physiology and sport science;

the volunteers for their participation and cooperation;

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Author's Declaration:

Unless otherwise indicated by the acknowledgment or reference to published literature, the present work in this thesis is the author's own and has not been submitted for a degree to any other university or institution.

Jérôme Durussel _____   Date _____

List of Publications:

Publications:

Jérôme Durussel, Evangelia Daskalaki, Martin Anderson, Tushar Chatterji, Diresibachew H. Wondimu, Neal Padmanabhan, Rajan K. Patel, John D McClure, Yannis P. Pitsiladis. Haemoglobin Mass and Running Time Trial Performance after Recombinant Human Erythropoietin Administration in Trained Men, PLoS One, 2013.

Jérôme Durussel, Ramzy Ross, Prithvi R. Kodi, Evangelia Daskalaki, Pantazis Takas, John Wilson, Bengt Kayser, Yannis P. Pitsiladis. Precision of the Optimized Carbon Monoxide Rebreathing Method to Determine Total Haemoglobin Mass and Blood Volume, European Journal of Sport Science, 13: 68-77, 2013.

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The 2013 Charles M. Tipton National Student Research Award of the American College of Sports Medicine (ACSM) which recognizes the student with the most outstanding research project of the year. **Jérôme Durussel**, John D. McClure, Martin W. McBride, Diresibachew H. Wondimu, Wendy Crawford, Evangelia Daskalaki, Tushar Chatterji, Neal Padmanabhan, Rajan K. Patel, Sandosh Padmanabhan, Yannis P. Pitsiladis. Blood Gene Expression Profiles of Kenyan and Caucasian Endurance Trained Males After rHuEpo Administration. American College of Sports Medicine (ACSM), 60th Annual Meeting and 4th World Congress on Exercise is Medicine, Indianapolis, United States, May 28 – June 1, 2013.

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Jérôme Durussel, Evangelia Daskalaki, Tushar Chatterji, Wendy Crawford, Jim McCulloch, Neal Padmanabhan, Rajan K. Patel, Sandosh Padmanabhan, Pawel Herzyk, Günter Gmeiner, Martin W. McBride, John D. McClure, Yannis P. Pitsiladis. Blood Gene Expression Profiles of Recombinant Human Erythropoietin in Endurance Trained Individuals. XXXII World Congress of Sports Medicine, Rome, Italy, September 27-30, 2012.

Jérôme Durussel, Evangelia Daskalaki, Tushar Chatterji, Wendy Crawford, Jim McCulloch, Neal Padmanabhan, Rajan K. Patel, Sandosh Padmanabhan, Pawel Herzyk**,** Günter Gmeiner, Martin W. McBride, John D. McClure, Yannis P. Pitsiladis. A Transcriptomic Based Approach to the Detection of Recombinant Human Erythropoietin Doping. Glasgow Polyomics Launch Symposium, Graham Kerr Building, University of Glasgow, Glasgow, UK, September 14, 2012.

Jérôme Durussel, Tushar Chatterji, Evangelia Daskalaki, Jim McCulloch, Wendy Crawford, Neal Padmanabhan, Sandosh Padmanabhan, Rajan K. Patel, Pawel Herzyk**,** Günter Gmeiner, Martin W. McBride, John D. McClure, Yannis P. Pitsiladis. Effects of Recombinant Human Erythropoietin on Blood Gene Expression Profiles in Endurance Trained Individuals. International Convention on Science, Education and Medicine in Sport (ICSEMIS), Scottish Exhibition & Conference Centre, Glasgow, UK, July 19-24, 2012.

Jérôme Durussel, Tushar Chatterji, Evangelia Daskalaki, Diresibachew Haile, Zeru Bekele, Neal Padmanabhan, Sandosh Padmanabhan, Rajan K. Patel, Timothy D. Noakes, Günter Gmeiner, John D. McClure, Yannis P. Pitsiladis. Endurance Performance, Total Haemoglobin Mass and Blood Volume Post Recombinant Human Erythropoietin Use in Trained Individuals.

American College of Sports Medicine (ACSM), 59th Annual Meeting and 3rd World Congress on Exercise is Medicine, San Francisco, United States, May 29 – June 2, 2012.

Related published conference communications:

Diresibachew H. Wondimu, **Jérôme Durussel**, Wondyfraw Mekonnen, Edwin Anjila, Neford Ongaro, Mathew Rutto, Tuwei Wilson, Martin Mooses, Evangelia Daskalaki, Yannis P. Pitsiladis. Blood Parameters and Running Performance of Kenyan and Caucasian Endurance Trained Males after rHuEpo Administration. American College of Sports Medicine (ACSM), 60th Annual Meeting and 4th World Congress on Exercise is Medicine, Indianapolis, United States, May 28 – June 1, 2013.

Evangelia Daskalaki, Liang Zheng, Gabriela Kalna, Eyal Gottlieb, **Jérôme Durussel**, Tushar Chatterji, Christian Frezza, Ann Hedley, Neal Padmanabhan, Sandosh Padmanabhan, Rajan K. Patel, Günter Gmeiner, David Watson, Yannis P. Pitsiladis. Effects of Recombinant Human Erythropoietin on Targeted Blood Metabolomic Profiles in Endurance Trained Individuals. Glasgow Polyomics Launch Symposium, Graham Kerr Building, University of Glasgow, Glasgow, UK, September 14, 2012.

Evangelia Daskalaki, Liang Zheng, Tushar Chatterji, **Jérôme Durussel**, Gabriela Kalna, Christian Frezza, Ann Hedley, Eyal Gottlieb, Neal Padmanabhan, Sandosh Padmanabhan, Rajan K. Patel, Günter Gmeiner, Yannis P. Pitsiladis. Effects of Recombinant Human Erythropoietin on Targeted Blood Metabolomic Profiles in Endurance Trained Individuals. International Convention on Science, Education and Medicine in Sport (ICSEMIS), Scottish Exhibition & Conference Centre, Glasgow, UK, July 19-24, 2012.

Andrew Bloodworth, Michael J. McNamee, Steve D. Mellalieu, **Jérôme Durussel**, Evangelia Daskalaki, Lauren Perterson, Yannis P. Pitsiladis. The Ethics and Psychology of Doping Healthy Endurance Trained Individuals. European College of Sport Science (ECSS), 17th annual Congress of the ECSS, Bruges, Belgium, July 4-7, 2012.

List of Abbreviations:

ABP	Athlete Biological Passport
CO	Carbon monoxide
Hb_{mass}	Total haemoglobin mass
RIN	RNA integrity number
rHuEpo	Recombinant human erythropoietin
$\dot{V}O_{2max}$	Maximal oxygen uptake
WADA	World Anti-Doping Agency

Chapter 1 : General Introduction

1.1 Erythropoietin and its therapeutic use:

Erythropoietin is a glycoprotein hormone produced primarily in the kidney that regulates red blood cell mass by stimulating the survival, proliferation and differentiation of erythrocytic progenitors (Figure 1.2) (Jelkmann 1992, 2011). Anaemia is a common complication of chronic kidney disease caused principally by a deficiency in the synthesis of endogenous erythropoietin (Eschbach *et al.* 2002). Recombinant human erythropoietin (rHuEpo) was first produced in the 1980s (Jacobs *et al.* 1985; Lin *et al.* 1985). In 1989 a 40 year-old patient with chronic kidney disease associated anaemia, who had undergone chronic haemodialysis for 7 years, unsuccessful kidney transplantation and received 313 units of red blood cells, was the first patient to receive rHuEpo treatment (Eschbach 1989; Eschbach *et al.* 1989)[1]. Within 8 weeks of rHuEpo therapy, his haematocrit increased from 15% to 38%, red blood cells transfusions were no longer required, he was able to play handball and resumed his physically demanding work (Eschbach 1989; Eschbach *et al.* 1989). Since this success of almost biblical proportions, many clinical trials have demonstrated the efficacy and short-term safety of rHuEpo for increasing and maintaining haemoglobin concentration, for reducing the need for red blood cell transfusion and improving the quality of life of patients with anaemia (Glaspy *et al.* 2010). However, the fact that rHuEpo has improved the quality of life of more than a million anaemic patients (Brines and Cerami 2005) has to be balanced against the concerns regarding an increased risk of venous-thromboembolic events and possible unknown adverse long-term effects in patients (Glaspy *et al.* 2010; Jelkmann 2010). rHuEpo is currently widely and mainly used to treat symptomatic anaemia caused by chronic kidney disease and anaemia with related symptoms in some cancer patients receiving chemotherapy, as well as to increase the amount of blood that can be taken from people donating their own blood before surgery.

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[1] See Preface.
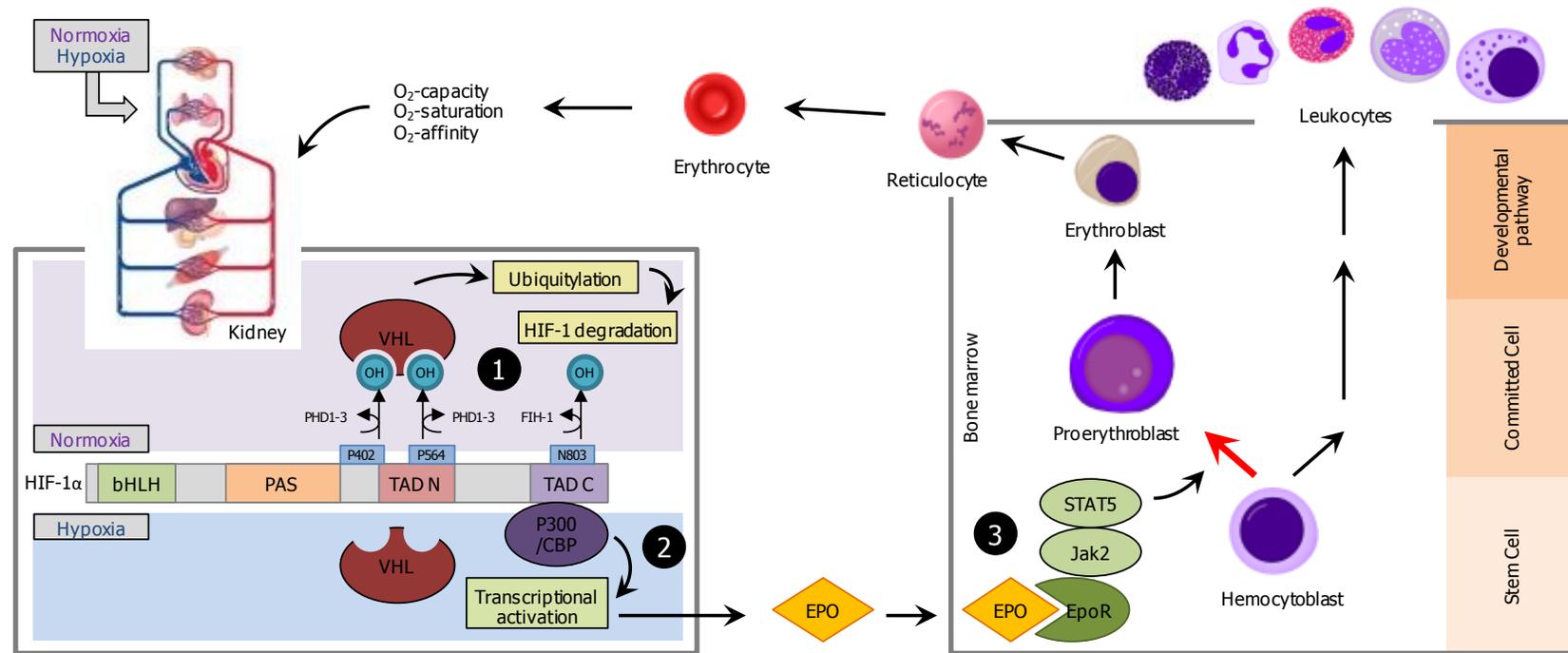
Figure 1.1: Simplified schematic diagram of the regulation of erythropoiesis.

Oxygen regulates the rate at which hypoxia-inducible factor 1α (HIF-1α) protein is degraded in the kidney. 1) Under normoxic conditions (normoxia), the von Hippel-Lindau (VHL) protein binds HIF-1α via the oxygen-dependent hydroxylation of proline residues (P402 and P564) by the enzymes prolyl hydroxylase-domain protein 1-3 (PHD1-3), resulting in the ubiquitylation of HIF-1α, its degradation and the inhibition of HIF-1α-mediated gene transcription. 2) Under hypoxic conditions (hypoxia), VHL cannot bind HIF-1α, resulting in a decreased rate of HIF-1α degradation, which, in turn, allows the transcriptional activation of HIF-1α target genes such as erythropoietin (*EPO*). 3) In the bone marrow, EPO binds to the erythropoietin receptor (EpoR) and activates the Jak2-STAT5 signalling cascade which then stimulates the survival, proliferation and differentiation (red arrow) of erythrocytic progenitors (proerythroblast, erythroblast and eventually reticulocyte) without affecting the leukocytes production.
Figure adapted from (Semenza 2003, 2004) and (Jelkmann 2011).

Images adapted from http://upload.wikimedia.org/wikipedia/commons/6/69/Hematopoiesis_(human)_diagram.png and http://www.netterimages.com/images/vtn/000/000/008/8732-150x150.jpg.

1.2 The emergence of blood doping and the misuse of erythropoietin:

During the Olympic Games in Mexico City in 1968, held at moderate altitude (2250 m a.s.l), the premise that blood adaptations, such as an increase in haemoglobin concentration, helped those athletes living at altitude to win most of the endurance races defined the new research focus in the field of sport physiology (Eichner 2007). "*We've just started to scratch the surface in athletic research*" reported Dr Faulkner, an exercise physiologist, in a newspaper article published in The Windsor Start on 6th December 1968, entitled "Altitude improved Olympic effort" (Herbert 1968). A few years later, the pioneering work of Ekblom *et al.* demonstrated that elevation in haemoglobin concentration increased maximal oxygen uptake ($\dot{V}O_{2max}$) and improved performance (Ekblom *et al.* 1972). Interestingly enough, soon after came the first accounts of blood manipulation in sports as well as the term "blood doping" in the media (Cazzola 2000; Leigh-Smith 2004; Lundby *et al.* 2012b). The newspapers Star-News and Pittsburgh Post-Gazette reported in an article published on 1st August 1976 entitled "Blood doping and other things" that "*The scientific scandal of the Olympics was as quiet as the flow of blood until Lasse Viren of Finland added the 5,000-meter gold medal to his 10,000-meter gold medal. 'He has got that extra blood in him,' said Ron Dixon of New Zealand. Lasse Viren and other European distance runners are suspected of being strengthened by what is known as 'blood doping' – the removal of a pint of the runner's blood, then the return of that same blood perhaps two weeks later. In that time his body has compensated for its absence. The runner now has an extra pint of blood in his system*" (Reporter unknown 1976). Indeed, there is evidence that blood transfusions were used with a certain success in the 1970s and 1980s by, among others, cyclists, marathoners and cross-country skiers (Eichner 2007; Lundby *et al.* 2012b).

Doping in sport seems to adapt rapidly to the new technology available. The appearance of rHuEpo, which was shown to successfully increase haemoglobin concentration in patients, radically transformed blood doping practices. Following the "transfusion era", rHuEpo doping took over in the 1990s, as was highlighted by the numerous doping cases during the Tour de France bicycle race and especially the Festina affair in 1998 (Lundby *et al.* 2012b). In 2000 Birkeland *et al.* scientifically demonstrated in his laboratory that rHuEpo administration for 4 weeks in 20 male athletes increased haematocrit (43% to 51%), $\dot{V}O_{2max}$ by 7% as well as time to

exhaustion by 9% (Birkeland *et al.* 2000). In the last decade, the practice of red cell transfusions has made a strong comeback in response to the development of anti-doping tests (see next paragraph). Based on testimonies from athletes who have been detected, it is now known that athletes are using rHuEpo in combination with blood transfusions (Lundby *et al.* 2012b).

1.3 Erythropoietin and anti-doping:

In 1990, erythropoietin was included on the list of prohibited substances by the International Olympic Committee because misuse by athletes was suspected, although no approved test existed at that time (Reichel 2011b). During these years, there were rumours that cheats used huge amount of rHuEpo which induced very high values of blood parameters. For instance, Bjarne Riis, the 1996 Tour de France winner (who admitted doping in 2007), acquired the nickname of "Mr 60%" which referred to his haematocrit level (Shermer 2008). Because rHuEpo was directly undetectable at the time, and as well as to protect the health of athletes, the 50% haematocrit rule was introduced in 1997 by the International Cycling Union (Sottas *et al.* 2010). Briefly, any racing cyclist who had a haematocrit above 50%[2] was declared ineligible and was excluded from the race. Despite this rule, the Festina scandal at the Tour de France in 1998 provided the proof of organized and widespread doping in professional cycling and highlighted the need for the creation of an independent international agency, which would set "unified standards for anti-doping work and coordinate the efforts of sports organizations and public authorities" (World Anti-Doping Agency 2010). The World Anti-Doping Agency (WADA) was consequently established in 1999.

Although the detection of rHuEpo doping is difficult because rHuEpo is structurally very similar to endogenous erythropoietin, a direct urinary test based on the presence of different isoforms in isoelectric profiles was developed and published by Lasne *et al.* in 2000 (Lasne and de Ceaurriz 2000; Lasne *et al.* 2002). This test became the first WADA approved detection method for rHuEpo doping. However, while the effects of rHuEpo administration can last for weeks, the test has a rather limited detection window, *i.e.* a few hours up to a few days depending on the dose used (Ashenden *et al.* 2006; Breidbach *et al.* 2003; Lundby *et al.* 2008a). At the same time as the

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[2] 50% for male athletes and 47% for female athletes.

development of the direct test, potential indirect detection of rHuEpo using blood markers of altered erythropoiesis was being investigated (Cazzola 2000; Parisotto *et al.* 2000). Notably, Parisotto *et al.* (2000) developed statistical models using indirect markers of altered erythropoiesis in order to detect rHuEpo doping during current (ON-model) and after repeated recent (OFF-model) administration, respectively. The OFF-model based on haemoglobin concentration and reticulocyte levels[3] identified at 12-21 days after the last rHuEpo injection approximately two-thirds of the subjects who had previously been administered with rHuEpo for 4 weeks without any false positives (Parisotto *et al.* 2000). However, despite these promising laboratory-based results, both direct and indirect detection methods have been shown to lead to unidentified doping due to inadequate detection (Ashenden *et al.* 2011; Borno *et al.* 2010; Lundby *et al.* 2008a) as well as unacceptable cases of false positives (Beullens *et al.* 2006; Parisotto *et al.* 2000; Schumacher *et al.* 2000). For instance, it is known that terrestrial or simulated altitude exposure can increase the risk of false positives by influencing the indices of rHuEpo doping (Ashenden 2004; Ashenden *et al.* 2003; Ashenden *et al.* 2001). There is therefore an urgent need for these methods to be revised in the hope of removing any possibility of athletes being incorrectly banned from competition or, conversely, avoiding sanctions due to broad definitions of legal reference limits.

1.4 Athlete Biological Passport:

The Athlete Biological Passport (ABP) was recently introduced as a new tool to indirectly detect erythropoiesis-stimulating agents, such as rHuEpo, and which can lead to a doping sanction imposed by a sports organization or its tribunal at the same time as intelligently targeting athletes for additional testing (Schumacher and d'Onofrio 2012; Schumacher *et al.* 2012; Zorzoli and Rossi 2012). The ABP approach relies on identifying intra-individual abnormal variability over time of selected haematological parameters (Figure 1.2) (Callaway 2011; Sottas *et al.* 2010). As nicely demonstrated by Zorzoli and Rossi, the behaviour of the athletes related to blood doping has changed since the introduction of the ABP (Zorzoli and Rossi 2010). Extreme reticulocyte values (*i.e.* <0.4% or >2.0%) have drastically decreased and, in that sense, the implementation of the ABP has undeniably been a step forward in the anti-doping field (Zorzoli and Rossi 2010).

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[3] OFFscore = haemoglobin concentration (g·L^{-1}) – 60√reticulocyte (%).

However, although this approach can potentially be suitable to identify abnormal enhanced erythropoiesis regardless of the method used, the sensitivity of the ABP has been questioned (Lippi and Plebani 2011). In order to minimise the risk of being caught via the ABP, it is well recognized that athletes are now using the so-called "micro doses" of rHuEpo which allegedly range from 10 to a maximum of 40 IU·kg^{-1} body mass (Ashenden *et al.* 2011; Ashenden *et al.* 2006; Morkeberg *et al.* 2013). These micro doses aim to increase haemoglobin mass (Hb$_{mass}$) while avoiding a large fluctuation in the ABP blood markers as well as minimising the detection window for conventional direct methods (Ashenden *et al.* 2011). Indeed, Ashenden *et al.* have demonstrated that the ABP did not reveal any suspicious doping activities when rHuEpo microdosing strategy was used (Ashenden *et al.* 2011). In addition, physiological variations of the haematological parameters of the ABP due to factors such as training or hypoxia exposure can conflict with the interpretation of the ABP results (Sanchis-Gomar *et al.* 2011). There is therefore a need for the ABP approach to be improved.
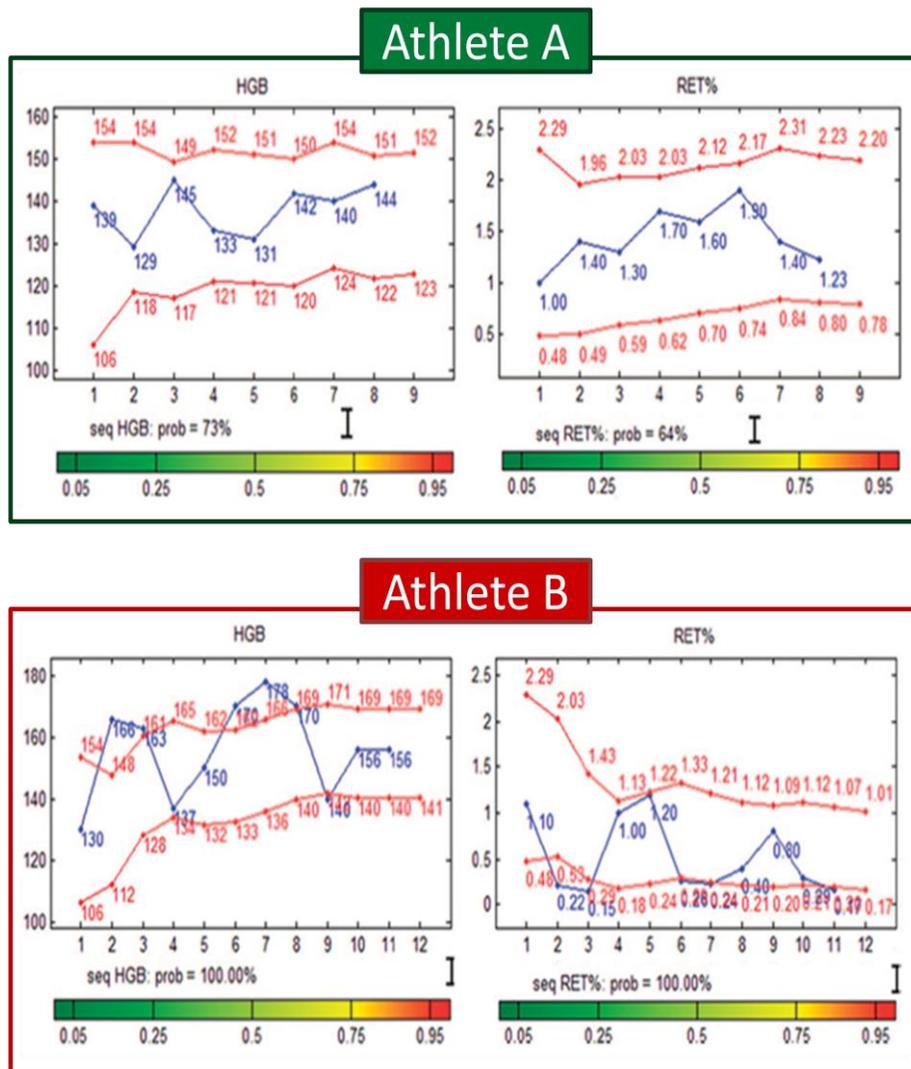
Figure 1.2: Examples of the Athlete Biological Passport (ABP) including haemoglobin concentration (HGB) and reticulocyte percentage (RET%).

Blue lines: actual tests results. Red lines: individual "normal" limits calculated by the ABP software. Athlete A presented normal variations. Conversely, athlete B presented abnormal variations, and was consequently suspected of doping. Figure adapted from Sottas *et al.* (2011).

1.5 Omics technologies – a transcriptomic approach:

A genome is defined as the entire collection of genetic information encoded by a particular organism (Snyder and Gerstein 2003). By semantic association, the words transcriptome, proteome and metabolome refer to the entirety of all transcripts, proteins and metabolites expressed by a genome at a specific time, respectively. The science related to these terms was named accordingly genomics, transcriptomics, proteomics and metabolomics and form the so-called "omics-cascade" (Reichel 2011a) (Figure 1.3). Using microarray technology, a characteristic gene expression signature of a particular stimulus can be revealed by assessing the transcriptional state of the cells of the experimental samples (Bilitewski 2009; Kurella *et al.* 2001). Therefore, if the gene expression profile following rHuEpo administration yields to a specific signature, new methods based on gene-expression profiles with potentially improved discriminatory power relative to current detection protocols can be developed. Data have emerged in support of this hypothesis (Varlet-Marie *et al.* 2009). Using the serial analysis of gene expression (SAGE) method, Varlet-Marie *et al.* identified 95 genes whose differential expression was subsequently tested by quantitative real-time polymerase chain reaction in two athletes (Varlet-Marie *et al.* 2009). These athletes were treated first with high doses of rHuEpo and then with micro doses. During the high dose regime, 33 marker genes for rHuEpo administration were identified and 5 remained differentially expressed during the micro dose regime. Moreover, this transcriptomic approach is not only confined to rHuEpo abuse, but can also be applied to other doping methods and substances used by the athletes. For instance, potential characteristic gene expression profiles have also been shown in 6 subjects after autologous blood transfusion (Pottgiesser *et al.* 2009; Pottgiesser *et al.* 2007). These recent preliminary results, although from only a limited number of subjects, provide the first confirmatory data to support the hypothesis that gene expression profiles may provide a sensitive method for the detection of rHuEpo and blood doping.
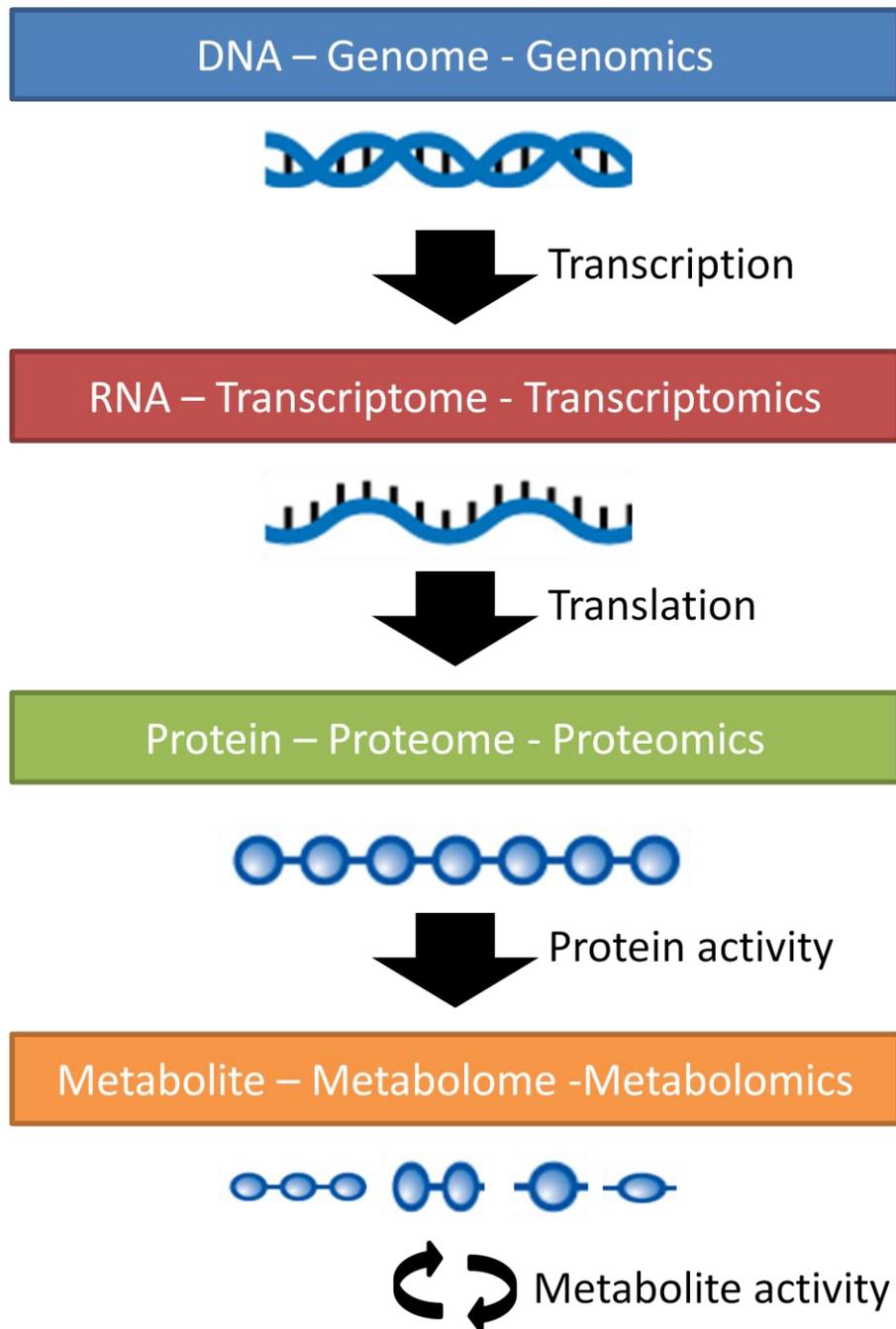
Figure 1.3: The "omics" cascade.

The cascade depicts the flow of information from the gene to the metabolism.

1.6 Current anti-doping policy and criticisms:

Following its creation, WADA drafted and implemented the World Anti-Doping Code which has been effective since 2004. The Code was revised in 2009, but its purposes remained unchanged, *i.e.* "to protect the athlete's fundamental right to participate in doping-free sport and thus promote health, fairness and equality for athletes worldwide", as well as "to ensure harmonized, coordinated and effective anti-doping programs at the international and national level with regards to detection, deterrence and prevention of doping". The World Anti-Doping Code defined doping as the occurrence of one or more of the anti-doping rule violations, in brief: the presence of a prohibited substance or its metabolites or markers in an athlete's sample; the use or attempted use of a prohibited substance or method; refusing or failing to submit to sample collection; possession of prohibited substances or methods (World Anti-Doping Agency 2009). A substance or method is considered for inclusion on the Prohibited List if WADA determines that the substance or method meets any two of the following three criteria: in brief, the substance or method 1) has the potential to enhance sport performance, 2) represents a health risk to the athlete, 3) violates the spirit of sport (World Anti-Doping Agency 2009).

While discussion of the WADA policy is beyond the scope of this thesis, it should be noted that criticisms have been addressed to the anti-doping policy (Kayser and Broers 2012; Kayser *et al.* 2007; Kayser and Smith 2008; Smith and Stewart 2008). For instance, Kayser and Broers argued that. although at first glance the current anti-doping policy appears reasonable and simple, it seems not to be able to attain its objectives using a zero-tolerance approach and possibly leads to more harm to society than it can prevent (Kayser and Broers 2012). Their possible alternative would be a pragmatic model based on regulation and harm reduction inspired by the experience gained with alternative drug policies (Kayser and Broers 2012; Smith and Stewart 2008). Another alternative (not necessarily incompatible with the latter) to the current anti-doping policy suggested by Lippi *et al.* would be to focus the effort on education and prevention as well as on biochemical or haematological identification and monitoring of "pathological" deviations rather than on all-out repression (Lippi *et al.* 2008). Although this debate on anti-doping policy (which is much broader than implied by the previous examples) is beyond the scope of this thesis, it is important to note that the knowledge gained from the longitudinal investigations of the effect of rHuEpo administration carried out during this doctoral research is valuable

regardless of the policy adopted. For instance, in addition to providing insights into the mechanism of the action of rHuEpo, the results of this research can be used not only for anti-doping repression but also for health screening.

1.7 Aims and objective of the research:

The overall aim of this doctoral research was to assess the effects of rHuEpo on blood gene expression profiles in endurance trained volunteers in order to identify a molecular signature of rHuEpo doping and hence provide a basis for the development of novel and robust testing models to detect blood doping. Using both phenotypic and molecular methods, this research was carried out firstly at sea-level in Glasgow (Scotland, UK) with Caucasian endurance trained males and then at moderate-altitude (~2,150 m) in Eldoret (Kenya) with Kenyan endurance runners who abstained from official sporting competition for the entire duration of the study. To achieve the overall aim of determining the blood molecular signature of rHuEpo doping, this research addressed the following objectives:

❖ To determine and compare the effects of rHuEpo administration on blood parameters and exercise performance in endurance trained males living and training at or near sea-level with another cohort living and training at moderate-altitude (~2,150 m) in Kenya. (Chapter 2 and 4).

❖ To establish adequate molecular methods and molecular workflow to enable valid and optimal assessment of blood gene expression (Chapter 3).

❖ To assess the effects of rHuEpo on blood gene expression profiles in endurance trained volunteers living and training at or near sea-level (Chapter 3).

❖ To replicate and compare the effects of rHuEpo administration on blood gene expression profiles in another cohort living and training at moderate-altitude (~2,150 m) in Kenya. (Chapter 5).

❖ To validate the gene microarray based findings using another specific and sensitive quantitative gene expression technology (Chapter 6).

❖ To evaluate and discuss the potential direct applications of the findings in the anti-doping field to detect rHuEpo doping (Chapter 7).

Chapter 2 : Haemoglobin Mass and Running Performance after Recombinant Human Erythropoietin Administration in Trained Men

2.1 Introduction and aim:

In healthy subjects, administration of rHuEpo increases haemoglobin concentration not only by the well known increase in red blood cell mass but also by a decrease in plasma volume (Lundby *et al.* 2007; Olsen *et al.* 2011). Theoretically, normal human red blood cells can persist in the circulation for approximately 17 weeks (Franco 2009). However, neocytolysis, the selective haemolysis of young circulating red blood cells, which contributes to the regulation of red cell mass, seems to appear during specific conditions that cause a rapid decrease in erythropoietin levels such as spaceflight, high altitude exposure or blood doping (Alfrey *et al.* 1997; Chang *et al.* 2009; Franco 2009; Rice and Alfrey 2005; Rice *et al.* 2001; Risso *et al.* 2007). Little is known about the time course of these mechanisms post rHuEpo administrations. In this study, Hb$_{mass}$ and related blood volumes were repeatedly determined using the optimized carbon monoxide (CO) rebreathing method (Durussel *et al.* 2013b; Schmidt and Prommer 2005) pre, during and post rHuEpo administration in healthy trained men.

Most studies investigating the effects of erythropoietin on exercise performance have used $\dot{V}O_{2max}$ tests. However, $\dot{V}O_{2max}$ tests alone may probably be of little help in predicting exercise performance among athletes of similar ability (Conley and Krahenbuhl 1980; Costill *et al.* 1973; Faria *et al.* 2005; Noakes 2008b; Shephard 2009). One study assessed submaximal performance in healthy male subjects and reported improvements of more than 50% in time to exhaustion on a cycle ergometer at a given 80% of $\dot{V}O_{2max}$ whereas the increase in $\dot{V}O_{2max}$ was approximately 12% (Thomsen *et al.* 2007). Despite being a very interesting finding which questions the mechanisms by which rHuEpo improves submaximal exercise performance (Noakes 2008a), the validity of the time to exhaustion test in assessing human exercise performance has been questioned (Jeukendrup and Currell 2005). In addition to being more variable than time trial tests (Laursen *et al.* 2007), time to exhaustion tests are less likely to mimic the exercise performance environment (Jeukendrup and Currell 2005; Laursen *et al.* 2007). There are indeed only minimal occasions where an athlete is required to maintain a constant intensity until volitional exhaustion compared with self-paced exercise (Jeukendrup and Currell 2005; Laursen *et al.* 2007).

The main purpose of the present study was to investigate the time course of Hb$_{mass}$ and related blood parameters as well as changes in running 3,000 m time trial performance following 4 weeks of rHuEpo administration in healthy trained men in order to determine whether the augmented $\dot{V}O_{2max}$ observed in laboratory-based experiments would translate into actual improvements in running performance in the field.

2.2 Methods:

Twenty healthy non-smoking physically active men (mean ± SD, age: 26.5 ± 5.0 yr, body mass: 74.8 ± 7.7, height: 179.5 ± 5.4 cm) participated in this study. All subjects underwent a medical assessment and provided written informed consent to participate. The subjects were regularly engaged in predominantly endurance-based activities such as running, cycling, swimming, triathlon and team sports. Subjects were requested to maintain their normal training but abstain from official sporting competition for the duration of the research protocol (Howman 2013; Wagner 2013). One subject dropped out of the study because he wished to participate in the London marathon, which was scheduled during the last week of the research protocol and although three rHuEpo injections had been given, seven weeks of washout were deemed sufficient to return to competition at this event (his performance in this marathon was 2h 57min 26s; his best performance is 2h 48min 23s). The subjects were divided into two groups for exercise performance analysis. The first group included subjects who had a history of running (n = 10) and the second group included the remaining subjects who were involved in other sporting activities (n = 9). This study was approved by University of Glasgow Ethics Committee and conformed to the Declaration of Helsinki. One of the subjects in the running group suffered from nasal obstruction and coughing during the study and could not perform the exercise tests post rHuEpo administration. The running performance data of this subject was excluded from the analyses.

2.2.1 Experimental design:

Each subject subcutaneously self-injected 50 IU·kg^{-1} body mass of rHuEpo (NeoRecormon, Roche, Welwyn Garden City, UK) every second day for 4 weeks. Daily oral iron supplementation (~ 100 mg of elemental iron, Ferrous Sulphate Tablets, Almus, Barnstaple, UK) was given during the 4 weeks of rHuEpo administration. None of the subjects reached the predetermined safety haematocrit limit of 55% and all subjects tolerated the rHuEpo administration well with no signs of hypertension (see Results and Discussion sections of this chapter). Venous blood samples from an antecubital vein were obtained in triplicate at baseline (over 2 weeks prior to the first rHuEpo administration), during rHuEpo administration (on days 2, 4, 8, 10, 14, 16, 20, 22, 26, 28) and for 4 weeks after rHuEpo administration (on days 30, 35, 41, 43, 48, 50, 57) (Figure 2.1 and Figure 2.2). All blood samples were taken after 10 minutes of

rest in the supine position (Ahlgrim *et al.* 2010) (Figure 2.3). Blood samples were homogenized using a roller mixer and then analysed in triplicate using a Sysmex XT-2000i (Sysmex UK, Milton Keynes, UK). Blood-count variables such as haemoglobin concentration, haematocrit and reticulocyte percentage were measured by the fluorescence method and flow cytometry of the Sysmex. The mean value of the triplicate was reported. Resting blood pressure and heart rate were recorded three times on both arms in the supine position before blood sampling using an automated cuff oscillometric device (Boso-Medicus, Bosch & Sohn GmbH, Jungingen, Germany).

2.2.2 Measurements of haemoglobin mass and related blood volumes:

Hb$_{mass}$ was determined in triplicate prior to the first rHuEpo injection and then weekly up to 4 weeks post rHuEpo administration (Figure 2.1) using the optimized CO-rebreathing method as previously described (Durussel *et al.* 2013b; Schmidt and Prommer 2005) (Figure 2.4). Briefly, a bolus of chemically pure CO dose of 1.0 mL·kg^{-1} body mass was administered with the first breath through a spirometer and rebreathed for 2 min with 4 L of oxygen. Change in carboxyhaemoglobin percentage in venous blood samples from baseline to 8 min after CO administration was measured using a blood gas analyzer (ABL 725, Radiometer, Copenhagen, Denmark). CO concentration was measured using the Pac 7000 Draeger CO-analyzer (Draeger Safety, Northumberland, UK) and an estimated alveolar ventilation of 7.5 l·min^{-1} was used for calculations. Hb$_{mass}$ as well as blood, red cell and plasma volume were calculated as previously described elsewhere (Durussel *et al.* 2013b). Blood volume was derived by dividing Hb$_{mass}$ by the haemoglobin concentration. Red cell volume was obtained by multiplying blood volume by the haematocrit and plasma volume was then calculated by subtracting red cell volume from blood volume. The typical error of measurement for Hb$_{mass}$ calculated from the two first baseline measurements was 1.5% (95% confidence interval (CI) 1.1 to 2.2%). The typical error of measurement for blood, red cell and plasma volume between the two weeks prior to the first rHuEpo administration were 2.4%, 1.6% and 3.6% (CI 1.8 to 3.5, 1.2 to 2.4 and 2.7 to 5.3), respectively.
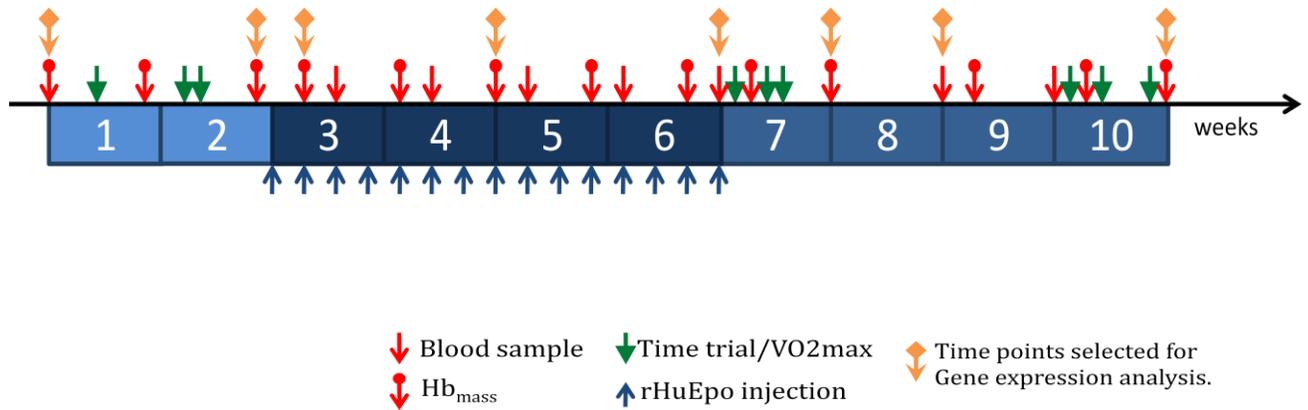
Figure 2.1: Experimental design.

Figure 2.2: Example of the subject's schedule.
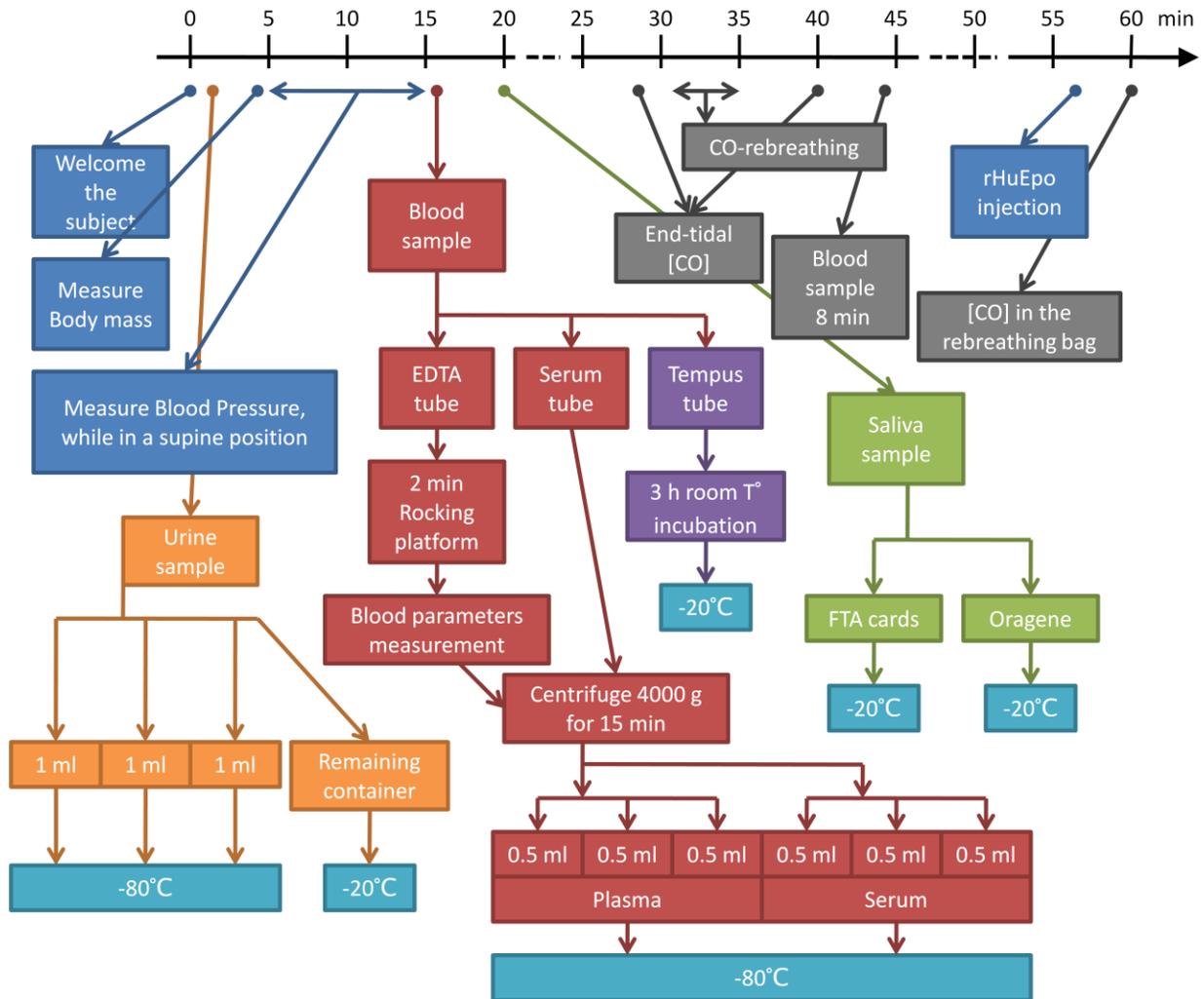
Figure 2.3: Standardised samples collection protocol.

Figure 2.4: Picture of the carbon monoxide rebreathing procedure.

2.2.3 Running performance assessment:

Two 3,000 m time trials separated by at least one day of rest were performed on a 200 m indoor athletic track (Kelvin Hall, Glasgow, UK) pre, post rHuEpo administration and at the end of the study (Figure 2.1). Verbal encouragement was given with feedback provided for the split time and remaining laps. The best performance on each occasion was used for analysis. The typical error of measurement for time trial performance calculated from the two tests on each phase was 1.3% (CI 1.1 to 1.7). Borg's rating of perceived exertion (RPE) was recorded at the completion of the time trial (Borg 1970). Temperature and humidity were recorded using a hygrometer. In addition to the time trial, $\dot{V}O_{2max}$ was determined pre, post rHuEpo administration and at the end of the study using an incremental test to exhaustion on a motorised treadmill. Both continuous or discontinuous protocols were used because of other purposes of the study (the speed was increased by 1 km·h^{-1}·min^{-1} or by 2 km·h^{-1} every 3 min with 3 min of active recovery at a walking pace between each bout, respectively). Following a 30 min recovery, $\dot{V}O_{2max}$ was verified using a square-wave protocol to exhaustion at a speed equivalent to the end speed attained during the incremental test minus 1 km·h^{-1} (Kirkeberg *et al.* 2011; Pettitt *et al.* 2013). Gas exchange was measured breath by breath using an automated metabolic gas analysis system (Cosmed Quark b2, Cosmed, Rome, Italy).

2.2.4 Statistical analysis:

Individual mean value was calculated when more than one blood sample was collected per week before further analysis. Changes over time in Hb_{mass}, blood, red cell and plasma volume were assessed using t-test to determine whether the slope of these parameters was significantly different from zero; the slope coefficient for each individual came from a linear regression of his measurements, using time as the predictor. Time trial performance, $\dot{V}O_{2max}$ and blood parameters data for the key stages of the study were analyzed using repeated measures ANOVA. Relationships between time trial performance, $\dot{V}O_{2max}$ and Hb_{mass} were assessed using Pearson's product moment correlation coefficients. Analyses were performed using Statistical Package for Social Sciences (SPSS, version 19). In the results section, data are described as mean ± SD and p values are from the repeated measures ANOVA analysis, unless mentioned otherwise.

2.3 Results:

2.3.1 Running performance (Table 2.1):

Running performance data of both groups combined (n=18, see Table 4.3) are presented in this paragraph (Table 2.1 for separate groups). Irrespective of running history, time trial performance significantly improved by ~6% post administration (10:30 ± 1:07 min:sec vs. 11:08 ± 1:15 min:sec, p < 0.001) and remained significantly enhanced by ~3% 4 weeks after rHuEpo compared to baseline (10:46 ± 1:13 min:sec vs. 11:08 ± 1:15 min:sec, p < 0.001). RPE did not significantly differ between the time trials pre, post rHuEpo administration and 4 weeks after rHuEpo (p > 0.111). Temperature (19.4 ± 2.8 °C) and humidity (46.6 ± 9.0%) remained relatively constant (p > 0.441). Relative to baseline, $\dot{V}O_{2max}$ significantly increased post administration (60.7 ± 5.8 mL·min^{-1}·kg^{-1} vs. 56.0 ± 6.2 mL·min^{-1}·kg^{-1}, p < 0.001) and remained significantly increased 4 weeks after rHuEpo (58.0 ± 5.6 mL·min^{-1}·kg^{-1} vs. 56.0 ± 6.2 mL·min^{-1}·kg^{-1}, p = 0.021). $\dot{V}O_{2max}$ was at least moderately correlated with Hb$_{mass}$ as well as time trial performance throughout the study (range, r = 0.48 to r = 0.88, p < 0.045). However, a significant correlation in individual responses compared to baseline between these three parameters was only observed for Hb$_{mass}$ and time trial performance at the end of the study (r = -0.68, p = 0.002).

2.3.2 Haematological parameters and resting blood pressure and heart rate (Table 2.2, Table 2.3 and Figure 2.5):

Table 2.2 illustrates the changes in the main haematological parameters for five key stages of the study. Relative to baseline values, both haematocrit and haemoglobin concentration gradually increased throughout the rHuEpo administration to reach a maximum approximately one week after the last injection (p < 0.001) and remained significantly elevated 4 weeks post rHuEpo administration (p < 0.001) (Figure 2.5A and Figure 2.5B). Reticulocyte percentage increased rapidly after the first two weeks of injections (p < 0.001) (Figure 2.5C). Nadir with a very low inter-subject variation was reached approximately two weeks after injections ceased, which was significantly lower compared with baseline values (p < 0.001) (Figure 2.5C). Body mass did not change during the study (p = 1.000). rHuEpo administration did not induce any significant changes in resting systolic (p > 0.441) and diastolic (p = 1.000) blood pressure as well as in resting heart rate (p = 1.000) throughout the study.

2.3.3 Haemoglobin mass and blood volumes (Table 2.2, Table 2.4 and Figure 2.6):

Relative to baseline, Hb$_{mass}$ and plasma volume were significantly increased ($p < 0.001$) and decreased ($p = 0.004$) at the end of the rHuEpo administration, respectively. Hb$_{mass}$ and red cell volume gradually increased by approximately 40 g·wk^{-1} (t-test, $p < 0.001$) and 135 mL·wk^{-1} (t-test, $p < 0.001$), respectively (Figure 2.6A and Figure 2.6B). Plasma volume decreased significantly by approximately 100 mL·wk^{-1} (t-test, $p < 0.001$) (Figure 2.6C) while blood volume remained relatively unchanged (t-test, $p = 0.318$) (Figure 2.6D). From week 1 to week 4 post rHuEpo administration, the rate of decrease in Hb$_{mass}$ and red cell volume toward baseline values was similar to that of the increase during administration (t-test, $p = 0.888$ and $p = 0.928$, respectively) (Figure 2.6A and Figure 2.6B) but both Hb$_{mass}$ and red cell volume were still significantly elevated 4 weeks post administration compared to baseline ($p < 0.001$). Plasma volume was restored to pre-injection levels 4 weeks post administration ($p = 0.108$) (Figure 2.6C).

2.3.4 Carboxyhaemoglobin (Table 2.2 and Figure 2.7):

Relative to baseline values, basal percentage of carboxyhaemoglobin prior to the CO-rebreathing procedure was significantly elevated at the end of the rHuEpo administration ($p < 0.001$). Percentage of carboxyhaemoglobin returned to pre-injection values 4 weeks post rHuEpo administration ($p = 0.851$).
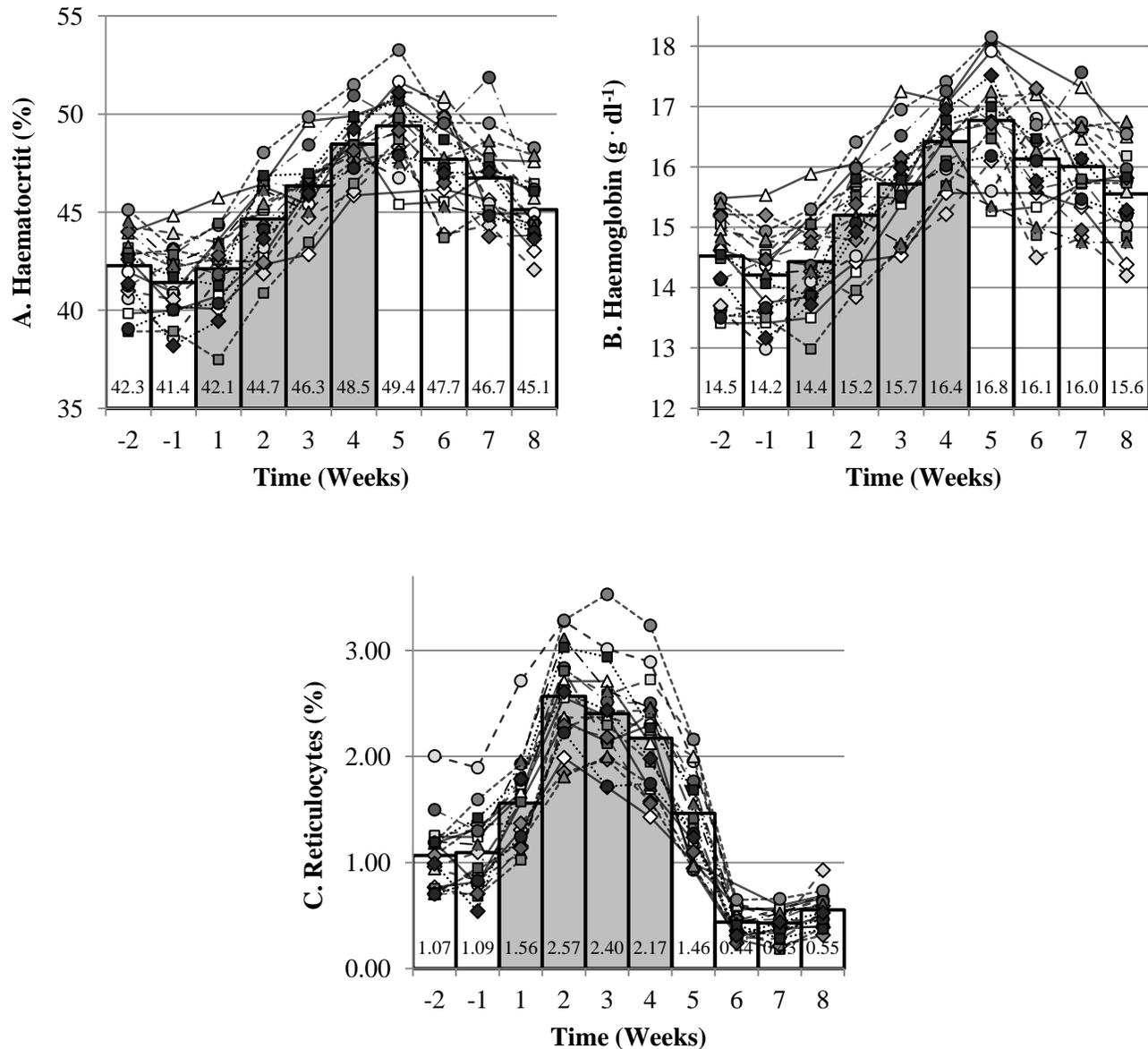
Figure 2.5: Individual changes in haematocrit (A), haemoglobin concentration (B) and reticulocytes (C).

Each line corresponds to one subject (n = 19) and each symbol corresponds to the same subject in all figures. Individual mean value was calculated when more than one blood sample was collected per week. The bar graphs represent the mean values of the 19 subjects. The grey bars represent the 4 weeks of rHuEpo administration.
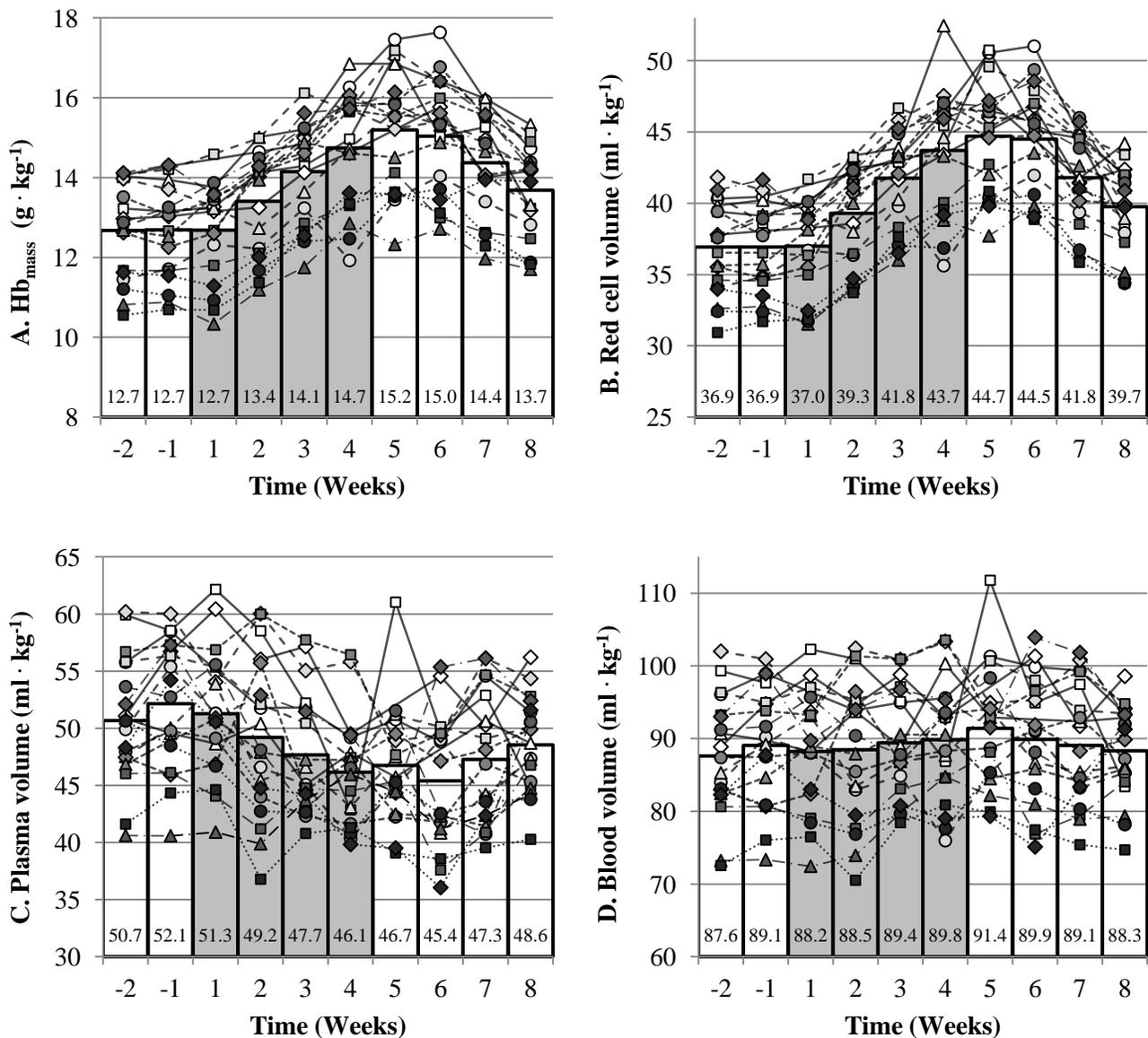
Figure 2.6: Individual changes in Hb_{mass} (A), red cell (B), plasma (C) and blood (D) volume.

Each line corresponds to one subject (n = 19) and each symbol corresponds to the same subject in all figures. Individual mean value was calculated when more than one blood sample was collected per week. The bar graphs represent the mean values of the 19 subjects. The grey bars represent the 4 weeks of rHuEpo administration.
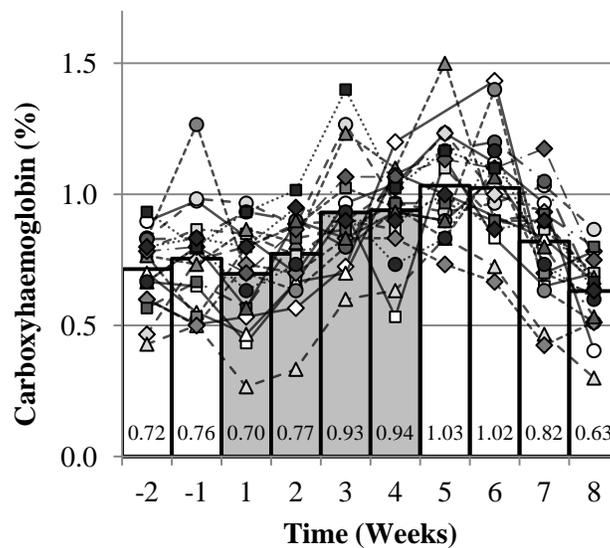
Figure 2.7: Individual changes in carboxyhaemoglobin.

Each line corresponds to one (n = 19) subject. Individual mean value was calculated when more than one blood sample was collected per week. The bar graphs represent the mean values of the 19 subjects. The grey bars represent the 4 weeks of rHuEpo administration.

Table 2.1. Running 3,000 m time trial performance and maximal oxygen uptake

	Group 1			Group 2		
	Baseline	End of rHuEpo	End of the study	Baseline	End of rHuEpo	End of the study
3,000 m total time (min:sec)	10:12 ± 0:42	9:40 ± 0:37[**]	9:53 ± 0:43[*]	12:05 ± 0:55	11:19 ± 0:53[***]	11:39 ± 0:58[**]
1st 1,000 m split (min:sec)	3:16 ± 0:13	3:10 ± 0:12[*]	3:13 ± 0:13	3:53 ± 0:27	3:36 ± 0:14[*]	3:41 ± 0:20
2nd 1,000 m split (min:sec)	3:26 ± 0:16[††]	3:15 ± 0:14[**†]	3:20 ± 0:14[††]	4:05 ± 0:17	3:54 ± 0:26[†]	3:59 ± 0:20[††]
3rd 1,000 m split (min:sec)	3:29 ± 0:15[†]	3:15 ± 0:13[**]	3:20 ± 0:16[**]	4:07 ± 0:16	3:49 ± 0:15[**††]	3:59 ± 0:19[††]
RPE scale (6-20)	18.0 ± 1.7	18.4 ± 0.9	19.0 ± 1.2	17.7 ± 2.2	18.4 ± 1.6	18.6 ± 1.4
$\dot{V}O_{2max}$ (mL·min^{-1}·kg^{-1})	60.3 ± 5.0	64.4 ± 3.9[*]	61.8 ± 3.4	51.6 ± 3.5	57.0 ± 5.1[***]	54.2 ± 4.7[**]

Group 1 included subjects who had a history of running (n = 9) and group 2 included the other subjects who were involved in other activities (n = 9). Values are means ± SD. Data were analyzed using repeated measures ANOVA (SPSS, version 19). Significant differences compared to baseline values are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Significant differences compared to the first 1,000 m split time within the same time trial are indicated by † $p < 0.05$, †† $p < 0.01$ and ††† $p < 0.001$. $\dot{V}O_{2max}$: Maximal oxygen uptake.

Table 2.2. Haematocrit, haemoglobin concentration, reticulocytes, haemoglobin mass, blood volumes, carboxyhaemoglobin and body mass before, during and 4 weeks post rHuEpo administration

	Baseline	Week 2 of rHuEpo	End of rHuEpo	Week 2 post rHuEpo	Week 4 post rHuEpo
Haematocrit (%)	41.9 ± 1.8	$44.7 \pm 2.0^{***}$	$49.2 \pm 2.0^{***}$	$47.7 \pm 2.2^{***}$	$45.1 \pm 1.7^{***}$
Haemoglobin (g·dl^{-1})	14.4 ± 0.7	$15.2 \pm 0.7^{***}$	$16.7 \pm 0.9^{***}$	$16.1 \pm 0.9^{***}$	$15.6 \pm 0.7^{***}$
Reticulocytes(%)	1.07 ± 0.31	$2.57 \pm 0.44^{***}$	$1.46 \pm 0.41^{**}$	$0.44 \pm 0.13^{***}$	$0.55 \pm 0.15^{***}$
Hb$_{mass}$ (g·kg^{-1})	12.7 ± 1.2	$13.4 \pm 1.3^{***}$	$15.2 \pm 1.5^{***}$	$15.1 \pm 1.4^{***}$	$13.7 \pm 1.1^{***}$
Hb$_{mass}$ (g)	947 ± 109	$1001 \pm 127^{***}$	$1131 \pm 131^{***}$	$1126 \pm 136^{***}$	$1023 \pm 132^{***}$
Blood volume (L)	6.6 ± 0.9	6.6 ± 1.0	6.8 ± 0.8	6.7 ± 0.9	6.6 ± 0.8
Red cell volume (L)	2.8 ± 0.3	$2.9 \pm 0.3^{***}$	$3.3 \pm 0.4^{***}$	$3.3 \pm 0.4^{***}$	$3.0 \pm 0.4^{***}$
Plasma volume (L)	3.8 ± 0.6	3.7 ± 0.7	$3.5 \pm 0.4^{**}$	$3.4 \pm 0.6^{***}$	3.6 ± 0.5
Carboxyhaemoglobin (%)	0.73 ± 0.15	0.77 ± 0.16	$1.04 \pm 0.19^{***}$	$1.01 \pm 0.20^{***}$	0.65 ± 0.15
Body mass (kg)	75.1 ± 8.4	74.7 ± 7.8	75.0 ± 7.9	74.6 ± 7.6	74.6 ± 7.5

N = 19. Values are means ± SD. Data were analyzed using repeated measures ANOVA (SPSS, version 19). Significant differences compared to baseline values are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Hb$_{mass}$: Haemoglobin mass.

Table 2.3. Resting blood pressure and heart rate before, during and 4 weeks post rHuEpo administration

	Baseline	Week 2 of rHuEpo	End of rHuEpo	Week 2 post rHuEpo	Week 4 post rHuEpo
Systolic blood pressure (mmHg)	128 ± 9	126 ± 7	126 ± 6	126 ± 7	124 ± 7
Diastolic blood pressure (mmHg)	70 ± 8	69 ± 8	69 ± 7	70 ± 7	69 ± 8
Heart rate (beats·min^{-1})	62 ± 8	60 ± 7	60 ± 7	59 ± 7	61 ± 8

N = 19. Values are means ± SD. Data were analyzed using repeated measures ANOVA (SPSS, version 19). No significant differences compared to baseline values were observed.

Table 2.4. Changes in haemoglobin mass, blood, red cell and plasma volume during and for 4 weeks post rHuEpo administration

	rHuEpo	Post rHuEpo
Haemoglobin mass (g·kg^{-1}·wk^{-1})	0.54 (0.46 to 0.63)***	-0.53 (-0.68 to -0.38)***
Blood volume (mL·kg^{-1}·wk^{-1})	0.34 (-0.36 to 1.05)	-0.60 (-1.20 to 0.00)
Red cell volume (mL·kg^{-1}·wk^{-1})	1.80 (1.50 to 2.10)***	-1.77 (-2.22 to -1.33)***
Plasma volume (mL·kg^{-1}·wk^{-1})	-1.30 (-1.94 to -0.66)***	0.50 (0.10 to 0.90)*

N = 19. Values are means (95% confidence interval). Changes over time were assessed using t-test to determine whether the slope of Hb$_{mass}$, blood, red cell and plasma volume was significantly different from zero; the slope coefficient for each individual came from a linear regression of his measurements, using time as the predictor. Significant changes are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

2.4 Discussion:

The aim of this study was to investigate the time course of Hb$_{mass}$ and related blood parameters as well as changes in running 3,000 m time trial performance following 4 weeks of rHuEpo administration in order to determine whether the augmented $\dot{V}O_{2max}$ would translate into actual improvements in running performance in the field. The main findings of this study are that, relative to baseline, running performance was significantly improved following 4 weeks of rHuEpo administration and remained significantly elevated 4 weeks post administration and that these performance effects coincided with significantly rHuEpo-induced elevated $\dot{V}O_{2max}$ and Hb$_{mass}$.

2.4.1 Running performance:

The effects of rHuEpo on exercise performance have principally been investigated using $\dot{V}O_{2max}$ tests. Despite slight differences in the frequency of injections and in the dosage used, rHuEpo administration for 4 to 6 weeks in healthy subjects has previously been shown to increase $\dot{V}O_{2max}$ by approximately 8% (Audran *et al.* 1999; Berglund and Ekblom 1991; Birkeland *et al.* 2000; Connes *et al.* 2003; Parisotto *et al.* 2000; Russell *et al.* 2002; Wilkerson *et al.* 2005), which was confirmed by our observations. However, $\dot{V}O_{2max}$ varies considerably among professional athletes (Lucia *et al.* 2002; Mujika and Padilla 2001) and $\dot{V}O_{2max}$ tests alone may probably be of little help in determining and ranking exercise performance when athletes of similar endurance ability are compared (Conley and Krahenbuhl 1980; Costill *et al.* 1973; Faria *et al.* 2005; Noakes 2008b; Shephard 2009). Time trial performance more closely reproduces competition conditions and more importantly allows the subjects to choose their own pace. It has previously been observed that blood doping via one unit of autologous blood transfusion, which increased the haematocrit by 5%, improved 10,000 m running performance by approximately one minute in 6 highly trained male distance runners, which corresponded to a ~3% improvement (Brien and Simon 1987). However, to our knowledge, no study has measured running time trial performance in order to determine whether the augmented $\dot{V}O_{2max}$ after rHuEpo administration would translate into actual improvements in performance in the field.

Relative to baseline, running performance improved post rHuEpo administration and remained enhanced 4 weeks after administration by approximately 6% and 3%, respectively. Following rHuEpo administration, subjects were able to maintain a faster pace throughout the 3,000 m time trial compared

to baseline. While the augmented oxygen transport capacity illustrated by the increase in Hb$_{mass}$ can explain the increase in $\dot{V}O_{2max}$ post rHuEpo administration, it cannot really explain the significant improvement in running performance. Indeed, as oxygen supply and demand are adequately matched during submaximal exercise in healthy men, the rHuEpo-induced augmented oxygen transport capacity cannot be the reason for the improvement in time trial running performance observed in our study (Noakes 2008a, b). In addition, failure to observe a strong correlation between individual changes in $\dot{V}O_{2max}$, Hb$_{mass}$ and running performance may imply that rHuEpo administration improves submaximal exercise performance by other mechanisms, although it could also be partly explained by the limited sensitivity of the methods of measurement and by the limitations of the study (see next paragraph). Other mechanisms than the increase in oxygen transport capacity have been proposed to explain the effects of rHuEpo on exercise performance (Boning *et al.* 2008, 2011). For instance, it has been demonstrated that self-reported mood, cognitive function and perceived physical condition were improved following rHuEpo administration (Miskowiak *et al.* 2008; Ninot *et al.* 2006). rHuEpo may indeed exert effects in nonhematopoietic tissues including the brain (Breidbach *et al.* 2003; Charrier *et al.* 2003; Jelkmann *et al.* 2009). Admittedly, two different studies conducted by the same research group did not reveal any measurable nonhematopoietic ergogenic effect of rHuEpo on exercise performance and the authors therefore concluded that the increased oxygen carrying capacity is the main if not the only reason why rHuEpo enhances exercise performance in healthy men (Chapman *et al.* 2011; Lundby *et al.* 2008b). On the other hand, the central governor model can give an alternative view (Noakes 2008b). As the perception of effort of the subjects did not change and because the subjects were not limited by an inadequate oxygen supply either before or after administration as they ran at submaximal levels, the central governor model would argue that the subjects ran faster after rHuEpo administration because of greater recruitment of motor units allowed for by a control mechanism of feed-forward and feedback to the brain (Noakes 2008b; especially Fig. 2 of that paper).

This study was part of a larger research project funded by WADA (see following chapters) and was designed primarily for other purposes. The present study was not blinded, did not include a control group and the subjects, whilst all were involved in endurance activities, were not all runners. As such, we were unable to adequately assess changes in the training load during the study due to the absence of a placebo group and the heterogeneity of sporting/training activity of the subjects. It is not impossible that the performance enhancements observed may, therefore, be partly due to placebo effect, to an

increase in the training load and to an improvement in pacing strategy, especially for the non-runners. It was, however, reassuring that the typical error of measurement for the two time trials conducted during each phase was low and that the amplitude of the improvement observed was well above the 95% confidence interval for the two attempts during each phase.

2.4.2 Haemoglobin mass and haematological parameters:

The level of Hb$_{mass}$ prior to the intervention reflected the regular participation in endurance activities of the subjects (Heinicke *et al.* 2001). Following the first week of rHuEpo administration, Hb$_{mass}$ gradually increased and reached its maximum one week after the last injection. The weekly increase of approximately 40 g is equivalent to the haemoglobin contained in one bag of 450 mL stored blood (Morkeberg *et al.* 2009). The delay of approximately one week between rHuEpo injections and the observed responses in Hb$_{mass}$ and red cell volume is explained by the reticulocyte maturation time of 1-4 days (Banfi 2008). While normal human red blood cells can theoretically persist in the circulation for approximately 17 weeks (Franco 2009), Hb$_{mass}$ and red cell volume started to decrease toward baseline values from approximately the second week post rHuEpo administration with a similar rate to that of the increase during administration. This decrease in Hb$_{mass}$ and red cell volume post rHuEpo administration entailed the haemolysis of circulating red blood cells and therefore the release of haem from haemoglobin (Belcher *et al.* 2010). The haem degradation is catalyzed by haem oxygenase and generates one molecule of CO per molecule of haem oxidized (Ryter and Otterbein 2004). The CO produced by haem metabolism represents more than 85% of the endogenous CO production in healthy non-smoking subjects (Ryter and Otterbein 2004). Although measurement of blood carboxyhaemoglobin is influenced by environmental factors such as air pollution (Franco 2009), carboxyhaemoglobin can be used as an index of haemolysis (Engel *et al.* 1971). Indeed, basal carboxyhaemoglobin levels increased at the end of the rHuEpo administration and remained elevated for 2 weeks post administration, reflecting accelerated haemolysis. Despite being only an indirect marker, these results are in agreement with the negative regulation of red cell mass by neocytolysis when supraphysiologic red cell mass and endogenous Epo suppression occur (induced by rHuEpo administration, for instance) (Chang *et al.* 2009; Rice and Alfrey 2005). In addition, this study confirmed that 4 weeks of rHuEpo administration sufficient to induce a significant increase in haematocrit did not increase resting blood pressure in healthy subjects when blood pressure was assessed by standard techniques compared to intra-arterial pressure transducers (Berglund and Ekblom 1991; Lundby and Olsen 2011; Lundby *et al.* 2008b;

Wilkerson *et al.* 2005). This study also demonstrates that the optimized CO-rebreathing method performed weekly for ten weeks is safe and well tolerated by healthy subjects.

2.4.3 Plasma volume:

Apart from confirming previous observations that rHuEpo administration increases haemoglobin concentration by increasing Hb$_{mass}$ and by decreasing plasma volume (Lundby *et al.* 2007; Olsen *et al.* 2011), the present study showed that plasma volume returned to pre-injection values only approximately 4 weeks post rHuEpo administration. Previous studies have concluded that plasma volume returned rather rapidly toward baseline value thus restoring pre-administration haemoglobin concentration values without affecting red cell volume (Lundby and Olsen 2011; Olsen *et al.* 2011). Olsen *et al.* (2011) used injections of 5,000 IU rHuEpo administered every second day for 2 weeks and thereafter only once a week for the 2 consecutive weeks to demonstrate that rHuEpo down-regulates the renin-angiotensin-aldosterone system as well as the rate of proximal renal tubular reabsorption and glomerular filtration. The longer and more contrasted rHuEpo administration regimen used in the present study with a more abrupt erythropoiesis stimulation termination may have induced a more profound down-regulation of the renin-angiotensin-aldosterone system and therefore, may explain why plasma volume had not been restored until 4 weeks after rHuEpo administration. In agreement with previous findings which used a similar rHuEpo regimen than the present study, pre-injection haemoglobin concentration values were not restored 4 weeks post administration (Parisotto *et al.* 2000).

2.4.5 Conclusion:

In conclusion, relative to baseline, running performance was significantly improved following 4 weeks of rHuEpo administration in trained men and remained significantly elevated 4 weeks after administration by approximately 6% and 3%, respectively. These performance effects coincided with significantly rHuEpo-induced elevated $\dot{V}O_{2max}$ and Hb$_{mass}$.

Chapter 3 : A Novel Transcriptomic Based Approach to the Detection of Recombinant Human Erythropoietin Doping

The Initial Cohort

3.1 Introduction and aim:

Administration of rHuEpo improves endurance performance (Durussel *et al.* 2013a; Thomsen *et al.* 2007). Hence rHuEpo is frequently subject to abuse by athletes as revealed by the declarations of confessed dopers such as Lance Armstrong (Walsh 2012) and Tyler Hamilton (Hamilton and Coyle 2012), although the use of rHuEpo is prohibited by WADA. The ABP was recently introduced as a new tool to indirectly detect erythropoiesis-stimulating agents, such as rHuEpo, which can lead to a doping sanction imposed by a sports organization or its tribunal at the same time as intelligently targeting athletes for direct testing (Schumacher and d'Onofrio 2012; Schumacher *et al.* 2012; Zorzoli and Rossi 2012). The ABP approach relies on identifying intra-individual abnormal variability over time of selected haematological parameters (Callaway 2011; Sottas *et al.* 2010). Although this approach appears to be suitable to identify abnormal enhanced erythropoiesis regardless of the method used, the sensitivity of the ABP has been questioned (Lippi and Plebani 2011). In order to induce only minor fluctuations in the ABP blood markers and/or to "normalize" these markers after blood manipulations such as autologous blood transfusion, athletes are now using the so-called "micro doses" of rHuEpo which allegedly range from 10 to a maximum of 40 IU·kg^{-1} body mass (Ashenden *et al.* 2011; Ashenden *et al.* 2006; Morkeberg *et al.* 2013). Indeed, Ashenden *et al.* have demonstrated that the ABP did not reveal any suspicious doping activities when a rHuEpo microdosing strategy was used (Ashenden *et al.* 2011). In addition, physiological variations of the haematological parameters of the ABP due to factors such as training or hypoxia exposure can conflict with the interpretation of the ABP results (Sanchis-Gomar *et al.* 2011). There is therefore a need for the ABP approach to be improved. Using omics technologies, a specific molecular signature of a particular stimulus (rHuEpo doping for instance) can be revealed. Molecular markers, such as gene markers, characteristic of rHuEpo doping have the potential to improve further the ABP (Reichel 2011a). The aim of the current study was to assess the effects of rHuEpo on blood gene expression profiles in order to identify a "molecular signature" of rHuEpo doping and hence provide a basis for the development of novel testing models to detect rHuEpo doping.

3.2 Methods:

3.2.1 Experimental design:

Nineteen healthy trained men (mean ± SD, age: 26.0 ± 4.5 yr, body mass: 74.8 ± 7.9 kg, height: 179.8 ± 5.4 cm) participated and completed the study. All subjects underwent a medical assessment and provided written informed consent to participate. The subjects were regularly engaged in predominantly endurance-based activities such as running, cycling, swimming, triathlon and team sports. Subjects were requested to maintain their normal training but abstain from official sporting competition for the duration of the research protocol (Howman 2013; Wagner 2013). This study was approved by University of Glasgow Ethics Committee and conformed to the Declaration of Helsinki. Each subject subcutaneously self-injected 50 IU·kg^{-1} body mass of rHuEpo (NeoRecormon, Roche, Welwyn Garden City, UK) every second day for 4 weeks. Daily oral iron supplementation (~ 100 mg of elemental iron, Ferrous Sulphate Tablets, Almus, Barnstaple, UK) was given during the 4 weeks of rHuEpo administration. As the sample capacity on Illumina HumanHT-12 v4.0 Expression BeadChips meant that the sample sizes should be a multiple of 3, the subject who suffered from nasal obstruction and coughing during the study and could not perform the time trials post rHuEpo administration was not included in the microarray experiment.

3.2.2 Blood sampling, RNA extraction and processing for microarray analysis (Figure 3.1):

After 10 min of rest in the supine position, 3 milliliters of whole blood were collected into Tempus Blood RNA tubes (Applied Biosystems, Foster City, USA) from an antecubital vein in triplicate at baseline, during rHuEpo administration and for 4 weeks after rHuEpo administration. The Tempus tubes were vigorously mixed immediately after collection, incubated at room temperature for 3 hours, and stored at -20°C before RNA extraction. Eight time points per subjects were selected for gene expression analysis, *i.e.* two baseline samples, then one sample each at 2 days, 2 weeks and 4 weeks after the first rHuEpo injection and 1 week, 2 weeks and 4 weeks after rHuEpo administration (Figure 2.1). RNA was extracted using the MagMAX for stabilized blood tubes RNA isolation kit (Ambion, Austin, USA). RNA yield was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and the RNA integrity number (RIN) was assessed using an Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, USA). The RIN is a value between 1 (totally degraded) and 10 (intact)

which describes the quality of the RNA sample. Samples with RIN below 7 are more likely to generate poor-quality arrays with low percentage of present calls (Kiewe *et al.* 2009). RNA was stored at -80°C until further analysis. The labelled cRNA was then produced using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, USA). The procedure consists in synthesizing cRNA by *in vitro* transcription from the cDNA produced by reverse transcription from 500 ng of RNA. For the Illumina microarray, 750 ng of the purified labeled cRNA samples were randomly hybridized to the Illumina HumanHT-12 v4.0 Expression BeadChip Kits following the manufacturers' recommended procedures (Illumina, San Diego, Canada). The Bead arrays were scanned on the Illumina BeadArray Reader and raw intensity values were saved in Illumina GenomeStudio software.
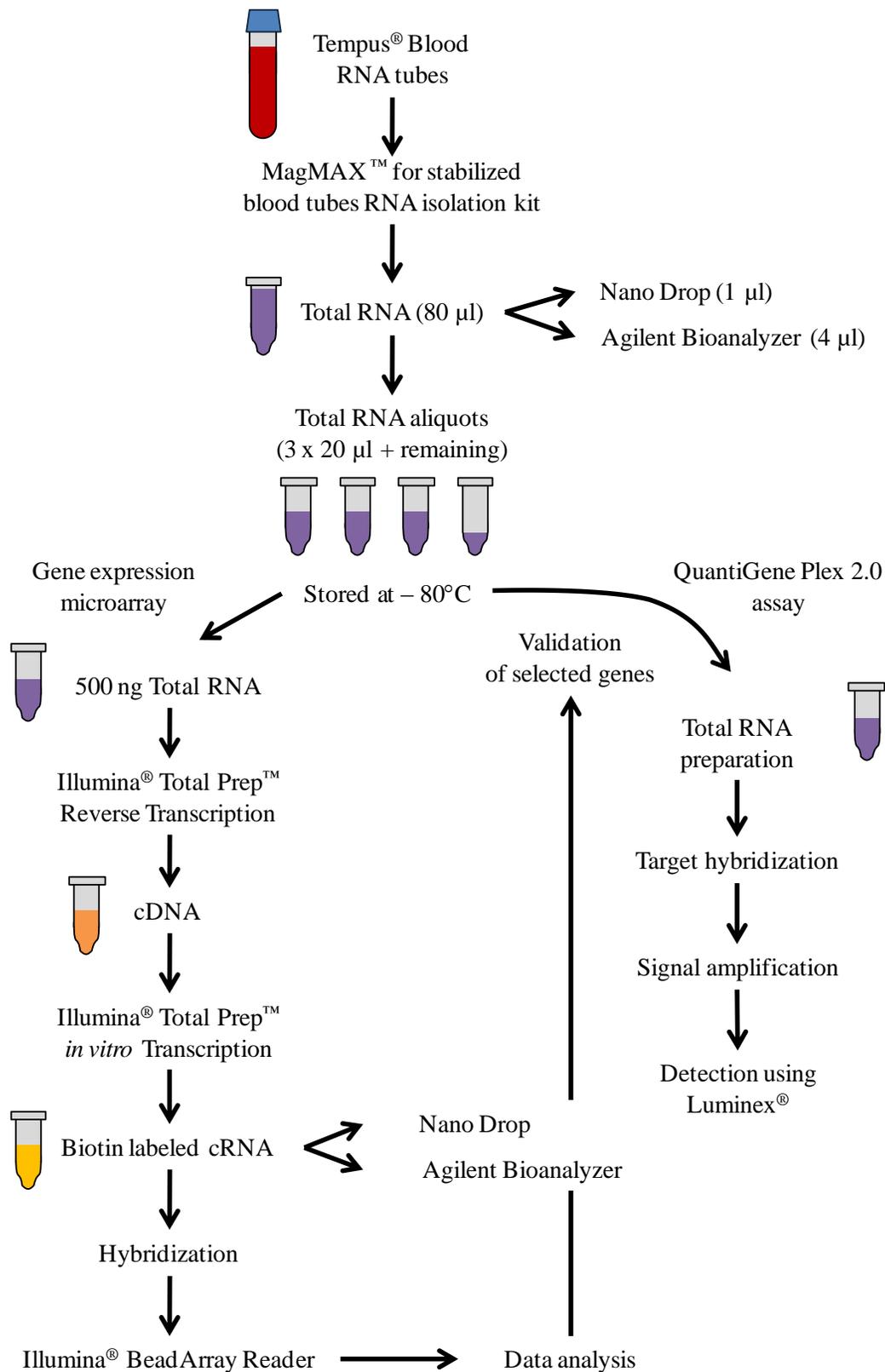
Figure 3.1: Molecular workflow.

3.2.3 Microarray data analysis:

Data were log2 transformed after adding a small constant of 51 to avoid negative values as well as to reduce the influence of probes with low signal intensities close to the background noise. The constant's value was chosen after inspection of scatter plots of the two baseline samples from each individual. To remove the array effect a linear model was fitted to the each gene's data using array as the predictor, and the coefficients for each array were removed from each value (Figure 3.2 and Figure 3.3). Rank Products analysis (Breitling *et al.* 2004) with a 5% false discovery rate (Benjamini and Hochberg 1995) was used to identify the differentially expressed transcripts during and post rHuEpo administration. An additional 1.5 fold-change threshold was applied for a more stringent analysis. Ingenuity Pathway Analysis was used to further analyze the data by incorporating biologically valid relationships (in a similar manner to Gene Ontologies).

3.2.4 Housekeeping genes:

In order to determine suitable housekeeping genes, transcripts were ranked according to three criteria: 1) high relative expression level, 2) low maximum fold change across the samples and 3) low variation over time using the Z score of generalized linear model. Rank products were used to determine the best housekeeping genes.
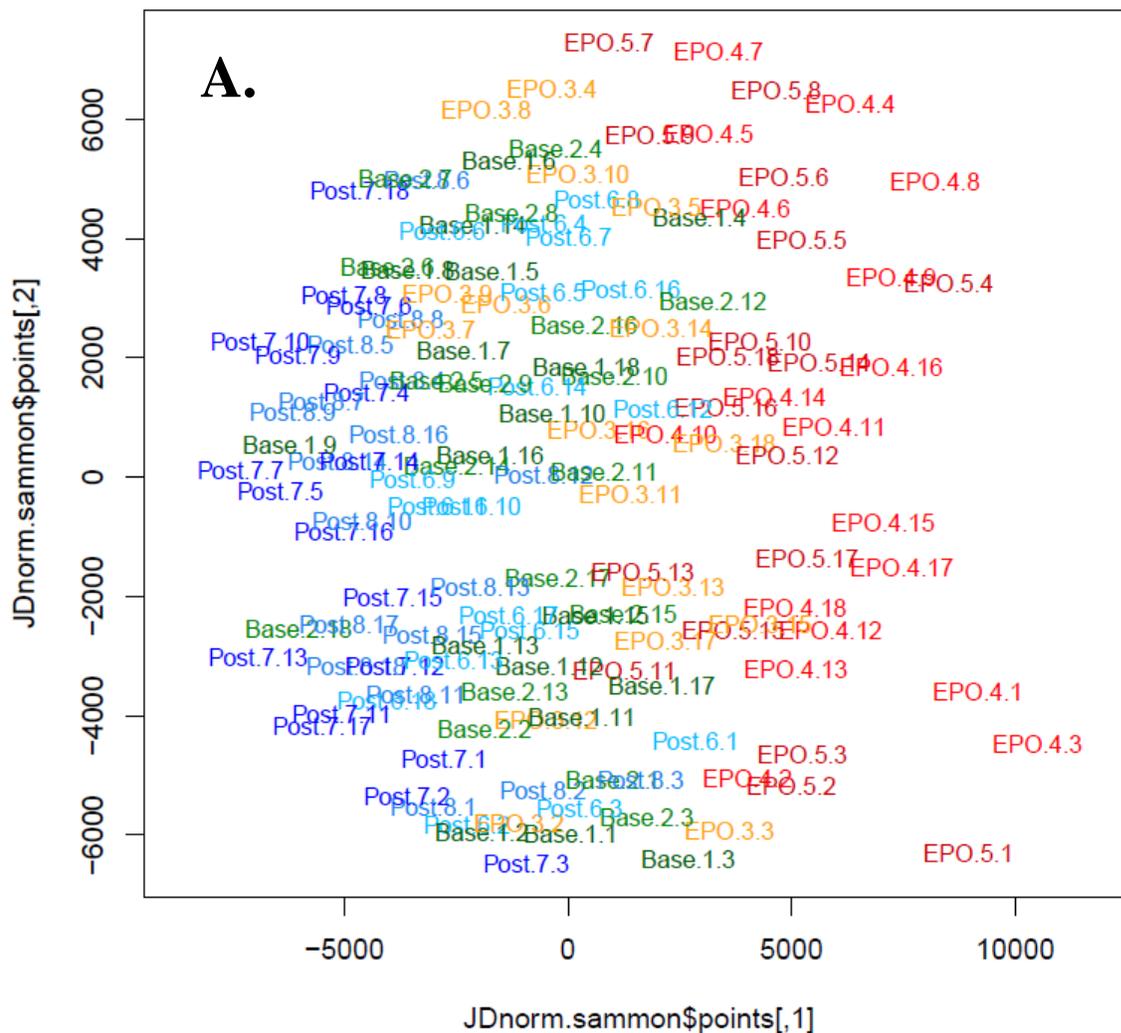
Figure 3.2: Principal component analysis (PCA) of the data before removing the array effect.

A) Each point is represented by the phase, time point and subject number (n = 18). The colours (dark green, green, orange, red, dark red, light blue, dark blue and blue) represent the 8 time points, respectively: two baseline (Base.1 and Base.2); three during rHuEpo administration, 2 days (EPO.3), 2 weeks (EPO.4) and 4 weeks (EPO.5) after the first rHuEpo injection; and three after rHuEpo administration, 1 week (Post.6), 2 weeks (Post.7) and 4 weeks (Post.8).
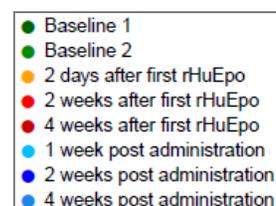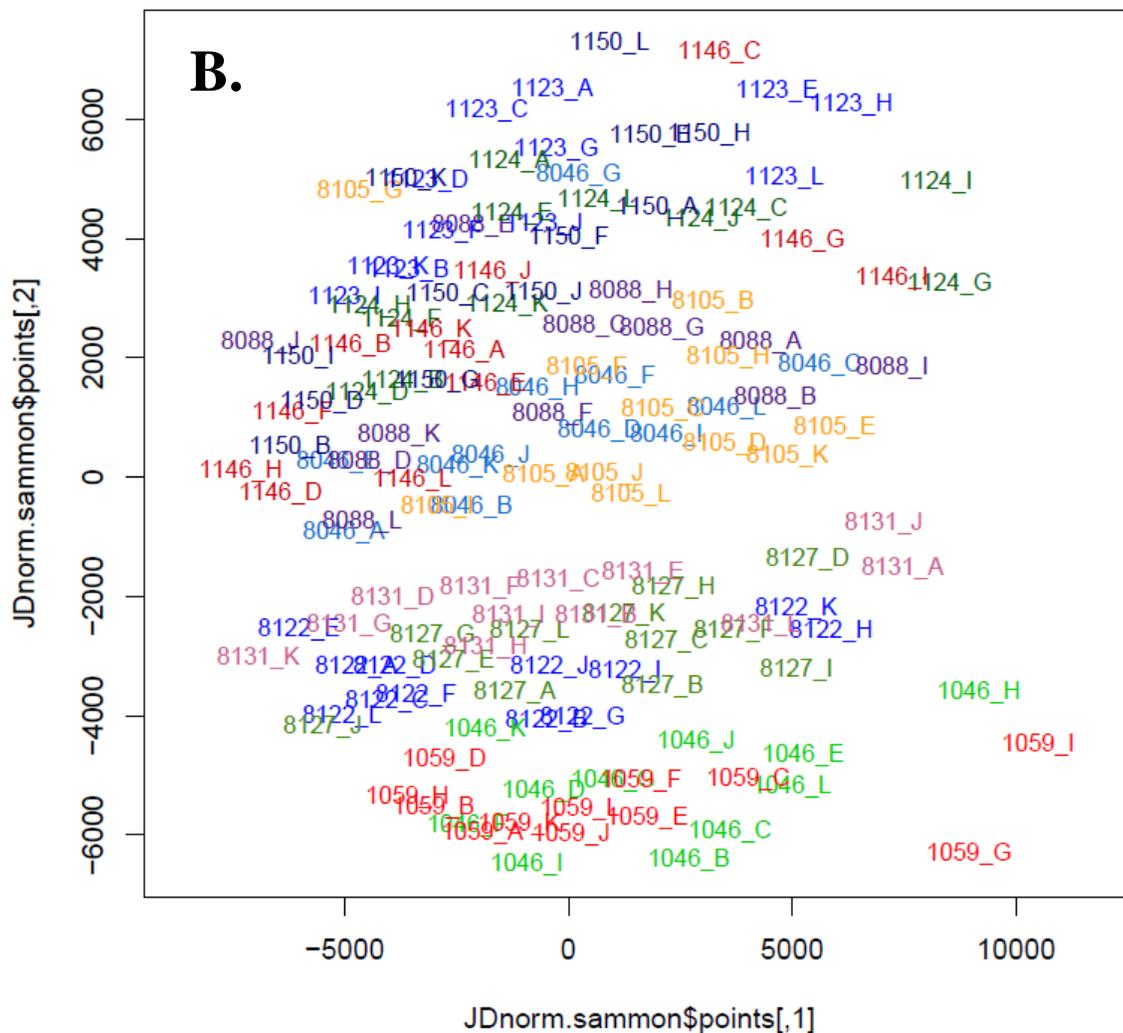
Figure 3.2 (continued):

B) Each point is represented by the array ID number and the array lane (n = 18 subjects x 8 time points). The 12 different colours represent the different arrays (n = 12). An array effect can be observed.
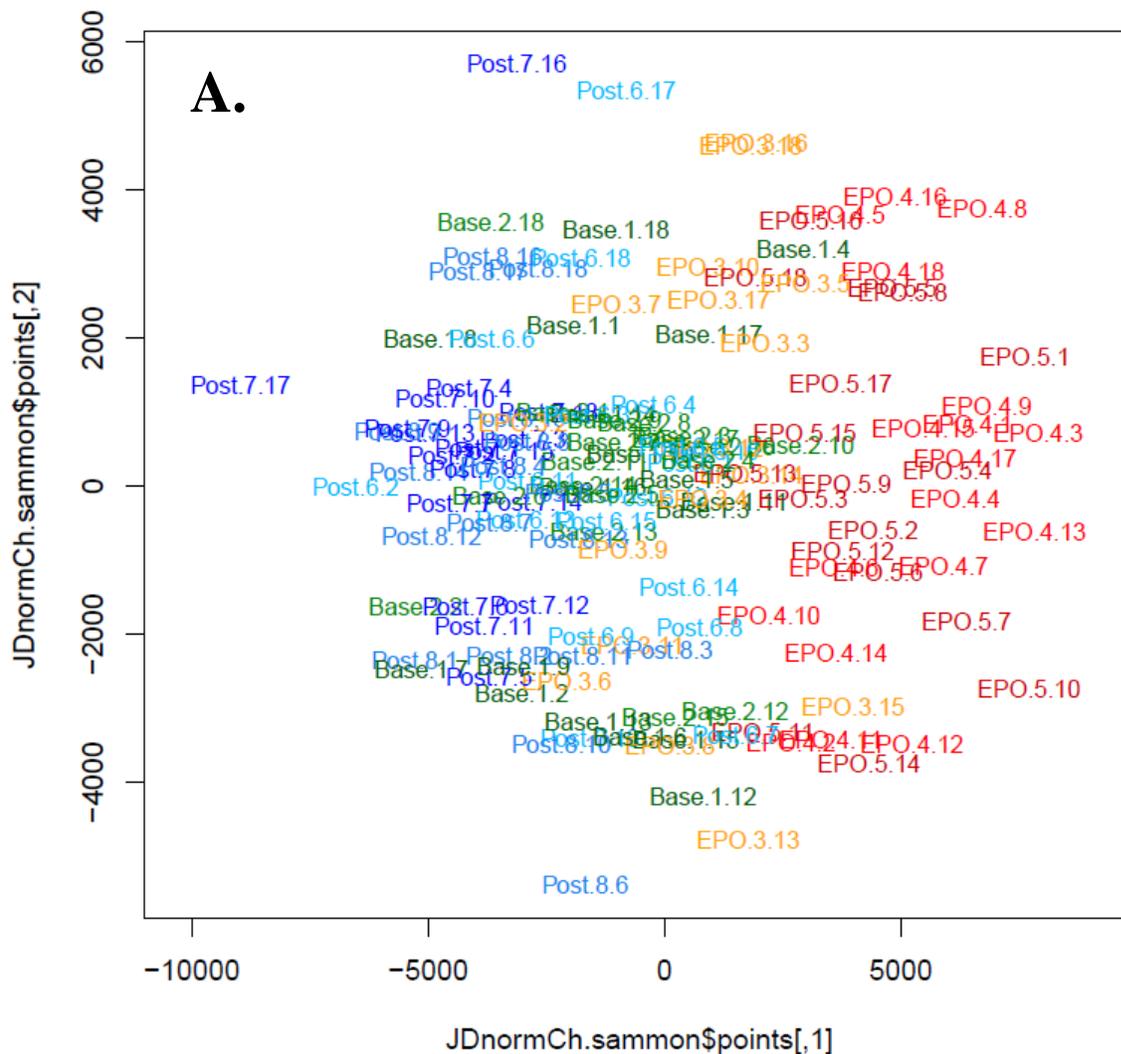
Figure 3.3: Principal component analysis (PCA) of the data after removing the array effect.

A) Each point is represented by the phase, time point and subject number (n = 18). The colours (dark green, green, orange, red, dark red, light blue, dark blue and blue) represent the 8 time points, respectively: two baseline (Base.1 and Base.2); three during rHuEpo administration, 2 days (EPO.3), 2 weeks (EPO.4) and 4 weeks (EPO.5) after the first rHuEpo injection; and three after rHuEpo administration, 1 week (Post.6), 2 weeks (Post.7) and 4 weeks (Post.8).
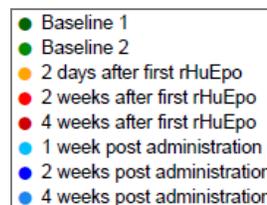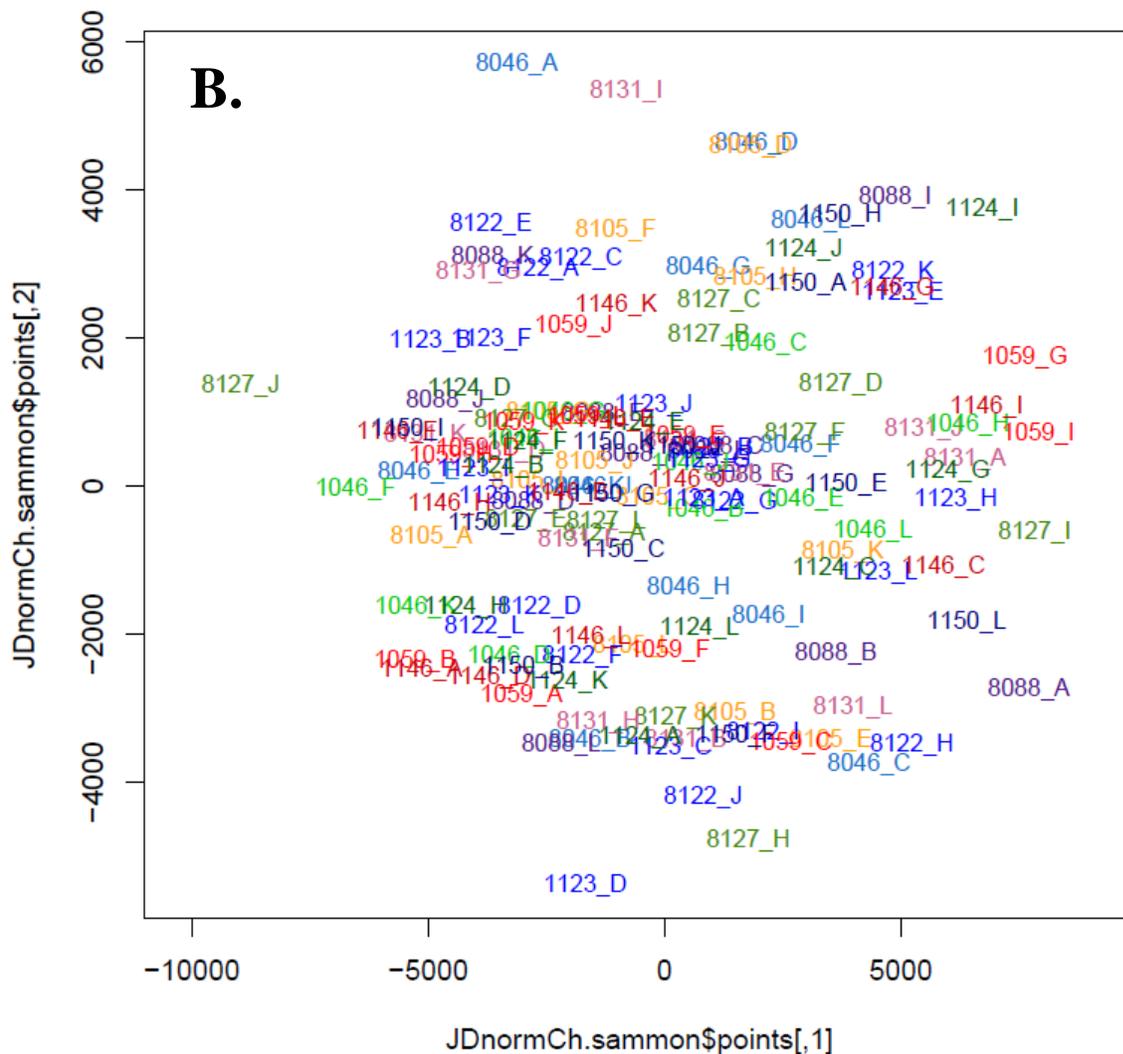
Figure 3.3 (continued):

A) Each point is represented by the array ID number and the array lane (n = 18 subjects x 8 time points). The 12 different colours represent the different arrays (n = 12). The array effect has been removed.

3.3 Results:

3.3.1 RNA yield and cRNA:

Relative to baseline (9.2 ± 2.9 μg), the RNA yield significantly increased during the last 2 weeks of rHuEpo administration (18.1 ± 7.0 μg, p < 0.001) and significantly decreased 2 and 4 weeks post rHuEpo administration (6.2 ± 2.2 μg and 7.0 ± 1.9 μg, p = 0.003 and p = 0.034), respectively (Figure 3.4). There was a good agreement between RNA yield and reticulocyte percentage (Figure 3.5 and Figure 2.5). The average RIN was 8.3 ± 1.1 (Figure 3.6). The quality of the cRNA samples was assessed by electrophoresis (Agilent 2100 Bioanalyzer, Agilent technologies, Santa Clara, USA) and as expected for RNA isolated from whole blood, the labelled cRNA profile showed a typical high concentration of globin mRNA (Figure 3.6). As a qualitative observation, the peak of globin mRNA was usually more prominent in samples collected during rHuEpo administration while it was less prominent post rHuEpo administration (see Appendix D).

3.3.2 Transcripts altered by rHuEpo administration:

Using the additional 1.5 fold-change threshold, 392 (389 up- and 3 down-regulated) transcripts were found to be differentially expressed during the last 2 weeks of rHuEpo administration compared to baseline (Figure 3.7). It is worth noting that 41 of these transcripts were found to be already up-regulated 2 days after only one single rHuEpo injection and remained so throughout administration (Figure 3.7). 135 (down-regulated) transcripts were differentially expressed at both 2 weeks and 4 weeks after the last rHuEpo injection (Figure 3.8). 133 transcripts were found to be differentially expressed in the same way during (up-regulated) and subsequently post (down-regulated) rHuEpo administration (Figure 3.9 and Figure 3.10). Using Ingenuity Pathway Analysis, the discovered genes were found to be mainly related to either the functional or structural properties of the erythrocyte or to the cell cycle and its regulation (see Appendix C for the list of these genes).
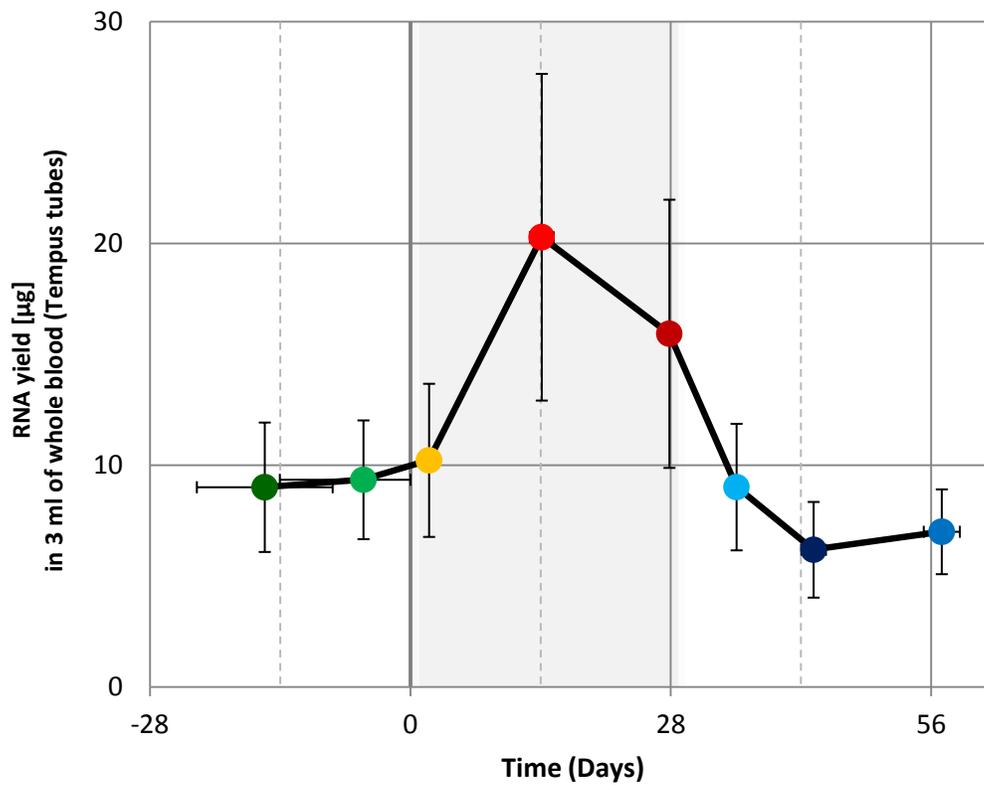
Figure 3.4: Changes in total RNA yield extracted from 3 ml of whole blood stabilized in Tempus tubes (n = 18 subjects x 8 time points).

Values are means ± SD. Time point 0 represents the first rHuEpo injection. The grey zone represents the 4 weeks rHuEpo administration.
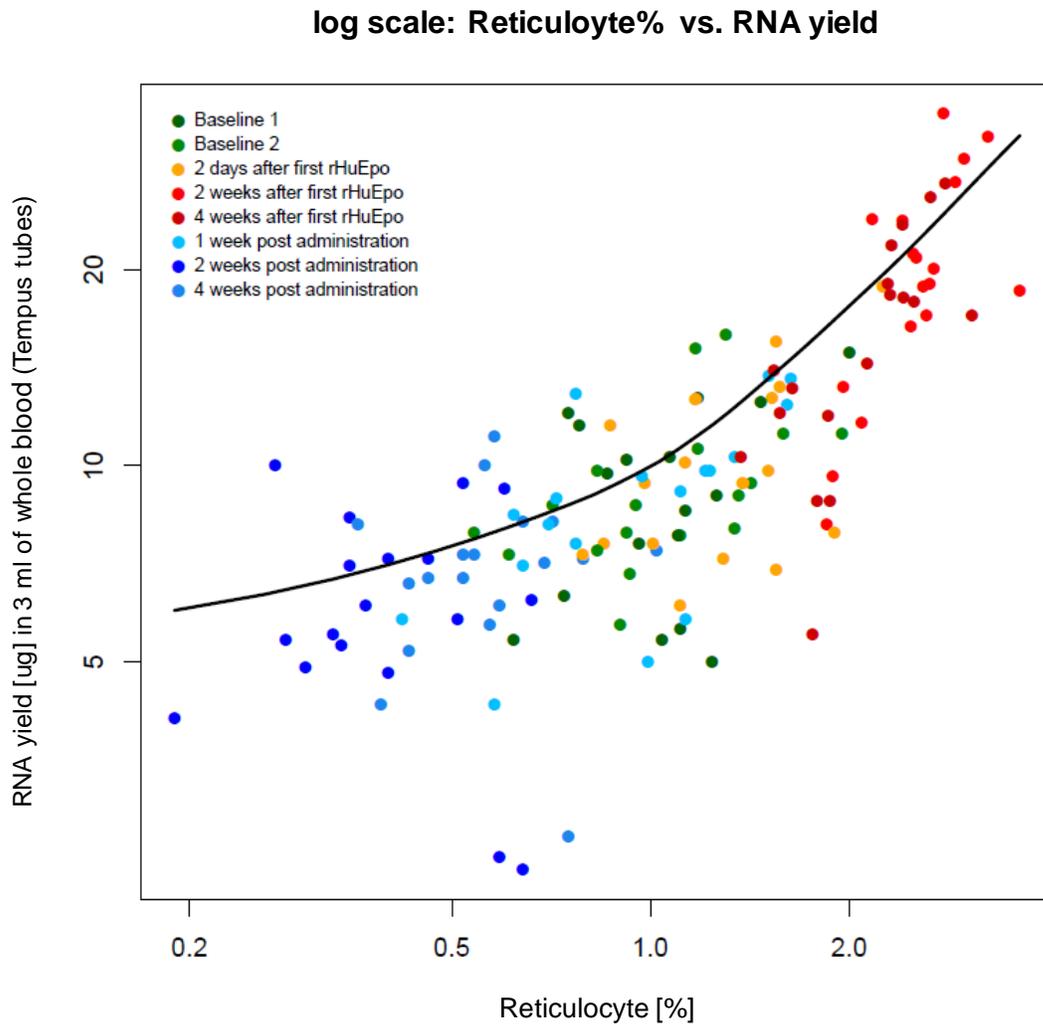
Figure 3.5: Relationship between reticulocyte percentage and RNA.

Each point represents one sample (n = 144) collected from 18 subjects at the 8 different time points which are represented by the different colours. The curve was fitted using polynomial regression.
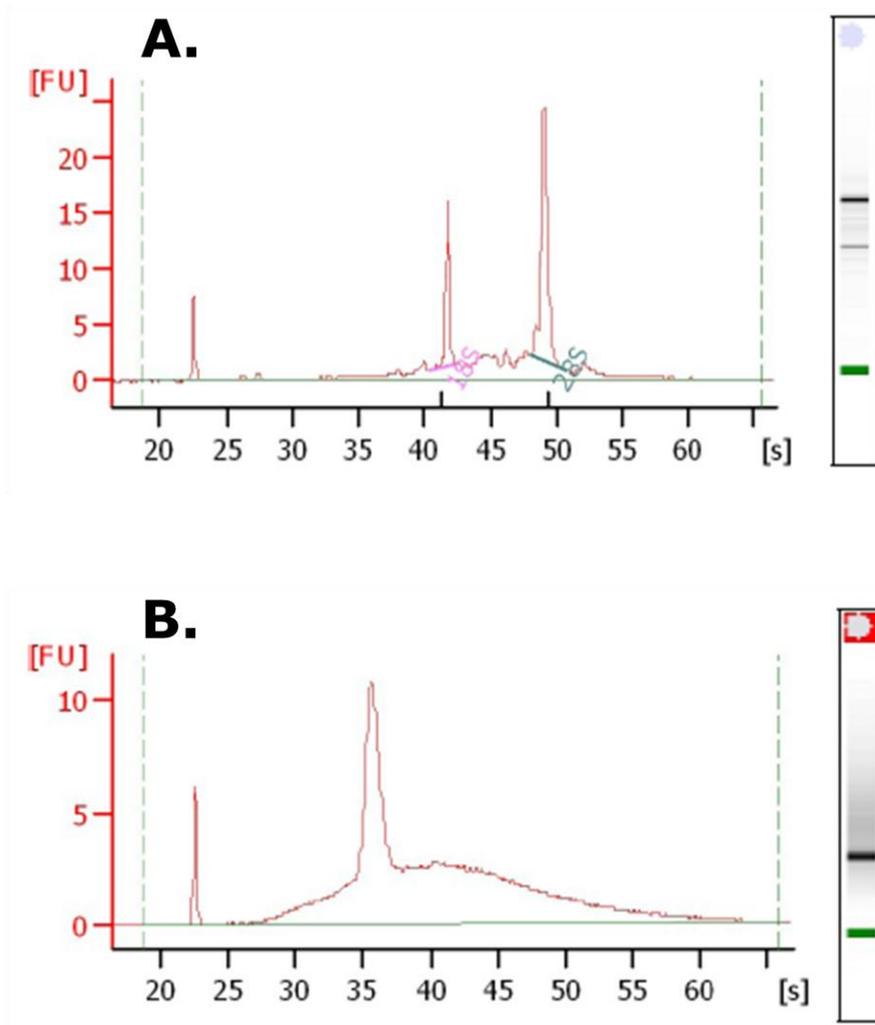
Figure 3.6: Quality assessment of total RNA and cRNA.

A representative electropherogram (Agilent 2100 Bioanalyzer, Agilent technologies, Santa Clara, USA) of total RNA (A) and cRNA (B).
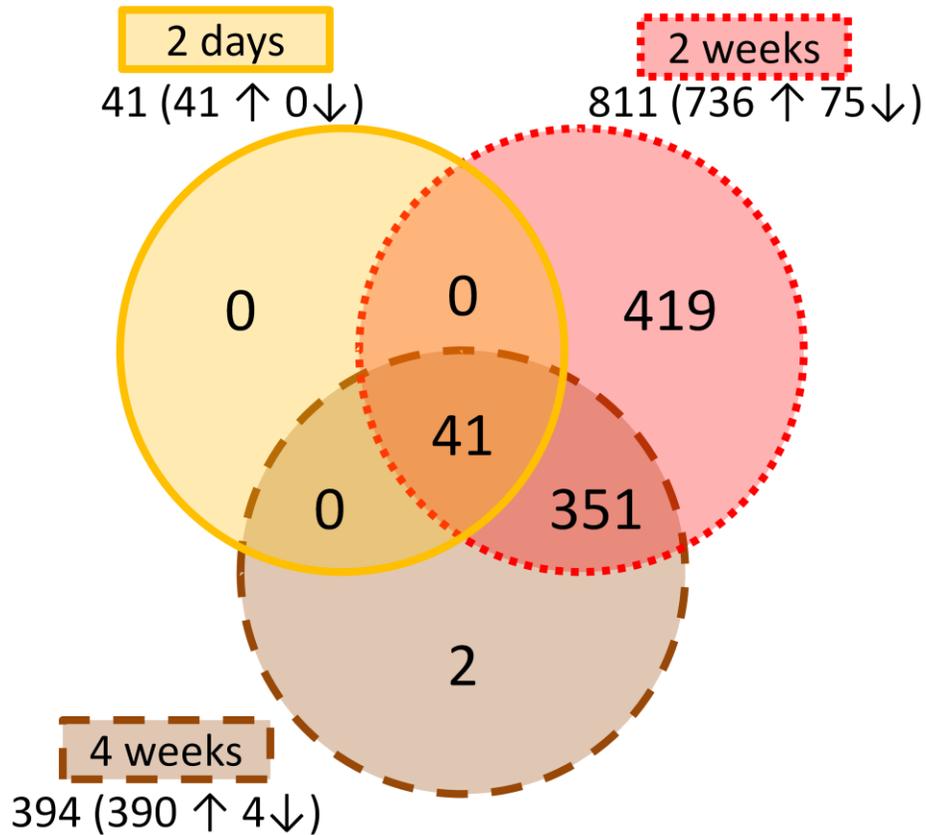
Figure 3.7: Venn diagram of transcripts differentially expressed during rHuEpo compared to baseline (n = 18 subjects).

The Venn diagram depicts the transcripts differentially expressed (5% false discovery rate and 1.5 fold-change threshold) 2 days (yellow, solid line circle), 2 weeks (red, dotted line circle) and 4 weeks (brown, dashed line circle) after the first rHuEpo injection compared to baseline, respectively. Values in brackets are number of transcripts up- (↑) and down-regulated (↓) compared to baseline.
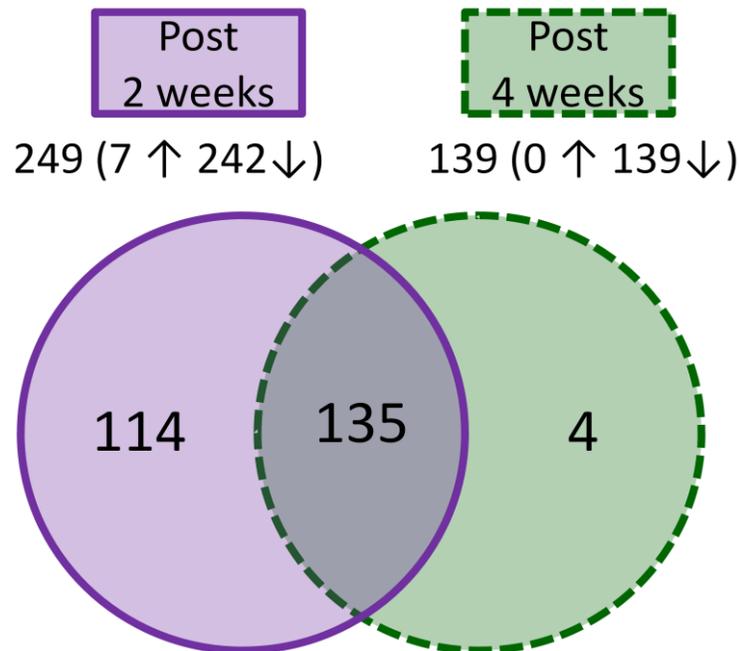
Figure 3.8: Venn diagram of transcripts differentially expressed post rHuEpo compared to baseline (n = 18 subjects).

The Venn diagram depicts the transcripts differentially expressed (5% false discovery rate and 1.5 fold-change threshold) 2 weeks (purple, solid line circle) and 4 weeks (green, dashed line circle) post rHuEpo administration compared to baseline, respectively. Values in brackets are number of transcripts up- (↑) and down-regulated (↓) compared to baseline.
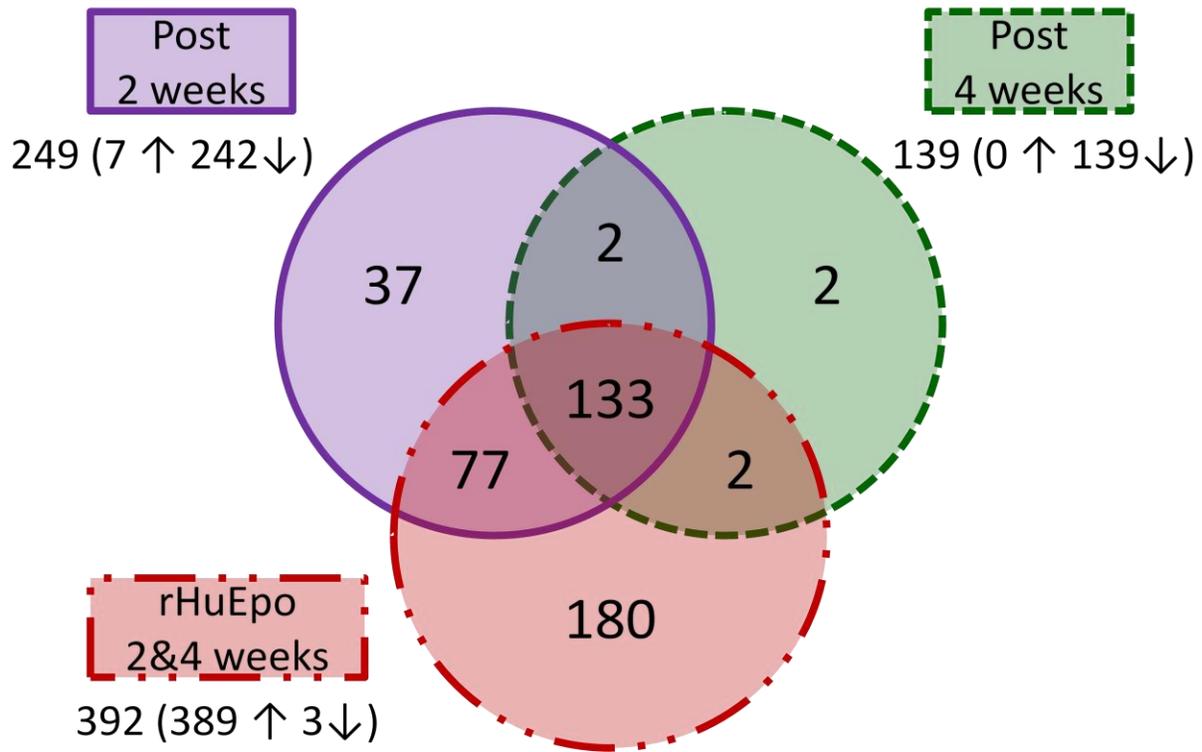
Figure 3.9: Venn diagram of transcripts differentially expressed during and post rHuEpo compared to baseline (n = 18 subjects).

The Venn diagram depicts the transcripts differentially expressed (5% false discovery rate and 1.5 fold-change threshold) during the last 2 weeks of rHuEpo (dark red, dashed and dotted line circle) as well as 2 weeks (purple, solid line circle) and 4 weeks (green, dashed line circle) post rHuEpo administration compared to baseline, respectively. Values in brackets are number of transcripts up- (↑) and down-regulated (↓) compared to baseline.
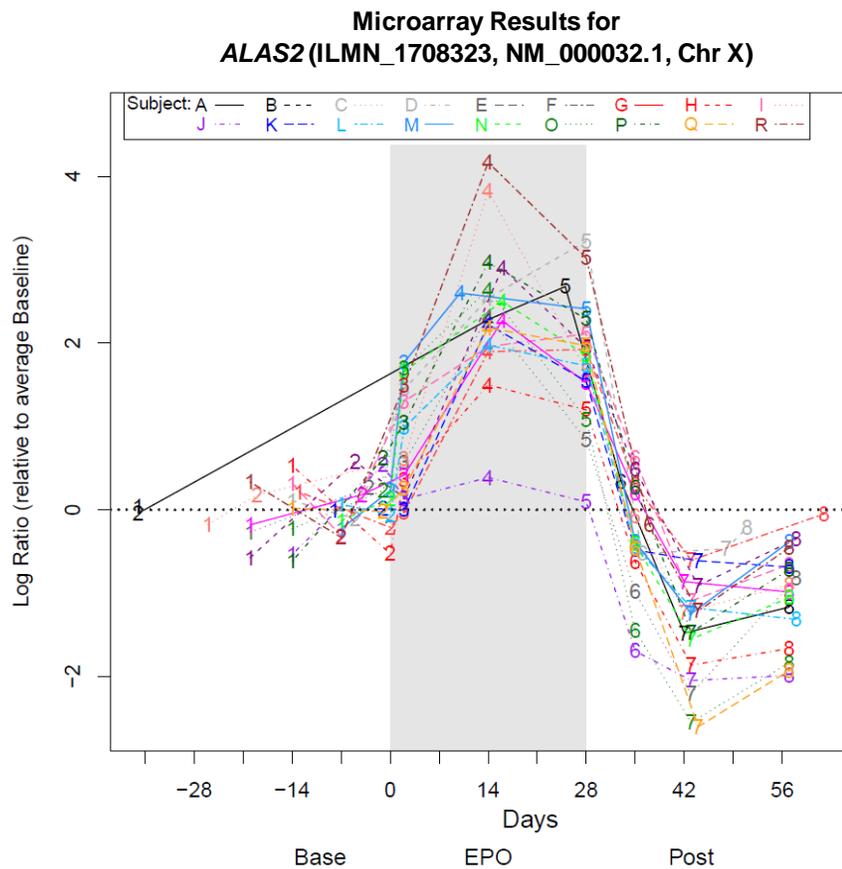
Figure 3.10: Typical blood gene expression profile following rHuEpo.

Individual changes in gene expression in Aminolevulinic acid synthase 2 (*ALAS2*) gene, typical of dozens of genes differentially expressed following rHuEpo. ALAS2 catalyzes the initial step in the haem pathway (Sadlon *et al.* 1999). Changes are reported in log ratio compared to the average baseline values. Time point 0 represents the first injection. The grey zone represents the rHuEpo administration. Each line corresponds to one subject (n = 18). Each number corresponds to the sample time point.

3.4 Discussion:

In the present study, we successfully used gene expression profiling in whole blood to identify genes that are differentially regulated following rHuEpo administration in trained male subjects. For instance, 133 transcripts were strongly up-regulated during rHuEpo and subsequently down-regulated up to 4 weeks after administration in all subjects. In addition, 41 transcripts were sensitive to rHuEpo injections as they were found to be already differentially expressed 2 days after only one single rHuEpo injection. These transcripts are therefore promising candidates to detect microdose rHuEpo doping strategies used by athletes (Ashenden *et al.* 2011; Ashenden *et al.* 2006). By providing the initial blood "molecular signature" of rHuEpo doping, the results of the present study suggest that longitudinal changes in the expression of specific biomarker genes have the potential to add a new dimension to the ABP to detect rHuEpo doping. However, these promising initial results remain to be replicated and validated. This will be considered in Chapters 5 and 6.

3.4.1 RNA yield and cRNA

The increase and subsequent decrease in total RNA yield during and post rHuEpo administration, respectively, is likely to be directly related to the changes in reticulocytes. Reticulocytes contain RNA which is progressively lost during the differentiation into mature red blood cells (Sysmex 2010). Of note, this feature enables the measurement of reticulocytes by flow cytometry (Sysmex 2010; Tanke *et al.* 1983). However, the changes in RNA yield in the present study did not influence downstream analysis as the total RNA input was standardised prior to amplification and hybridization (*i.e.* 500 and 750 ng of RNA and cRNA, respectively). It has been shown that a high proportion of globin mRNA reduced the sensitivity of detection of transcripts in a microarray experiment and that using globin reduction methods in the preparation of cRNA can reduce this negative impact (Liu *et al.* 2006; Raghavachari *et al.* 2009; Thach *et al.* 2005; Vartanian *et al.* 2009). Nevertheless, the effect of removing globin mRNA on the overall microarray performance seems to be relatively modest. Firstly, the vast majority of the transcripts were commonly detected between methods regardless of whether globin mRNA was removed or not (Debey *et al.* 2006; Liu *et al.* 2006; Raghavachari *et al.* 2009; Vartanian *et al.* 2009). Secondly, not removing globin mRNA generates highly reproducible data (Vartanian *et*

al. 2009). Thirdly, good housekeeping genes whose expression was consistent throughout the study were found (see Chapters 5 and 6).

3.4.2 A first insight into the functions of the genes altered by rHuEpo administration:

The function of the genes altered by rHuEpo will be discussed in more detail in the following chapters. However, in order to give a first insight, it was observed that a large number of these genes were unsurprisingly related to the structural or functional properties of haemoglobin or erythrocytes. Of note, haemoglobin alpha and beta were not included in the analysis because of the overabundance of their mRNA. Aminolevulinic acid synthase 2 (ALAS2) catalyzes the initial step in the haem pathway. In order to accommodate the rHuEpo induced increased demand for haem molecules which assembled with globin chains form haemoglobin, ALAS2 expression increased (Sadlon *et al.* 1999). A similar explanation of the changes in expression is applicable to the following genes and related proteins which are all found in abundance in erythrocytes. Haemoglobin delta (HBD) is part of the adult haemoglobin type A2 ($\alpha_2\delta_2$) which represents 2.5 to 3.5% of Hb$_{mass}$ (Mosca *et al.* 2009). Haemoglobin epsilon 1 (HBE1) is part of the embryonic haemoglobins Gower 1 and 2 and is then normally supplanted by fetal and adult haemoglobin (Manning *et al.* 2007). The erythrocyte membrane protein band 4.2 (EPB42) is an abundant protein component of the erythrocyte membrane and is involved in maintaining the membrane organization and structure in erythrocytes (Satchwell *et al.* 2009). Carbonic anhydrase 1 (CA1) is a member of the carbonic anhydrase family which catalyzes the reversible hydration/dehydration reaction between carbon dioxide and bicarbonate (Imtaiyaz Hassan *et al.* 2012). CA1 is the most abundant non-haemoglobin protein in erythrocytes (Imtaiyaz Hassan *et al.* 2012). Solute carrier family 4 anion exchanger member 1 (SLC4A1) is the major integral membrane protein of erythrocytes and serves to increase the total carbon dioxide carrying capacity of the blood (Perrotta *et al.* 2005). Alpha haemoglobin stabilizing protein (AHSP) is an abundant erythroid protein that binds with free alpha haemoglobin and forms a stable complex (Kihm *et al.* 2002; Weiss *et al.* 2005).

Interestingly, *CA1* and *HBD*, as well as other genes related to erythrocyte functions which were differentially expressed during rHuEpo such as 2,3-bisphosphoglycerate mutase (*BPGM*), ferrochelatase (*FECH*), glycophorin B (*GYPB*), erythrocyte membrane protein band 4.1 (*EPB41*)

and haemoglobin mu (*HBM*) were also found to be differentially expressed in sickle cell disease patients compared to healthy controls (Raghavachari *et al.* 2009). BPGM occurs only in the erythrocyte and is a multifunctional enzyme which plays a key role in the dissociation of oxygen from haemoglobin (Fujita *et al.* 1998). FECH catalyzes the final steps in the haem pathway (Sadlon *et al.* 1999). GYPB is an erythrocyte membrane protein which bears antigens of blood groups (Blumenfeld and Huang 1995). Like EPB42, EPB41 is protein of the erythrocyte membrane involved in cytoskeletal connectivity (Satchwell *et al.* 2009). *HBM* transcript represents approximately 0.1% of the normal adult alpha globin in erythroid tissues (Goh *et al.* 2005).

The action of erythropoietin was at first thought to be limited to the regulation of erythropoiesis (Jelkmann *et al.* 2009). However, it has been shown that erythropoietin also has biological functions in nonhematopoietic tissues including the brain (Brines and Cerami 2005; Jelkmann *et al.* 2009). Several studies demonstrated and confirmed that erythropoietin is involved in the mechanisms of neuroprotection in hypoxic-ishaemic, traumatic, excitotoxic and inflammatory injuries, modulation of nitric oxide synthesis and neurotransmitter release, as well as protection of the blood-brain barrier against permeability and inflammation induced by vascular endothelial growth factor (Brines and Cerami 2005). In addition, it has been demonstrated that self-reported mood, depression, memory, cognitive function and perceived physical condition were improved following rHuEpo administration (Miskowiak *et al.* 2008; Miskowiak *et al.* 2007; Miskowiak *et al.* 2009, 2010; Miskowiak *et al.* 2012; Ninot *et al.* 2006). Furthermore, promising recent results showed that erythropoietin therapy, by its neuroprotective and neurogenerative action, can be effective against pathophysiological features of schizophrenia and other brain diseases (Charrier *et al.* 2003; Fuchs *et al.* 2008; Kastner *et al.* 2012; Sargin *et al.* 2010; Venda *et al.* 2010; Wustenberg *et al.* 2011). Although the relation should be considered with caution, several genes which were strongly up-regulated during rHuEpo and subsequently down-regulated up to 4 weeks after administration seemed to be related to the nervous system and/or cognitive function. Synuclein alpha (SNCA), which was found to be highly expressed in the brain, is suggested to play a role in the regulation of dopamine production and transport as well as in neurodegenerative disorders such as Parkinson's disease (Fuchs *et al.* 2008; Miller *et al.* 2004; Venda *et al.* 2010). Although SNCA was initially thought to be "expressed only in nervous

system tissue" (Maroteaux *et al.* 1988), it has recently been demonstrated that SNCA was also highly present in the erythrocyte (Bartels *et al.* 2011; Fauvet *et al.* 2012; Scherzer *et al.* 2008). In particular, *SNCA* and haem metabolism genes such as *ALAS2* and *FECH* (see above) were found to be co-induced by the transcription factor GATA1 (Scherzer *et al.* 2008). Based on these observations, the authors hypothesized that haem metabolism may be a missing link between alpha-synuclein aggregation and iron deposition, which are two common events of some neurodegenerative disorders (Scherzer *et al.* 2008). Dihydropyrimidinase-like 5 (DPYSL5) or collapsin response mediator protein 5 (CRMP5) is a member of the CRMP family (Charrier *et al.* 2003). CRMPs are strongly expressed in the developing nervous system and are thought to play a role in the different events of brain development, myelination and adult neuronal plasticity (Charrier *et al.* 2003). Of note, CRMPs (including CRMP5) protein level was found to be increased in hypoxia-ischemia mice brain (Zhou *et al.* 2008). Polymorphisms in lamin (LMNA), which is involved in nuclear processes such as replication and transcription (Goldman *et al.* 2002) and near the serpin peptidase inhibitor clade A member 13 (SERPINA13) were found to be associated with cognitive function in a British elderly population (Cluett *et al.* 2010). However, this finding was not confirmed in a Chinese group (Yeh *et al.* 2011). Selenium binding protein 1 (SELENBP1) was found to be increased in blood and brain of patients with schizophrenia and is potentially associated with growth and remodeling of neuritis (Kanazawa *et al.* 2008).

3.4.3 Conclusion:

In the present study, we demonstrated that blood gene expression profiles were profoundly and significantly altered during rHuEpo administration and for at least 4 weeks after the rHuEpo administration leading to the initial "molecular signature" of rHuEpo doping. A large number of the identified genes were related to the functional or structural properties of the erythrocyte. These very promising initial data suggest that omics technologies such as gene expression have the potential to add a new dimension to the ABP for rHuEpo detection. However, these results still need to be replicated and validated (see Chapters 5 and 6).

Chapter 4 : Replication in Another Cohort (part 1):

Blood Parameters and Running Performance in Kenyan Runners Living and Training at Altitude after Recombinant Human Erythropoietin Administration

4.1 Introduction:

Administration of rHuEpo increases blood oxygen carrying capacity and endurance performance in normoxic environments. It has been shown that 4 to 6 weeks of rHuEpo administration increased $\dot{V}O_{2max}$ by ~8% (Audran *et al.* 1999; Berglund and Ekblom 1991; Birkeland *et al.* 2000; Connes *et al.* 2003; Durussel *et al.* 2013a; Parisotto *et al.* 2000; Russell *et al.* 2002; Wilkerson *et al.* 2005) as well as submaximal cycling capacity at 80% of $\dot{V}O_{2max}$ by an astonishing ~54% using time to exhaustion (Thomsen *et al.* 2007) and submaximal running performance by ~6% using 3,000 m time trial (Durussel *et al.* 2013a). While these performance studies using rHuEpo are difficult to compare given the variation in exercise protocols and rHuEpo administration regimes between studies, a 2 to 6% improvement in middle-distance (1,500-5,000 m) running performance is probably a reasonable estimate for the ergogenic effect of rHuEpo (Amann *et al.* 2008; Hopkins *et al.* 1999). Although caution is required when extrapolating these findings to the elite athlete, it should be noted that only a small fraction of this improvement would likely be seen as a worthwhile performance enhancement (Hopkins *et al.* 1999). Hence, rHuEpo is, allegedly, frequently subject to abuse by athletes, even though the use of rHuEpo is prohibited by WADA and that the ergogenic effects of rHuEpo in top elite performance have yet to be scientifically confirmed.

When evaluating the ergogenic effects of rHuEpo during normobaric hypoxia, the relative improvement in $\dot{V}O_{2max}$ induced by rHuEpo administration was greater at a simulated altitude up to 3,500 m compared to normoxia, and consequently, the progressive decrease of $\dot{V}O_{2max}$ due to hypoxia was blunted up to this "altitude threshold" (Robach *et al.* 2008). On the other hand, above 3,500 m, $\dot{V}O_{2max}$ remained unaltered by rHuEpo administration (Lundby and Damsgaard 2006; Robach *et al.* 2008). Administration of rHuEpo increased the arterial oxygen content regardless of the severity of hypoxia, however, peak oxygen delivery directed to the exercising legs was only enhanced during normoxia (Robach *et al.* 2008). This blood flow redistribution may explain why $\dot{V}O_{2max}$ is increased during normoxia but not during severe hypoxia. However, it remains unknown why the relative improvement in $\dot{V}O_{2max}$ was more than doubled (*i.e.* 17.5% vs. ~8%) at a moderate altitude up to 3,500 m compared to normoxia (Robach *et al.* 2008). It may be noteworthy to mention that non-haematological mechanisms such as augmented buffer

capacity, mood improvement and placebo effect have all been proposed to account for the observed changes in exercise performance in response to either hypoxia or rHuEpo administration (Boning *et al.* 2008, 2011; Gore *et al.* 2007; Lundby *et al.* 2012a; Miskowiak *et al.* 2008; Ninot *et al.* 2006). Further insights into the interaction between rHuEpo administration, hypoxia exposure and exercise performance are needed to better understand the underlying mechanisms.

In response to the extraordinary success of the Kenyan and Ethiopian middle- and long-distance runners since the 1968 Mexico City Olympics, several factors have been investigated in order to explain this east African running supremacy including the fact that most of these runners live and train at an altitude of approximately 2,000 to 2,500 m in eastern Africa (Onywera *et al.* 2006; Wilber and Pitsiladis 2012). For instance, the homeland of the Kalenjin tribe, which is known to produce the vast majority of the best runners in Kenya, is situated at 1,830 to 2,450 m (Wilber and Pitsiladis 2012). Although chronic hypobaric and acute normobaric hypoxia can lead to very different physiological responses, it can be speculated that athletes residing at moderate-altitude may benefit even more from rHuEpo administration compared to sea-level athletes based on the potential enhanced effects of rHuEpo on $\dot{V}O_{2max}$ in this altitude range (Robach *et al.* 2008). As illustrated by the recent first positive test for rHuEpo in a Kenyan runner who previously won the Seoul marathon, Kenya is not spared by doping cases (BBC 2013a). In this part of the world where champions are continuously emerging and where being a successful runner is associated with substantial socioeconomic rewards, athletes are subjected to an environment with extreme pressure to perform (Wilber and Pitsiladis 2012). This specific social, psychological and economical situation may increase the likelihood of doping behaviour (Ehrnborg and Rosen 2009). The ergogenic effect of rHuEpo administration associated with hypoxia has only been investigated using acute normobaric hypoxia with volunteers living at or near sea-level (Lundby and Damsgaard 2006; Robach *et al.* 2008). Hence, it remains unknown whether similar effects in haematological parameters and exercise performance will be found in athletes exposed chronically to moderate altitude. Furthermore, in the anti-doping field, the ABP uses intra-individual abnormal variability over time of selected haematological parameters in order to indirectly detect erythropoiesis-stimulating agents, such as rHuEpo (Callaway 2011; Sottas *et al.* 2010). In this context, knowledge of the haematological response following rHuEpo

administration in athletes living and training at moderate altitude is required. Therefore, the main purpose of the present study was to define the time course of changes in haematological parameters, $\dot{V}O_{2max}$ and running time trial performance following 4 weeks of rHuEpo administration in Kenyan endurance runners from the Kalenjin tribe who live and train at moderate altitude (~2,150 m) and abstained from official sporting competition for the entire duration of the study (Howman 2013; Wagner 2013). These results were compared with observations made in the Caucasian cohort living and training at or near sea-level that underwent an identical rHuEpo administration regime (Chapter 2) (Durussel *et al.* 2013a).

4.2 Methods:

4.2.1 Subjects:

Twenty Kenyan endurance runners from the Kalenjin tribe based at moderate altitude (Eldoret, Kenya, 2,150 m, mean ± SD, age: 26.4 ± 4.1 yr, body mass: 56.8 ± 4.7 kg, height: 171.8 ± 6.4 cm) participated in the study. This cohort (KEN) was compared with a previously published cohort (SCO) of nineteen Caucasian endurance trained males based at or near sea-level (Glasgow, Scotland, age: 26.0 ± 4.5 yr, body mass: 74.8 ± 7.9 kg, height: 179.8 ± 5.4 cm) that underwent an identical rHuEpo regime (Chapter 2) (Durussel *et al.* 2013a). All subjects underwent a medical assessment and provided written informed consent to participate. Subjects were requested to maintain their normal training but abstain from official sporting competition for the duration of the research study (Howman 2013; Wagner 2013). This study was approved by the Ethics Committees of Moi University (Kenya) and University of Glasgow (Scotland, UK) and conformed to the Declaration of Helsinki. Diresibachew Haile Wondimu carried out the data collection in the Kenyan cohort (see Acknowledgment).

4.2.2 Experimental design:

Each subject subcutaneously self-injected under supervision 50 IU·kg^{-1} body mass of rHuEpo (NeoRecormon, Roche, Welwyn Garden City, UK) every second day for 4 weeks. Saline injections substituted rHuEpo when the predetermined safety haematocrit limit of 55% was reached by one subject in KEN on five occasions (days 14, 16, 18, 20 and 22 after the first injection). Thereafter, the subject resumed the standard rHuEpo regimem. Daily oral iron supplementation (~ 100 mg of elemental iron, Ferrous Sulphate Tablets, Almus, Barnstaple, UK) was given during the 4 weeks of rHuEpo administration. Venous blood samples from an antecubital vein were obtained in triplicate at baseline, during rHuEpo administration and for 4 weeks after rHuEpo administration as previously described (Durussel *et al.* 2013a). All blood samples were taken after 10 min of rest in the supine position (Ahlgrim *et al.* 2010). Blood samples were then measured in triplicate using a Sysmex XT-2000i (Sysmex UK, Milton Keynes, UK). The mean value of the triplicate was reported. OFF score was calculated as follows:

$$\text{haemoglobin concentration } \left(\text{g·L}^{-1}\right) \text{ - } 60\sqrt{\text{reticulocytes (\%)}} \text{ (Gore } \textit{et al. } 2003\text{)}.$$

Resting blood pressure and heart rate were recorded three times on both arms in the supine position before blood sampling. Due to practical reasons, the CO-rebreathing method (Schmidt and Prommer 2005) could not be set up for this present study and, unlike in SCO, Hb_{mass} was unfortunately not measured in KEN. Urine osmolality was measured using a portable osmometer (OsmoCheck, Vitech, UK).

4.2.3 Running performance assessment:

Two 3,000 m time trials separated by at least one day rest were performed on a 400 m outdoor athletics track (Chepkoilel Stadium, Eldoret, Kenya) at an altitude of 2,150 m pre, post rHuEpo administration and at the end of the study (Figure 4.1). Verbal encouragement was given with feedback provided for the split time and remaining laps. The best performance on each occasion was used for analysis. The typical error of measurement for time trial performance calculated from the two tests on each phase was 1.1% (95% confidence intervals 0.9 to 1.3). Borg's rating of perceived exertion (RPE) (Borg 1970) was recorded at the completion of the time trial. Temperature, humidity and wind speed were recorded via portable hygrometer and anemometer. In addition to the time trial, $\dot{V}O_{2max}$ was determined pre, post rHuEpo administration and at the end of the study using an incremental test to exhaustion on a motorised treadmill. Subjects were familiarized to run on the treadmill at all intensities on the day of the test. After 15 minutes of rest, the speed initially set at 9 $km \cdot h^{-1}$ for 4 min with an incline of 1% was then increased by 1 $km \cdot h^{-1} \cdot min^{-1}$ to reach 17 $km \cdot h^{-1}$. Thereafter, the speed remained constant and the incline was increased by 2.5% $\cdot min^{-1}$ until volitional exhaustion. Following a 30 min recovery, $\dot{V}O_{2max}$ was verified using a square-wave protocol to exhaustion at a speed equivalent to the end incline attained during the incremental test (Kirkeberg *et al.* 2011; Pettitt *et al.* 2013). Gas exchange variables were measured breath by breath using an automated metabolic gas analysis system (Cosmed K4b2 or FitMate, Cosmed, Rome, Italy). One subject did not perform the exercise performance tests at the end of the study because of an injury, while four subjects were unable to perform the $\dot{V}O_{2max}$ tests due to flooding of the laboratory and serious damage to equipment. Nineteen and fifteen subjects were therefore included for the statistical analysis of running time trial performance and $\dot{V}O_{2max}$, respectively.

200 m indoor athletics track, Kelvin Hall, Glasgow, Scotland, UK

400 m outdoor athletics track, Chepkoilel Stadium, Eldoret, Kenya, at an altitude of 2,150 m.

Figure 4.1: Pictures of the athletics tracks in Scotland (top) and Kenya (bottom) where the 3,000 m running time trials took place.

4.2.4 Statistical analysis:

The mean was calculated when more than one blood sample was collected per week before further analysis. Time trial performance, $\dot{V}O_{2max}$ and blood parameters data for the key stages of the study were analyzed using repeated measures ANOVA with Bonferroni correction for multiple comparisons. Relationships between haemoglobin concentration, time trial performance and $\dot{V}O_{2max}$ were assessed using the Pearson's product moment correlation coefficient. Data are described as mean ± SD. When values of the two groups are compared, the results of KEN precede SCO.

4.3 Results:

4.3.1 Haematological parameters (Table 4.1, Table 4.2, Figure 2.5, Figure 4.2 and Figure 4.3):

Table 4.1 illustrates the changes in the main haematological parameters for five key stages of the study in both groups. Haematocrit and haemoglobin concentration were significantly higher in KEN compared to SCO at baseline ($p < 0.001$ and $p = 0.004$, respectively) (Figure 4.3A and Figure 4.3B). However, although haematocrit and haemoglobin concentration significantly increased in both groups following rHuEpo ($p < 0.001$), KEN and SCO reached similar maximum values approximately one week after the rHuEpo administration ($p = 0.638$ and $p = 0.958$, respectively) (Figure 4.3A and Figure 4.3B). Reticulocyte percentage followed a similar pattern in both groups. From similar baseline values ($p = 0.146$), reticulocyte percentage increased rapidly after the first two injections ($p < 0.001$), and attained higher values in SCO compared to KEN during the rHuEpo administration ($p = 0.003$) (Figure 4.3C). Nadir was reached approximately two weeks after the last rHuEpo injection with similar values in both groups ($p = 0.101$), which were significantly lower compared to baseline values ($p < 0.001$) (Figure 4.3C). However, while the reticulocyte percentage remained decreased four weeks after the last rHuEpo injection compared to baseline in SCO ($p < 0.001$), pre-injection levels were restored in KEN ($p = 0.106$) (Figure 4.3C). OFF score significantly decreased during rHuEpo ($p < 0.001$) and then increased post administration ($p < 0.001$) in both groups (Figure 4.3D). However, OFF score in KEN was significantly elevated at baseline and at the middle point of rHuEpo administration compared to SCO ($p = 0.004$ and $p = 0.001$, respectively) (Figure 4.3D). Interestingly, in contrast to the mean group results, one Kenyan subject (white triangle and line highlighted in red) had a distinctive haematological response to rHuEpo administration (Figure 4.2). His haematocrit and haemoglobin concentration remained relatively stable throughout the study and the response in reticulocyte percentage was blunted. Table 4.2 illustrates the changes in the resting systolic and diastolic blood pressure as well as in resting heart rate for five key stages of the study in both groups. KEN had slightly lower baseline resting systolic blood pressure ($p = 0.005$) and heart rate ($p = 0.024$) compared to SCO but similar diastolic blood pressure ($p = 0.122$). No major changes were observed after rHuEpo administration in resting systolic and diastolic blood pressure as well as in resting heart rate. Urine osmolality remained relatively constant throughout the study period (378 ± 193 mOsmol·kgH$_2$O^{-1}, $p = 1.000$).
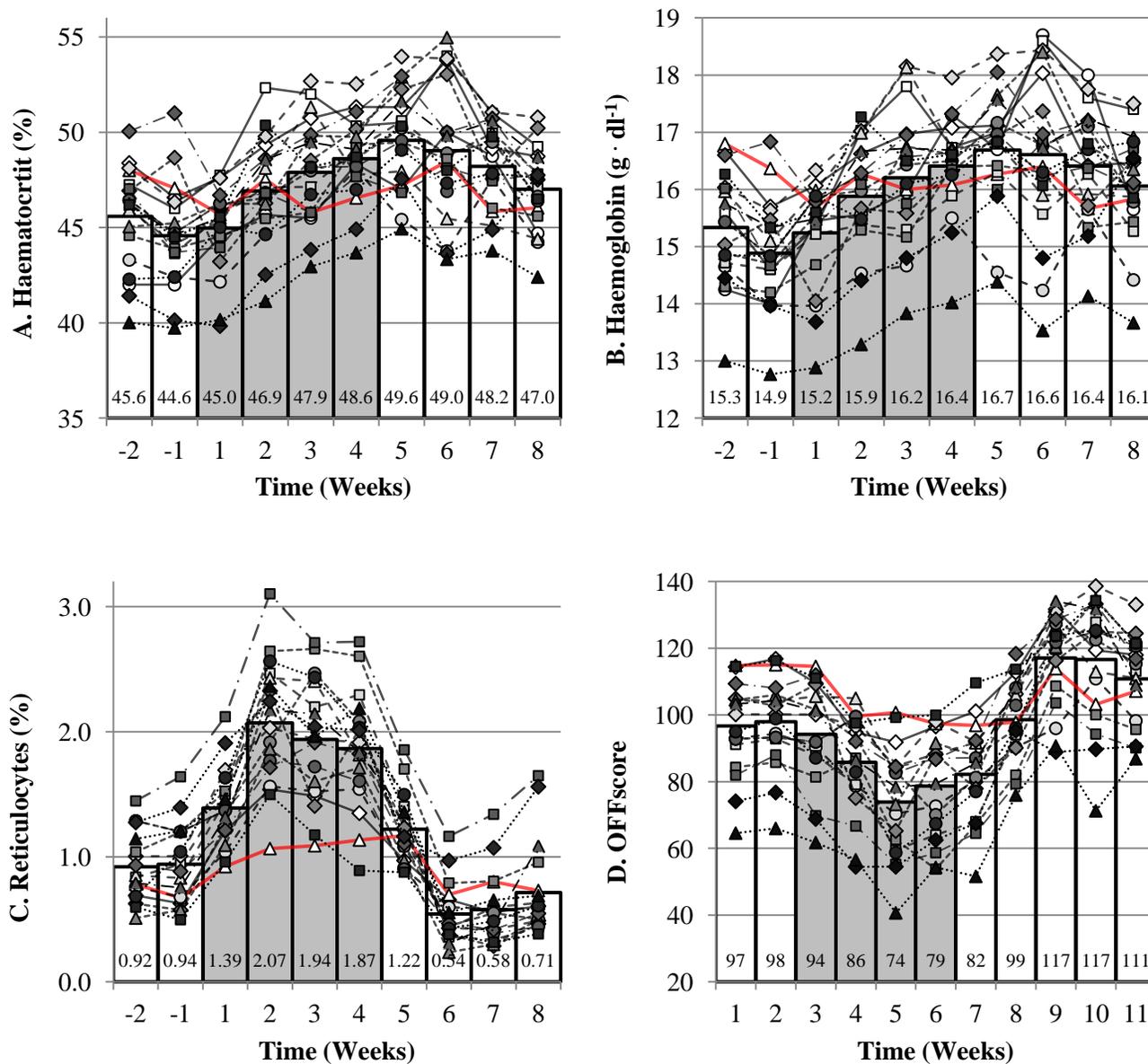
Figure 4.2: Individual changes in haematocrit (A), haemoglobin concentration (B), reticulocytes (C) and OFF score (D) in KEN.

Each line corresponds to one subject (the "non-responder" is highlighted in red) and each symbol corresponds to the same subject in all figures (n = 20). Individual mean value was calculated when more than one blood sample was collected per week. The bar graphs represent the mean values of the 20 subjects (KEN). The grey bars represent the 4 weeks of rHuEpo administration.
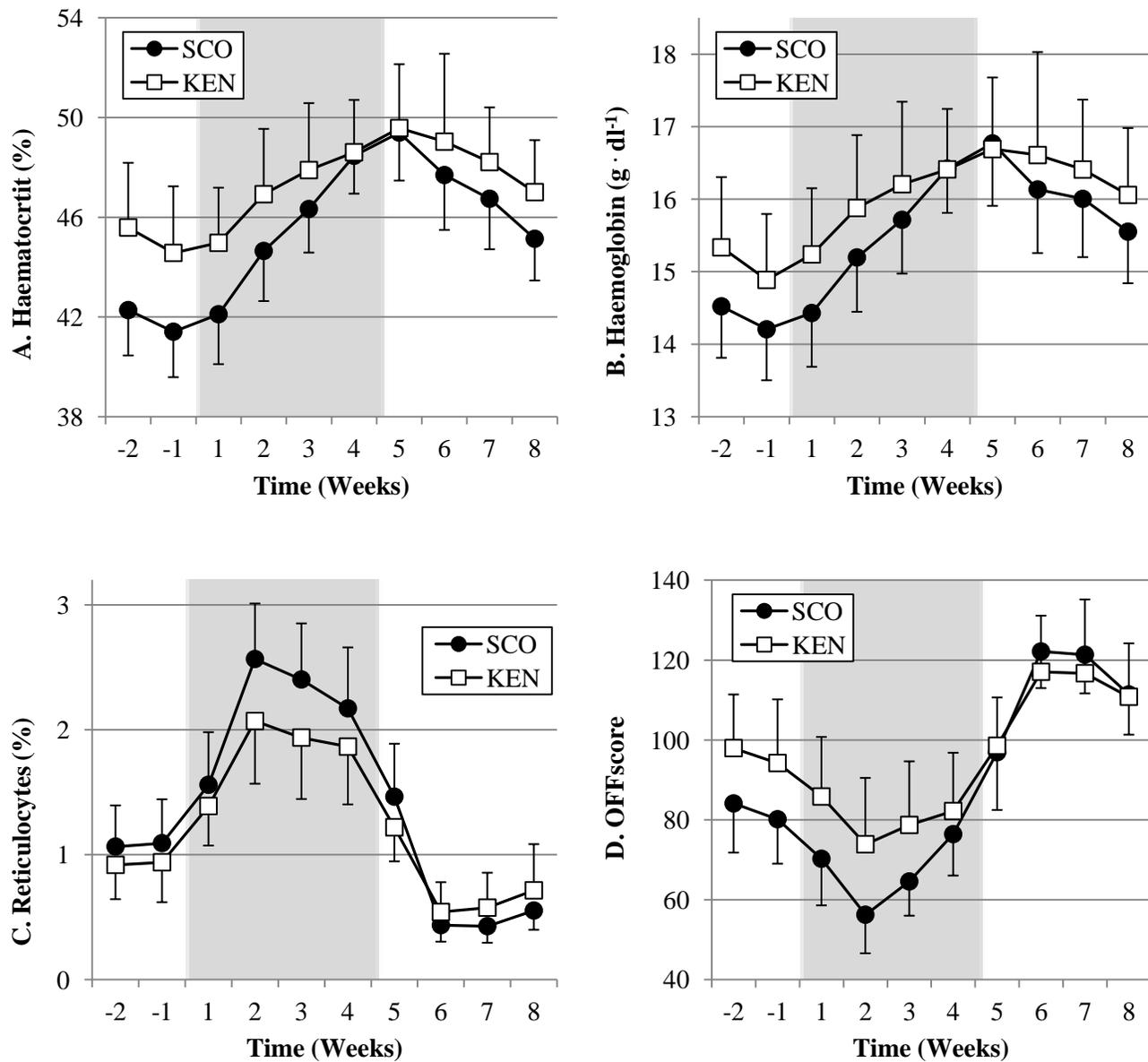
Figure 4.3: Changes in haematocrit (A), haemoglobin concentration (B), reticulocytes (C) and OFF score (D) in SCO (n = 19) and KEN (n = 20).

Results are mean ± SD. SCO and KEN are represented by the black circle and the white square, respectively. The grey zone represent the 4 weeks of rHuEpo administration.

Table 4.1. Haematocrit, haemoglobin concentration, reticulocytes and body mass before, during and 4 weeks post rHuEpo administration in SCO and KEN

	SCO (n = 19)				
	Baseline	Week 2 of rHuEpo	End of rHuEpo	Week 2 post rHuEpo	Week 4 post rHuEpo
Haematocrit (%)	41.9 ± 1.8	44.7[***] ± 2.0	49.2[***] ± 2.0	47.7[***] ± 2.2	45.1[***] ± 1.7
Haemoglobin (g·dl⁻¹)	14.4 ± 0.7	15.2[***] ± 0.7	16.7[***] ± 0.9	16.1[***] ± 0.9	15.6[***] ± 0.7
Reticulocytes (%)	1.07 ± 0.31	2.57[***] ± 0.44	1.46[**] ± 0.41	0.44[***] ± 0.13	0.55[***] ± 0.15
OFF score	83 ± 12	56[***] ± 10	97[**] ± 14	122[***] ± 9	111[***] ± 10
Body mass (kg)	75.1 ± 8.4	74.7 ± 7.8	75.0 ± 7.9	74.6 ± 7.6	74.6 ± 7.5
	KEN (n = 20)				
Haematocrit (%)	45.3[†††] ± 2.6	46. 9[*†††] ± 2.6	49.6[***] ± 2.6	49.0[***] ± 3.5	47.0[†††] ± 2.1
Haemoglobin (g·dl⁻¹)	15.2[††] ± 0.9	15.9[***†] ± 1.0	16.7[***] ± 1.0	16.6[**] ± 1.4	16.1[**] ± 0.9
Reticulocytes (%)	0.93 ± 0.28	2.07[***††] ± 0.50	1.22[**†] ± 0.27	0.54[***] ± 0.24	0.71 ± 0.37
OFF score	97[††] ± 14	74[***††] ± 17	99 ± 12	117[***] ± 14	111[***] ± 13
Body mass (kg)	56.7[†††] ± 4.9	56.7[†††] ± 4.9	56.7[†††] ± 4.6	56.5[†††] ± 4.6	56.5[†††] ± 4.7

Values are means ± SD. Data were analyzed using repeated measures ANOVA (SPSS, version 19). Significant differences compared to baseline values are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Significant differences in KEN compared to SCO are indicated by † $p < 0.05$, †† $p < 0.01$ and ††† $p < 0.001$. SCO: 19 Caucasian trained men living at sea level; KEN: 20 Kenyan runners living at moderate altitude.

Table 4.2. Resting blood pressure and heart rate before, during and 4 weeks post rHuEpo administration in SCO and KEN

	SCO (n = 19)				
	Baseline	Week 2 of rHuEpo	End of rHuEpo	Week 2 post rHuEpo	Week 4 post rHuEpo
Systolic blood pressure (mmHg)	128 ± 9	126 ± 7	126 ± 6	126 ± 7	124 ± 7
Diastolic blood pressure (mmHg)	70 ± 8	69 ± 8	69 ± 7	70 ± 7	69 ± 8
Heart rate (beats·min^{-1})	62 ± 8	60 ± 7	60 ± 7	59 ± 7	61 ± 8
	KEN (n = 20)				
Systolic blood pressure (mmHg)	$121 \pm 6^{\dagger\dagger}$	$118 \pm 7^{**\dagger\dagger}$	122 ± 8	121 ± 9	$119 \pm 7^{\dagger}$
Diastolic blood pressure (mmHg)	67 ± 5	65 ± 4	67 ± 4	66 ± 7	65 ± 5
Heart rate (beats·min^{-1})	$56 \pm 7^{\dagger}$	$54 \pm 8^{\dagger}$	$54 \pm 7^{*\dagger\dagger}$	56 ± 9	$55 \pm 7^{\dagger}$

Values are means ± SD. Data were analyzed using repeated measures ANOVA (SPSS, version 19). Significant differences compared to baseline values are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Significant differences in KEN compared to SCO are indicated by † $p < 0.05$, †† $p < 0.01$ and ††† $p < 0.001$. SCO: 19 Caucasian trained men living at sea level; KEN: 20 Kenyan runners living at moderate altitude.

4.3.2 Running performance (Table 4.3, Table 4.4, Figure 4.4, Figure 4.5, Figure 4.6 and Figure 4.7):

Table 4.3 illustrates the changes in time trial performance and $\dot{V}O_{2max}$. Both time trial performance and $\dot{V}O_{2max}$ were significantly better in KEN compared to SCO throughout the study ($p < 0.001$) (Figure 4.4 and Figure 4.6). However, interestingly, the relative improvements were similar between the two groups for time trial performance as well as for $\dot{V}O_{2max}$ post administration (~5%, $p = 0.188$ and ~8%, $p = 0.180$, respectively) and 4 weeks after rHuEpo (~3%, $p = 0.938$ and ~4%, $p = 0.692$, respectively) (Table 4.4, Figure 4.5 and Figure 4.7). Unlike SCO whose RPE post time trial remained relatively constant throughout the study, RPE in KEN increased after rHuEpo administration compared to pre-injections levels (16.1 ± 1.1 vs. 14.6 ± 1.5, $p < 0.001$) (Table 4.3). Temperature, humidity and wind records are reported in Table 1.

Time trial performance was significantly correlated with $\dot{V}O_{2max}$ throughout the study when KEN was grouped with SCO ($r > 0.78$, $p < 0.001$) but not when KEN alone was analyzed ($p > 0.337$) (Figure 4.8). A significant correlation in individual responses compared to baseline between parameter was observed for time trial performance and $\dot{V}O_{2max}$ post rHuEpo administration when KEN was analyzed alone and when grouped with SCO ($r = 0.86$, $p < 0.001$, $r = 0.62$, $p < 0.001$, respectively). No significant correlation was found between haemoglobin concentration and either time trial performance or $\dot{V}O_{2max}$.
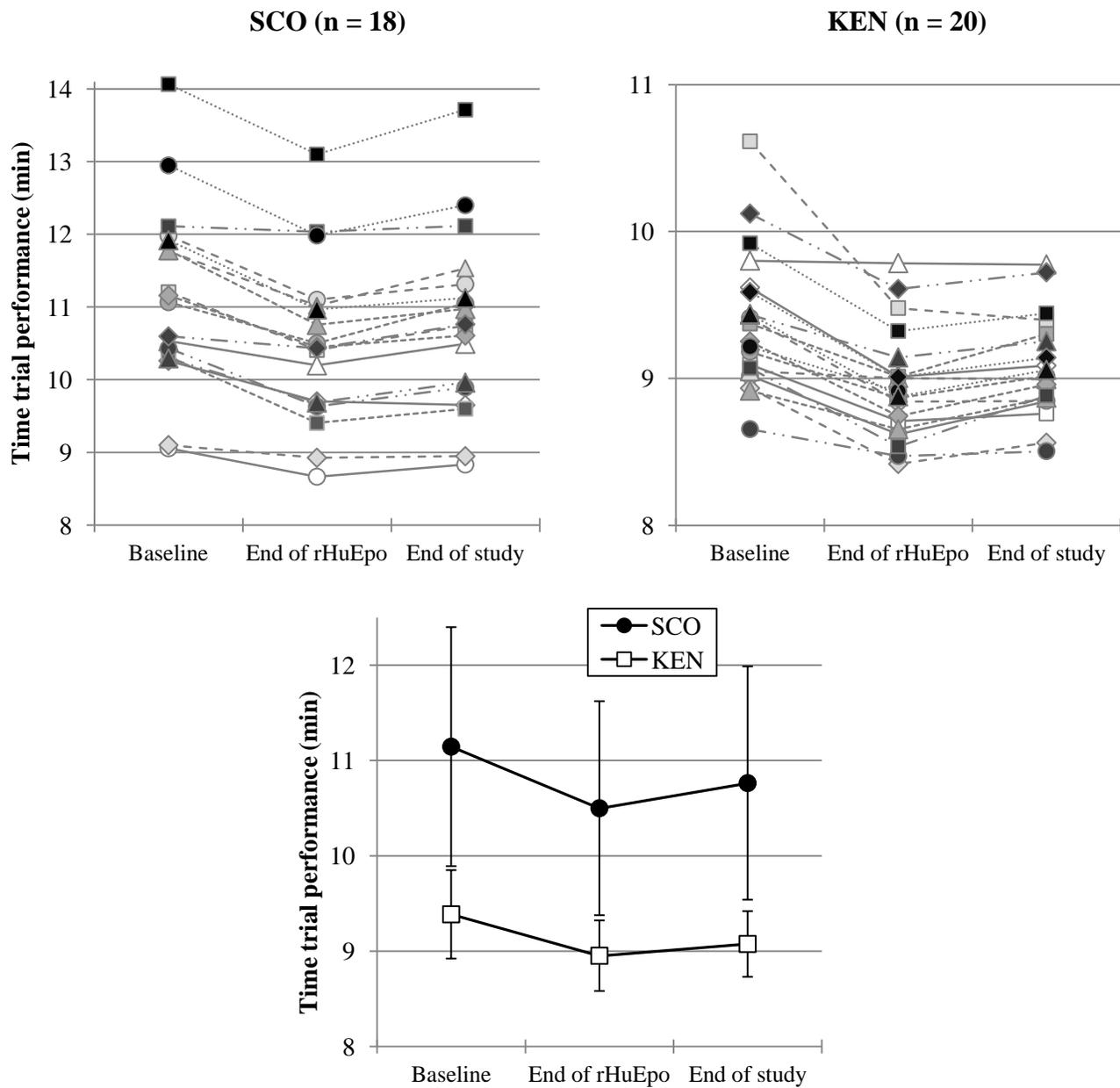
Figure 4.4: Changes in time trial performance in SCO and KEN.

The top panels represent individual changes in SCO (top left panel) and KEN (top right panel). The bottom panel illustrates the mean ± SD. SCO and KEN are represented by the black circle and the white square, respectively.
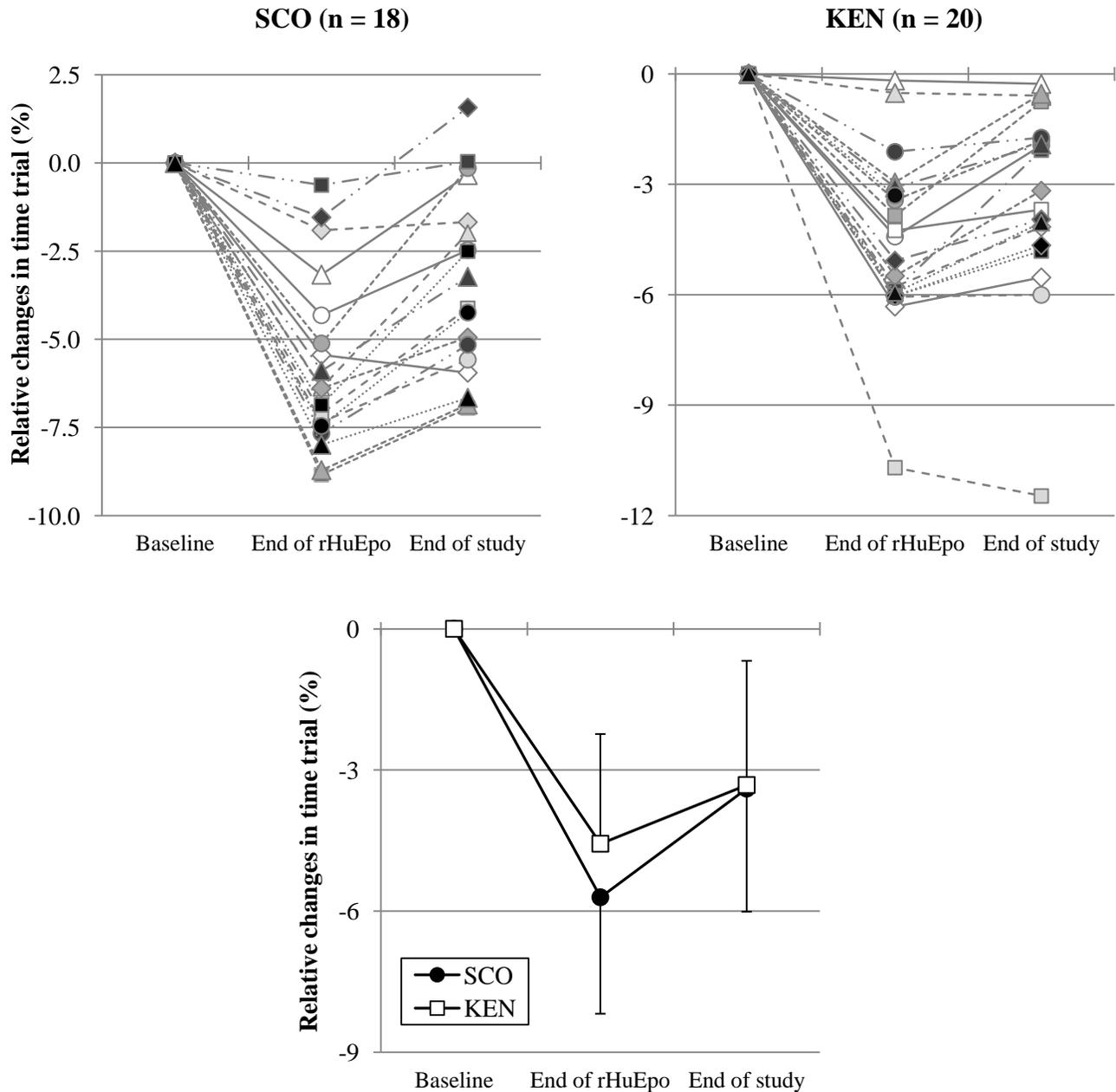
Figure 4.5: Relative changes in time trial performance in SCO and KEN.

The top panels represent individual relative changes in SCO (top left panel) and KEN (top right panel). The bottom panel illustrates the mean ± SD. SCO and KEN are represented by the black circle and the white square, respectively.

SCO (n = 18)   KEN (n = 16)
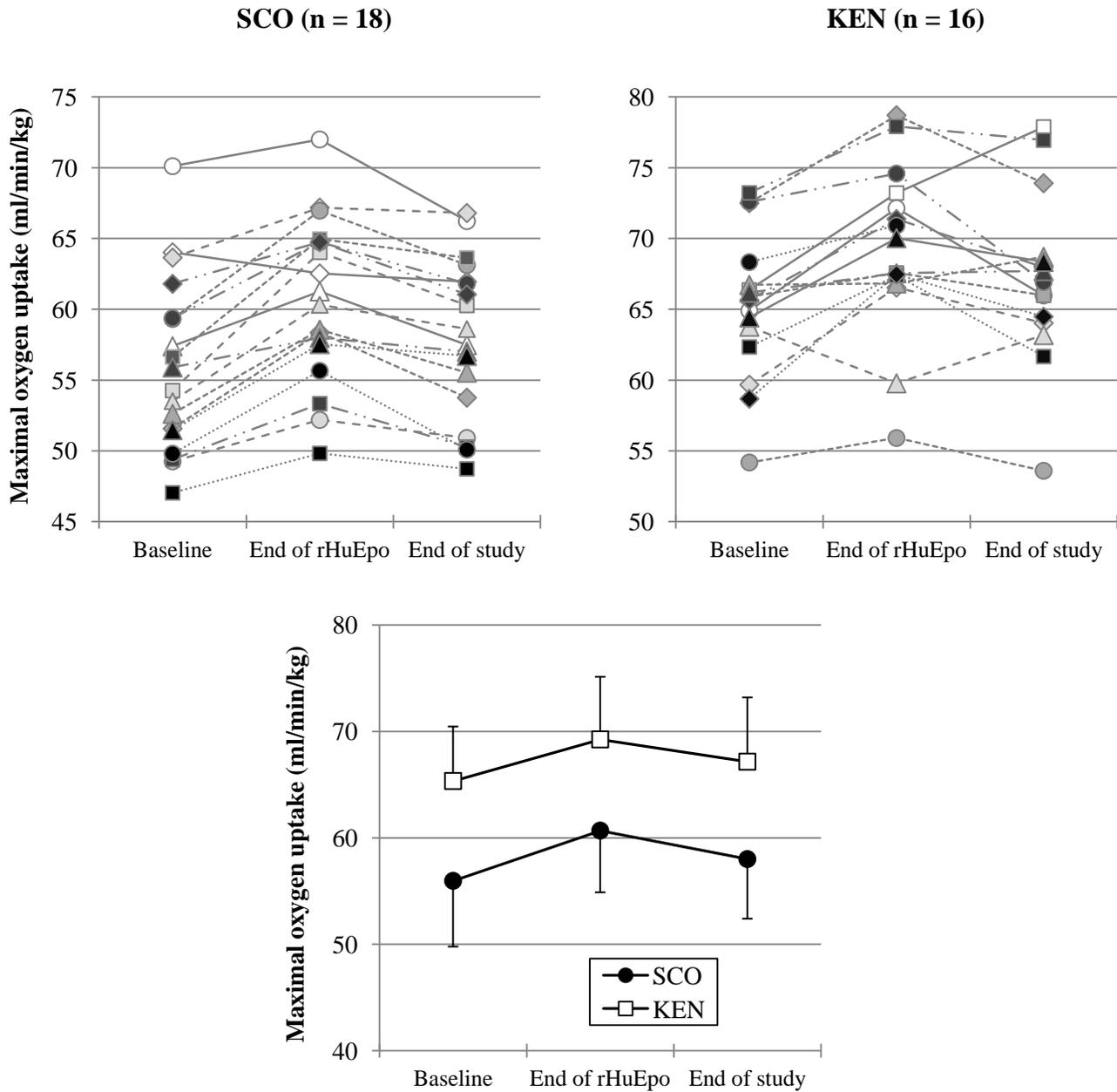
Figure 4.6: Changes in maximal oxygen uptake ($\dot{V}O_{2max}$) in SCO and KEN.

The top panels represent individual changes in SCO (top left panel) and KEN (top right panel). The bottom panel illustrates the mean ± SD. SCO and KEN are represented by the black circle and the white square, respectively.
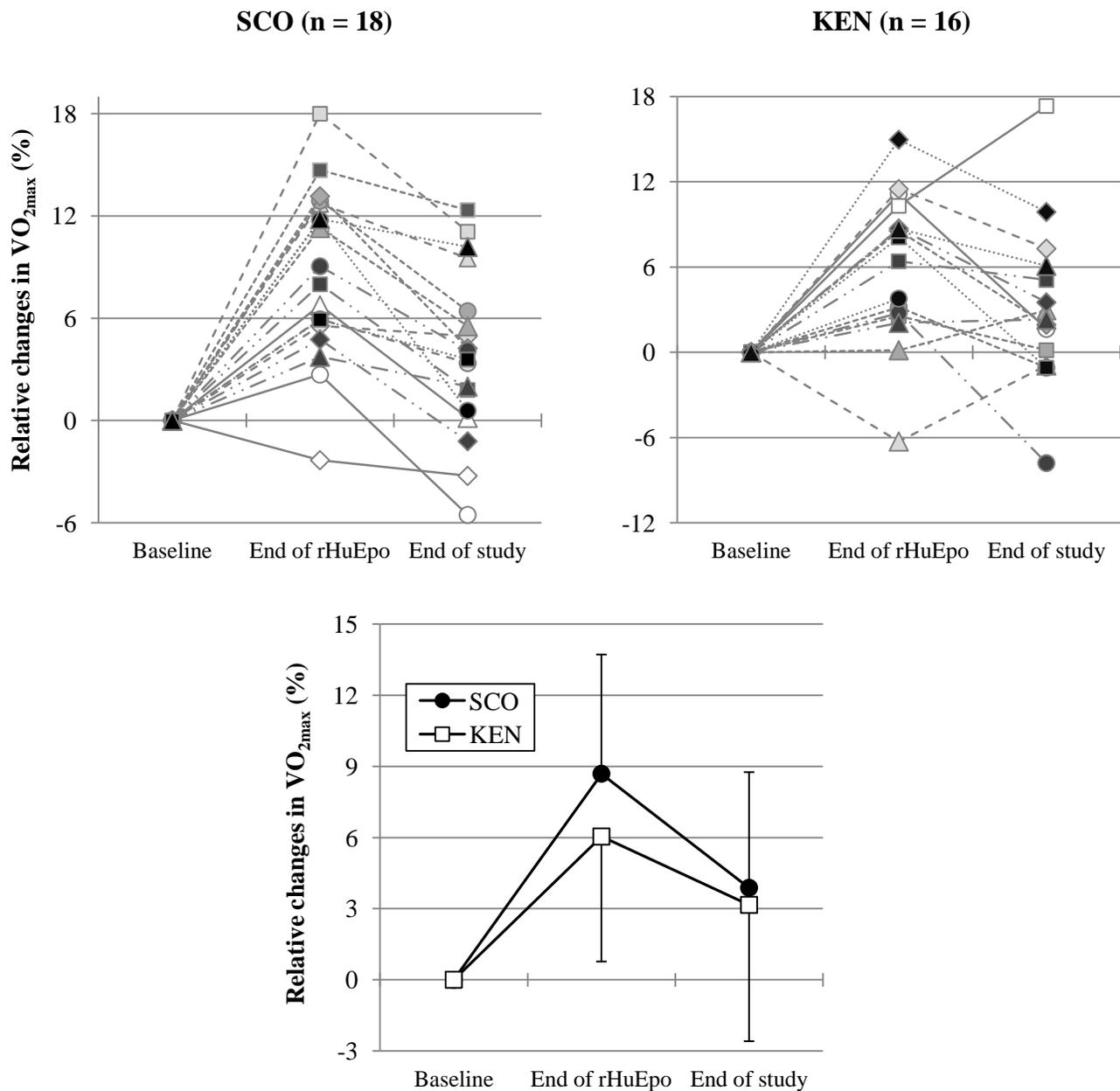
Figure 4.7: Relative changes in maximal oxygen uptake ($\dot{V}O_{2max}$) in SCO and KEN.

The top panels represent relative individual changes in SCO (top left panel) and KEN (top right panel). The bottom panel illustrates the mean ± SD. SCO and KEN are represented by the black circle and the white square, respectively.
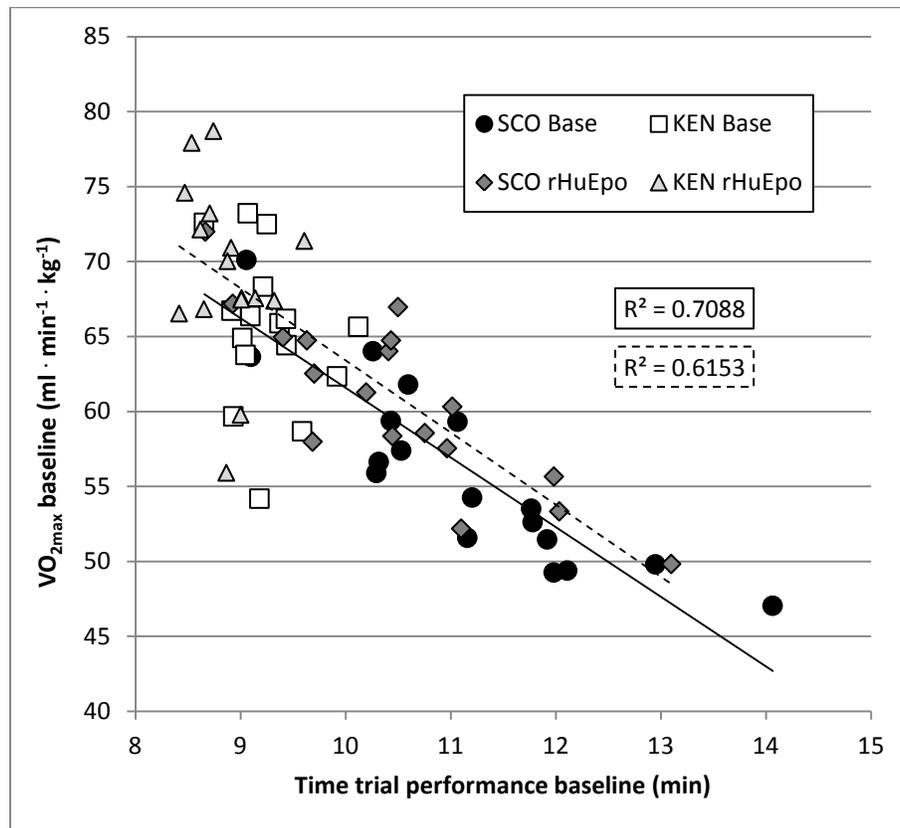
Figure 4.8: Relationship between maximal oxygen uptake ($\dot{V}O_{2max}$) and 3,000 m running time trial performance in KEN and SCO.

Relationship between absolute $\dot{V}O_{2max}$ and 3,000 m running time trial performance in KEN and SCO at baseline (white squares and black circles, respectively; solid line) and immediately after the rHuEpo administration (light grey triangles and dark grey diamonds, respectively; dotted line).

Table 4.3. Running 3,000 m time trial performance and maximal oxygen uptake in SCO and KEN

	SCO		
	Baseline	End of rHuEpo	End of the study
3,000 m total time (min:sec)	$11:08 \pm 1:15$	$10:30^{***} \pm 1:07$	$10:46^{***} \pm 1:13$
First 1 km split time (min:sec)	$3:35 \pm 0:28$	$3:23^{**} \pm 0:18$	$3:27^{*} \pm 0:22$
Second 1 km split time (min:sec)	$3:46 \pm 0:28$	$3:35^{***} \pm 0:28$	$3:40^{**} \pm 0:26$
Third 1 km split time (min:sec)	$3:49 \pm 0:25$	$3:32^{***} \pm 0:22$	$3:39^{**} \pm 0:26$
RPE scale (6-20)	17.8 ± 1.9	18.4 ± 1.2	18.8 ± 1.3
Temperature (Cº)	19.7 ± 2.5	19.0 ± 1.9	19.5 ± 3.8
Humidity (%)	48.9 ± 9.5	46.6 ± 8.7	44.6 ± 8.8
$\dot{V}O_{2max}$ (l·min^{-1})	4.2 ± 0.4	$4.6^{***} \pm 0.5$	$4.3^{*} \pm 0.5$
$\dot{V}O_{2max}$ (ml·min^{-1}·kg^{-1})	56.0 ± 6.2	$60.7^{***} \pm 5.8$	$58.0^{*} \pm 5.6$
	KEN		
3,000 m total time (min:sec)	$9:23^{†††} \pm 0:28$	$8:57^{***†††} \pm 0:22$	$9:04^{***†††} \pm 0:20$
First 1 km split time (min:sec)	$3:04^{†††} \pm 0:10$	$2:56^{**†††} \pm 0:07$	$2:57^{*†††} \pm 0:07$
Second 1 km split time (min:sec)	$3:17^{††} \pm 0:22$	$3:02^{*†††} \pm 0:08$	$3:04^{†††} \pm 0:08$
Third 1 km split time (min:sec)	$3:10^{†††} \pm 0:13$	$3.00^{†††} \pm 0.28$	$3:03^{*†††} \pm 0:08$
RPE scale (6-20)	$14.6^{†††} \pm 1.5$	$16.1^{***†††} \pm 1.1$	$15.3^{†††} \pm 1.2$
Temperature (Cº)	$24.0^{†††} \pm 2.5$	$25.4^{†††} \pm 2.4$	$23.3^{†} \pm 3.9$
Humidity (%)	55.4 ± 16.1	50.4 ± 9.2	$58.5^{††} \pm 15.9$
Wind speed (m·s^{-1})	1.6 ± 0.8	1.9 ± 0.8	$2.8 \pm 0.7^{**}$
$\dot{V}O_{2max}$ (l·min^{-1})	$3.6^{†††} \pm 0.4$	$3.9^{**†††} \pm 0.4$	$3.7^{†††} \pm 0.4$
$\dot{V}O_{2max}$ (ml·min^{-1}·kg^{-1})	$65.3^{†††} \pm 5.1$	$69.2^{**†††} \pm 5.9$	$67.2^{†††} \pm 6.0$

Values are means $\pm$ SD. Data were analyzed using repeated measures ANOVA (SPSS, version 19). Significant differences compared to baseline values are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Significant differences in KEN compared to SCO are indicated by † $p < 0.05$, †† $p < 0.01$ and ††† $p < 0.001$. SCO: Scottish trained individuals living and training at or near sea-level; 18 subjects were included in the analysis of both time trial performance and $\dot{V}O_{2max}$; KEN: Kenyan runners living and training at moderate altitude; 19 and 15 subjects were included in the analysis of time trial performance and $\dot{V}O_{2max}$; $\dot{V}O_{2max}$: Maximal oxygen uptake.

Table 4.4. Relative changes in running 3,000 m time trial performance, maximal oxygen uptake, haematocrit and haemoglobin concentration in KEN and SCO.

	KEN		SCO	
	End of rHuEpo	End of the study	End of rHuEpo	End of the study
3,000 m performance	-4.6 ± 2.4% (-5.8 to -3.5)	-3.3 ± 2.6% (-4.6 to -2.1)	-5.7 ± 2.5% (-6.9 to -4.5)	-3.4 ± 2.6% (-4.7 to -2.1)
Maximal oxygen uptake ($\dot{V}O_{2max}$)	6.2 ± 5.4% (8.9 to 3.4)	3.1 ± 5.7% (5.9 to 0.4)	8.7 ± 5.0% (11.2 to 6.2)	3.9 ± 4.9% (6.4 to 1.3)
Haematocrit	9.7 ± 4.7% (11.9 to 7.5)[***]	4.1 ± 5.0% (6.2 to 1.9)[*]	17.6 ± 4.9% (19.8 to 15.3)	7.8 ± 4.5% (10.0 to 5.6)
Haemoglobin concentration	10.1 ± 5.5% (12.5 to 7.6)[**]	5.9 ± 5.8% (8.4 to 3.4)	16.2 ± 5.2% (18.7 to 13.7)	8.2 ± 5.1% (10.7 to 5.7)

Values are means ± SD (95% confidence intervals). Data were analyzed using repeated measures ANOVA (SPSS, version 19). Significant differences in KEN compared to SCO are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. KEN: Kenyan runners living and training at moderate altitude; 19 and 15 subjects were included in the analysis of time trial performance and $\dot{V}O_{2max}$. SCO: Scottish trained individuals living and training at or near sea-level; 18 subjects were included in the analysis of both time trial performance and $\dot{V}O_{2max}$.

4.4 Discussion:

The main aim of this study was to determine whether the effects of rHuEpo administration on blood parameters, $\dot{V}O_{2max}$ and running time trial performance in Kenyan endurance runners living and training at moderate altitude (~2,150 m) would be similar to those observed in Caucasian trained males living and training at or near sea-level (Chapter 2) (Durussel *et al.* 2013a). The main finding of this study was that, although baseline values were markedly different between groups and blood parameters did not change as much in KEN as in SCO, the relative improvements in running performance post rHuEpo (~5%) and 4 weeks post administration (~3%) were not significantly different between both groups.

4.4.1 Haematological parameters:

On the basis of the limited literature of haematological profiles of the Kenyan distance runners, it seems that Kenyan endurance athletes living and training at moderate altitude (2,000-2,500 m) may have relatively high haemoglobin concentration and haematocrit but within or close to the levels typical of sea-level populations (Moore *et al.* 2007). The mean haemoglobin concentration and haematocrit reported in our study were slightly lower but in accordance with the values previously reported in the literature (Ashenden *et al.* 2003; Moore *et al.* 2007; Prommer *et al.* 2010). This small difference may be due to the posture adopted before blood sampling (Ahlgrim *et al.* 2010) which was not reported in the other studies. Regardless of these minor discrepancies, baseline haemoglobin concentration and haematocrit values were as expected higher in KEN compared to SCO. The administration of rHuEpo stimulated the bone marrow production in both groups as indicated by the significant increase in immature reticulocyte fraction and reticulocyte percentage. Subsequently, haemoglobin concentration and haematocrit significantly increased in both groups to a similar mean maximum of approximately 17 g·dl^{-1} and 50% one week after the cessation of rHuEpo. While increasing haematocrit enhances the oxygen carrying capacity of the blood, it is also associated with elevated blood viscosity which in turn can limit cardiac work and may hamper exercise performance (Boning *et al.* 2011; Schuler *et al.* 2010). It has been demonstrated *in vivo* that there is indeed an optimal haematocrit for systemic exercise in mice (Schuler *et al.* 2010). Some authors reported that the optimal haematocrit values for human exercise performance should be within the broad range of 43 to 55% (Boning *et al.* 2011; Robertson *et al.* 1982), while others speculated that it should be close to the measurements

reported in a Finnish cross-country skier three times Olympic gold medallist with an autosomal dominant mutation in erythropoietin receptor which was associated with remarkable haemoglobin concentration and haematocrit above 20 g·dl^{-1} and 65%, respectively (Juvonen *et al.* 1991; Schuler *et al.* 2010). Our results showed that the elevated blood viscosity after rHuEpo administration in our study illustrated by the increase in haematocrit and haemoglobin concentration which peaked at similar times and levels in both groups did not hinder significant improvement in running performance either in SCO or in KEN.

4.4.2 Running performance:

Although baseline values were markedly different between groups and blood parameters did not change as much in KEN as in SCO, the relative improvements in running performance as well as in $\dot{V}O_{2max}$ post rHuEpo and 4 weeks post administration were not significantly greater in SCO compared to KEN. It has been well established that, despite slight differences in the frequency of injections and in the dosage used, rHuEpo administration for 4 to 6 weeks increases $\dot{V}O_{2max}$ by approximately 8% in normoxia (Audran *et al.* 1999; Berglund and Ekblom 1991; Birkeland *et al.* 2000; Connes *et al.* 2003; Durussel *et al.* 2013a; Parisotto *et al.* 2000; Russell *et al.* 2002; Wilkerson *et al.* 2005). In the present study, improvements of similar magnitude were observed in Kenyan sub-elite endurance runners who live and train at moderate-altitude altitude (2,000-2,500 m). In addition, the changes in the laboratory based measurements translated into actual improvements in running performance in the field. On average, the subjects were 26 s faster during the 3,000 m run at an elevation of 2,150 m (corresponding to a ~5% improvement). While the blood oxygen content after rHuEpo was not enhanced as much in KEN as in SCO, illustrated by the increase in haemoglobin concentration and haematocrit of ~10% in KEN and ~17% in SCO, the relative improvements in running performance and $\dot{V}O_{2max}$ were approximately similar (~5 and ~7%, respectively). It has been previously reported that the relative improvement of $\dot{V}O_{2max}$ induced by rHuEpo administration was more than doubled (*i.e.* 17.5%) at a simulated moderate altitude up to 3,500 m compared to normoxia (Robach *et al.* 2008). Based on these results, the similar relative improvement in $\dot{V}O_{2max}$ associated with a smaller increase in blood oxygen content after rHuEpo in KEN compared to SCO may be explained by the fact that the experiments were carried out in moderate-altitude hypoxia and normoxia, respectively.

These findings question how rHuEpo administration enhances exercise performance, especially at moderate-altitude. As no measurable nonhematopoietic ergogenic effect of rHuEpo on exercise performance in normoxic conditions was observed in two different studies conducted by the same authors, they attributed the ergogenic effects of rHuEpo to the increase in oxygen carrying capacity (Lundby *et al.* 2008b; Rasmussen *et al.* 2010). The oxygen supply to exercising muscle depends on the haemoglobin concentration and cardiac output. However, haemoglobin concentration is only weakly related to $\dot{V}O_{2max}$ whereas the relationship between Hb_{mass} and $\dot{V}O_{2max}$ is much stronger (Kanstrup and Ekblom 1984; Schmidt and Prommer 2010). Unlike haemoglobin concentration, Hb_{mass} is not affected by changes in plasma volume and is therefore a key complementary parameter to evaluate blood oxygen carrying capacity (Schmidt and Prommer 2010). Unfortunately, Hb_{mass} and related blood volumes were not determined in KEN. As a result and although the urine osmolality of KEN, which can give some indication of the hydration status, did not significantly change throughout the study, it cannot be ruled out that the rHuEpo-induced change in Hb_{mass} and related blood volumes were different when comparing SCO and KEN. In addition, because the study was designed primarily for other purposes, the study was not blinded and did not include a control group. As such, factors unrelated to rHuEpo such as altered motivation (placebo, order effect, *etc.*) may partly explain the reported performance effects and reflected in the small but significant rise in RPE after rHuEpo in KEN. Nevertheless, it is tempting to speculate that the similar relative improvements in running performance and $\dot{V}O_{2max}$, despite haemoglobin concentration and haematocrit not changing as much in KEN as in SCO, may be due to mechanisms not directly related to the blood oxygen carrying capacity such as augmented buffering capacity (Boning *et al.* 2008, 2011) and mood improvement (Miskowiak *et al.* 2008; Ninot *et al.* 2006). Other potential explanations involving the central nervous system include the anticipatory model which would argue that greater recruitment of motor units was allowed during the 3,000 m run post rHuEpo administration because the control mechanism of feed-forward and feedback to the brain was modified (Kayser 2003; Noakes 2008a, b; Noakes *et al.* 2001). Despite some conjecture, the precise mechanism(s) underlying the apparently similar ergogenic effects of rHuEpo at moderate altitude as compared to normoxia (*i.e.* sea-level) remain speculative.

4.4.3 Non-responders to rHuEpo administration:

Based on the blunted response in the haematological parameters and exercise performance, one Kenyan subject did not respond to rHuEpo administration. It is well known that there is a marked variability in the sensitivity to rHuEpo in anaemic patients as well as in rHuEpo dose requirement (Eschbach *et al.* 1989; Macdougall 1995). Approximately 90% of patients receiving rHuEpo respond to rHuEpo therapy by increasing their haemoglobin concentration. The remaining 10% of patients show either no response or suboptimal response, *i.e.* failing to attain target haemoglobin concentration levels despite increasing the dose of rHuEpo to very high levels (Macdougall 1995). In addition, the sensitivity to hypoxia exposure during altitude training in healthy volunteers or athletes is also variable as reflected by significant but not clear-cut differences in plasma erythropoietin concentrations (Chapman *et al.* 1998). The athletes who responded to altitude training demonstrated higher erythropoietin levels which in turn seemed to trigger a sufficient increase in Hb_{mass} and associated improvements in exercise performance (Chapman *et al.* 1998; Robach and Lundby 2012; Wilber *et al.* 2007). The particular subject in KEN who demonstrated a blunted haematological and exercise performance response after rHuEpo may therefore be representative of the 10% of the treated patients or of the "non-responders" to altitude training. Further research is needed to determine the exact causes of this variability in responsiveness to rHuEpo administration and altitude training.

4.4.4 rHuEpo and anti-doping at altitude:

The use of rHuEpo is prohibited by WADA. The direct urinary test to detect rHuEpo was developed and published by Lasne *et al.* in 2000 (Lasne and de Ceaurriz 2000; Lasne *et al.* 2002). However, while the effects of rHuEpo administration on haematological parameters and exercise performance can last for weeks as shown in the present study, the test has a rather limited detection window, *i.e.* a few hours up to a few days depending on the dose used (Ashenden *et al.* 2006; Breidbach *et al.* 2003; Lundby *et al.* 2008a). At the same time as the development of the direct test, potential indirect detection of rHuEpo using blood markers of altered erythropoiesis is being explored (Cazzola 2000; Parisotto *et al.* 2000). The ABP was introduced as a new tool to indirectly detect blood doping which can lead to disciplinary sanctions imposed by a sports organization or its tribunal at the same time as intelligently targeting athletes for additional testing (Schumacher *et al.* 2012; Zorzoli and Rossi 2012). The

ABP approach relies on identifying intra-individual abnormal variability over time of selected haematological parameters including haemoglobin concentration, reticulocyte percentage and OFF score (Callaway 2011; Sottas *et al.* 2010). In this context, factors such as residence at altitude and ethnicity have been shown to significantly influence these haematological parameters (Ashenden *et al.* 2003; Ashenden *et al.* 2001; Sharpe *et al.* 2002) and confirmed in the present study as the magnitude of change in haemoglobin concentration, reticulocyte percentage and OFF score used in the ABP was reduced in KEN as compared with SCO in response to the same rHuEpo administration regime. As a result, it may be more difficult to detect rHuEpo doping at altitude compared to sea-level using the currently anti-doping methods due to the blunted haematological response. There are indeed concerns about the risk of the misuse of altitude exposure by some athletes in order to mask blood doping practices such as the administration of rHuEpo (Lundby *et al.* 2012b; Sanchis-Gomar *et al.* 2009). Interestingly, a recent published review concluded that the size of the benefit of rHuEpo administration in top elite athletes, which has yet to be scientifically confirmed, is likely to be small or nonexistent (Heuberger *et al.* 2012). The present findings are contrary to this conclusion. Although one still has to be cautious in extrapolating findings from sub-elite to truly elite athletes, the significant improvement in running performance observed in the present study would almost certainly translate into a worthwhile enhancement in elite performance (Hopkins *et al.* 1999). Moreover, in professional cycling stage races, such as the Tour de France, the mountain stages, which peak at altitudes ranging from approximately 1,500 m up to above 2,500 m, often separate winners from contenders (Jeukendrup *et al.* 2000). If the ergogenic effects of rHuEpo are indeed enhanced at moderate-altitude or can at least significantly reduce the hypoxia-induced impairment in exercise performance, rHuEpo administration has the potential to improve further the athlete's chance of winning the stage or the overall classification. However, further research is required to confirm these findings and related hypotheses.

In conclusion, this is the first study to demonstrate that 4 weeks of rHuEpo administration significantly increased the already relatively high basal haemoglobin concentration and haematocrit values of Kenyan endurance runners living and training at moderate-altitude by ~10%. Although the rHuEpo-induced increase in key haematological parameters in these altitude Kenyan endurance runners was not as high compared to Caucasian counterparts living and

training at or near sea-level, the relative improvements of ~5% and ~3% in running performance immediately after the rHuEpo administration and 4 weeks after the last injection were similar in both groups. These findings have important implications for current anti-doping practices.

Chapter 5 : Replication in Another Cohort (part 2):

Blood Gene Expression Profiles in Kenyan Runners Living and Training at Altitude after Recombinant Human Erythropoietin Administration

5.1 Introduction and aim:

Administration of rHuEpo improves endurance capacity and performance not only at sea-level (Chapter 2) (Durussel *et al.* 2013a; Thomsen *et al.* 2007) but also at moderate altitude (Chapter 4) (Robach *et al.* 2008). Hence rHuEpo is frequently subject to abuse by athletes as revealed by the declarations of confessed dopers such as Lance Armstrong (Walsh 2012) and Tyler Hamilton (Hamilton and Coyle 2012), although the use of rHuEpo is prohibited by WADA. The aim of the previous study described in Chapter 3 was to assess the effects of rHuEpo on blood gene expression profiles in order to identify a "molecular signature" of rHuEpo doping and hence provide a basis for the development of improved testing models to detect rHuEpo doping based on gene expression profiles. On the basis of the data generated by the study described in Chapter 3, blood gene expression profiles were profoundly and significantly altered during rHuEpo administration and for at least 4 weeks after the rHuEpo administration leading to the initial "molecular signature" of rHuEpo doping. For example, using a 1.5 fold-change threshold, 133 transcripts were found to be differentially expressed in the same way during (up-regulated) and subsequently post (down-regulated) rHuEpo administration. Also, 41 transcripts were found to be already up-regulated 2 days after only one single rHuEpo injection. These very promising data suggest that omics technologies such as gene expression have the potential to add a new dimension to the ABP for rHuEpo detection. However, part of the validation process of microarray data, these results need to be replicated (Allison *et al.* 2006; Chuaqui *et al.* 2002). The haematological and exercise performance results previously described in Chapter 4 confirmed that the perturbation involving rHuEpo worked effectively in both the Scottish and Kenyan groups. Using the same gene microarray molecular methods previously described in Chapter 3, the aim of the present study was to assess and compare the effects of rHuEpo on blood gene expression profiles discovered in the Scottish sea-level group with another cohort composed of Kenyan endurance runners living and training at moderate altitude who underwent an identical rHuEpo administration.

5.2 Methods:

5.2.1 Experimental design:

In order to replicate the findings in the previously described Scottish (SCO) cohort, twenty Kenyan endurance runners (KEN, mean ± SD, age: 26.4 ± 4.1 yr, body mass: 56.8 ± 4.7 kg, height: 171.8 ± 6.4 cm) based at moderate altitude (Eldoret, Kenya, ~2,150 m) participated in the study and underwent an identical rHuEpo administration regime in moderate hypoxic conditions, *i.e.* each subject subcutaneously self-injected 50 IU·kg^{-1} body mass of rHuEpo (NeoRecormon, Roche, Welwyn Garden City, UK) every second day for 4 weeks. All subjects underwent a medical assessment and provided written informed consent to participate. The subjects were requested to maintain their normal training but abstain from official sporting competition for the duration of the research protocol (Howman 2013; Wagner 2013). This study was approved by University of Glasgow Ethics Committee as well as the Moi University Committee and conformed to the Declaration of Helsinki.

5.2.2 Blood sampling, RNA extraction and processing for microarrays analysis (Figure 3.1):

After 10 min of rest in the supine position, 3 ml of whole blood were collected into Tempus Blood RNA tubes (Applied Biosystems, Foster City, USA) from an antecubital vein in triplicate at baseline, during rHuEpo administration and for 4 weeks after rHuEpo administration (Figure 2.1). The Tempus tubes were vigorously mixed immediately after collection, incubated at room temperature for 3 hours and stored at -20˚C. The frozen tubes were shipped to Glasgow (Scotland, UK) on ice and stored at -20˚C before RNA extraction. Eight time points per subjects were selected for gene expression analysis, *i.e.* two baseline samples, then one sample each at 2 days, 2 weeks and 4 weeks after the first rHuEpo injection and 1 week, 2 weeks and 4 weeks after rHuEpo administration. RNA was extracted using the Tempus Spin RNA isolation kit (Life Technologies, Paisley, UK) (see Appendix B.2). RNA yield was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and the RIN was assessed using an Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, USA). RNA was stored at -80°C until further analysis. RNA was then prepared using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, USA). The procedure consists in synthesizing cRNA by *in vitro* transcription from the cDNA produced by reverse transcription from 500 ng of RNA. For the Illumina microarray, 750 ng of the purified labeled cRNA samples were randomly hybridized

to the Illumina HumanHT-12 v4.0 Expression BeadChip Kits following the manufacturers' recommended procedures (Illumina, San Diego, Canada). The Bead arrays were scanned on the Illumina BeadArray Reader and raw intensity values were saved in Illumina GenomeStudio software.

5.2.3 Microarray data analysis:

The microarray data analysis used was the same as in Chapter 3. Briefly, data were log2 transformed after adding a small constant of 51 to avoid negative values as well as to reduce the influence of probes with low signal intensities close to the background noise. The regression coefficient between the arrays was determined and was used as a scaling factor to remove the array effect (Figure 3.2 and Figure 3.3). Rank Products analysis (Breitling *et al.* 2004) with a 5% false discovery rate (Benjamini and Hochberg 1995) was used to identify the differentially expressed transcripts during and post rHuEpo administration. An additional 1.5 fold-change threshold was applied for a more stringent analysis. Ingenuity Pathway Analysis was used to further analyze the data by incorporating biologically valid relationships (in a similar manner to Gene Ontologies).

5.3 Results:

5.3.1 RNA yield:

The average yield of the total RNA was 10.6 ± 6.6 μg in KEN. Although the RNA yield in KEN graphically followed a similar pattern to that in SCO (Figure 5.1), no significant difference was observed during or post rHuEpo administration compared to baseline in KEN (probably due to a lack of power, n = 6). Values between the two groups were similar until 4 weeks post rHuEpo administration when the RNA yield in KEN was higher than in SCO (p = 0.028). The average RIN value for KEN was 9.1 ± 0.2 (see Appendix B.2).

5.3.2 Transcripts altered by rHuEpo administration (Table 5.1, Table 5.2, Figure 5.2, Figure 5.3, Figure 5.4, Figure 5.5, Figure 5.6 and Figure 5.7):

Using the additional 1.5 fold-change threshold, there were 52 (up-regulated) transcripts which were already up-regulated two days after only the first rHuEpo injection, and remained so throughout administration in KEN (Figure 5.2). Among these transcripts, 30 were in common with SCO (Figure 5.2 and Table 5.1). The remaining 11 and 22 transcripts were also up-regulated in both groups but were not included in the intersection of the Venn diagram due to the stringent 1.5 fold-change threshold. Rap1 GTPase-activating protein 1 (*RAP1GAP*), family with sequence similarity 46 member C (*FAM46C*) and oxysterol-binding protein 2 (*OSBP2*) were the top three genes whose expression were altered by a mean fold change higher than 2 in both groups two days after only the first rHuEpo injection (Figure 5.3 and Table 5.1). However, unlike the other genes, there was an extremely high variability in the gene expression response in *RAP1GAP* (Figure 5.3). Fewer transcripts were differentially expressed post rHuEpo administration in KEN compared to SCO (Figure 5.4 and Table 5.2). However, the 15 transcripts which were differentially expressed during (up-regulated) as well as subsequently post (down-regulated) rHuEpo administration in SCO were also found in KEN (Figure 5.5 and Table 5.2). Delta-aminolevulinate synthase 2 (*ALAS2*), alpha-synuclein (*SNCA* − 2 transcripts) and ring finger protein 213 (*RNF213*) were the top three genes whose expression were down-regulated by a mean fold change higher than 4 in SCO and higher than 2 in KEN (Figure 5.6 and Table 5.2). Although the changes in gene expression were of lesser magnitude compared to the genes presented above, a few transcripts such as eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein) (*EEF1D*), ribosomal protein S29 (*RPS29*) and parkinson

protein 7 (*PARK7*) were, unlike the majority of the discovered transcripts, down-regulated during rHuEpo administration and then up-regulated post administration (Figure 5.7). Using Ingenuity Pathway Analysis, the dysregulated genes were found to be mainly related to either the functional or structural properties of the erythrocyte or to the cell cycle and its regulation.

5.3.3 Housekeeping genes:

The top 100 best housekeeping genes in SCO and KEN were compared and one transcript was found to be in common. The gene associated with this transcript was MORF4 family-associated protein 1 (*MRFAP1*). *MRFAP1* was a relatively abundant transcript with a maximum fold change across the samples lower than 1.5 and a low variation over time in both groups (Figure 5.8).
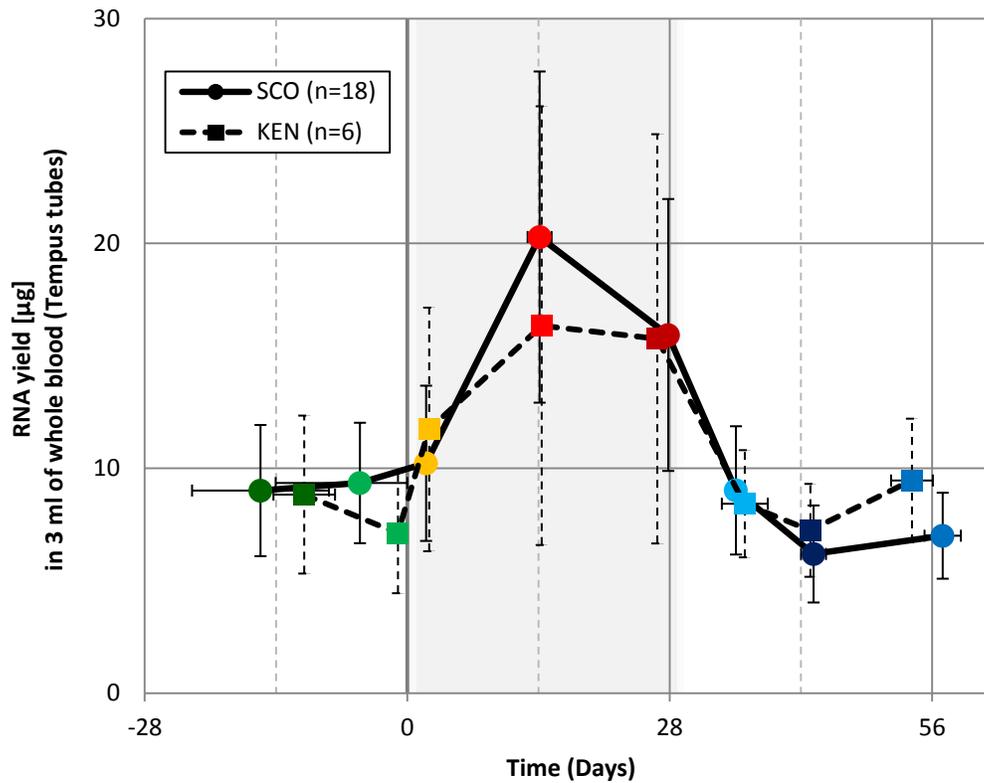
Figure 5.1: Changes in total RNA yield extracted from 3 ml of whole blood stabilized in Tempus tubes in SCO (n = 18) and KEN (n = 6)[4].

Values are means ± SD. Time point 0 represents the first rHuEpo injection. The grey zone represents the 4 weeks rHuEpo administration. SCO and KEN are represented by the circle and the square, respectively.

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[4] See Appendix A for KEN (n = 18).
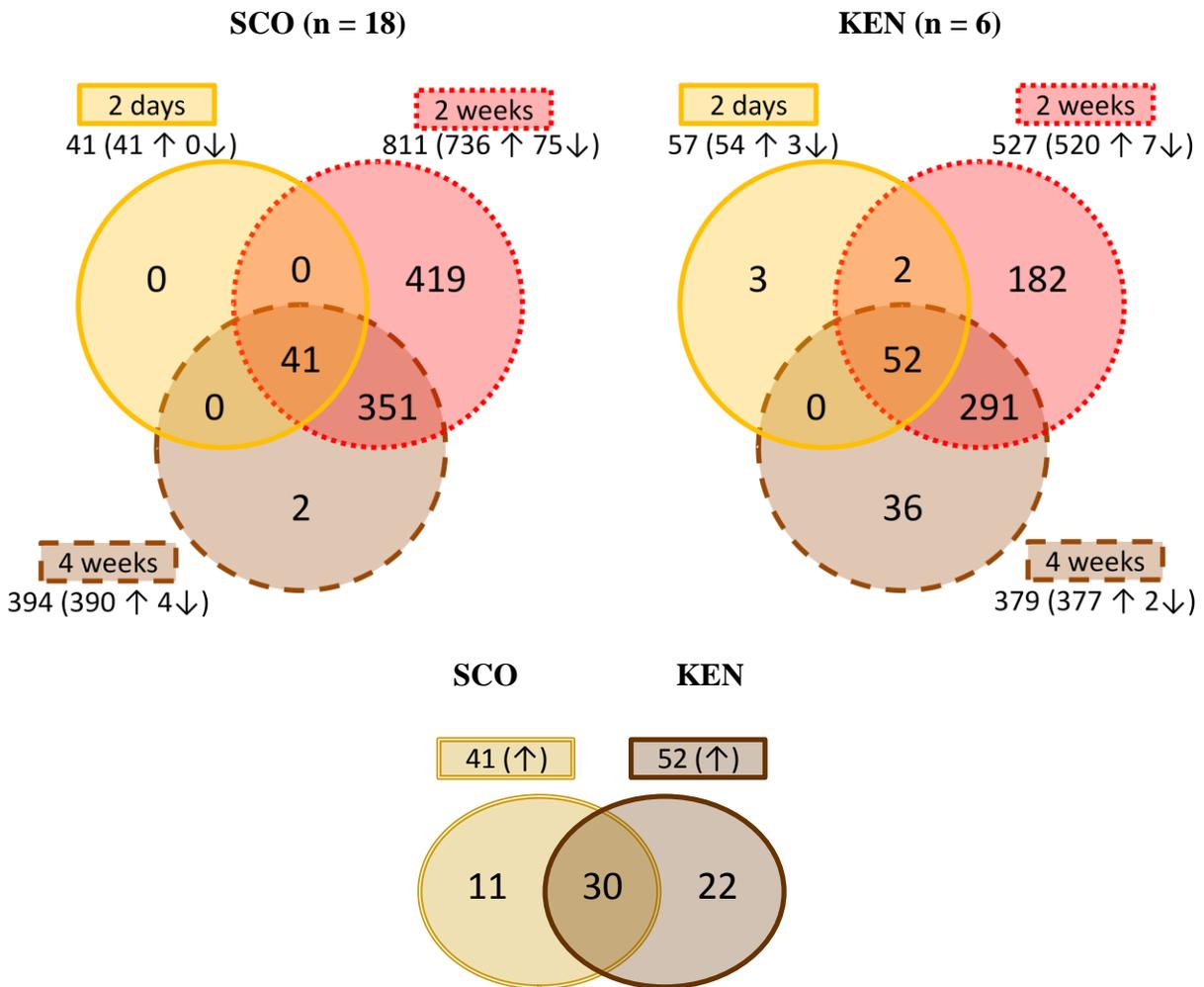
Figure 5.2: Venn diagrams of transcripts differentially expressed during rHuEpo compared to baseline in SCO and KEN.

The 3-way Venn diagrams (top panels) depicts the transcripts differentially expressed (5% false discovery rate and 1.5 fold-change threshold) 2 days (yellow, solid line circle), 2 weeks (red, dotted line circle) and 4 weeks (brown, dashed line circle) after the first rHuEpo injection compared to baseline, respectively. Values in brackets are number of transcripts up- (↑) and down-regulated (↓) compared to baseline. SCO (n = 18) and KEN (n = 6) are represented in the left and right top panels, respectively. The 2-way Venn diagram (bottom panel) depicts the intersection of the 3-way Venn diagrams.

Figure 5.3A: Individual changes in gene expression in the top three genes whose expression were altered by a mean fold change higher than 2 in both groups two days after the first rHuEpo injection.

Rap1 GTPase-activating protein 1 (*RAP1GAP*): Changes are reported in normalised intensity (left panels) and log ratio compared to the average baseline values (right panels). Time point 0 represents the first injection. The grey zone represents the rHuEpo administration. Each line corresponds to one subject. SCO (n = 18) and KEN (n = 6) are represented in green and red, respectively.
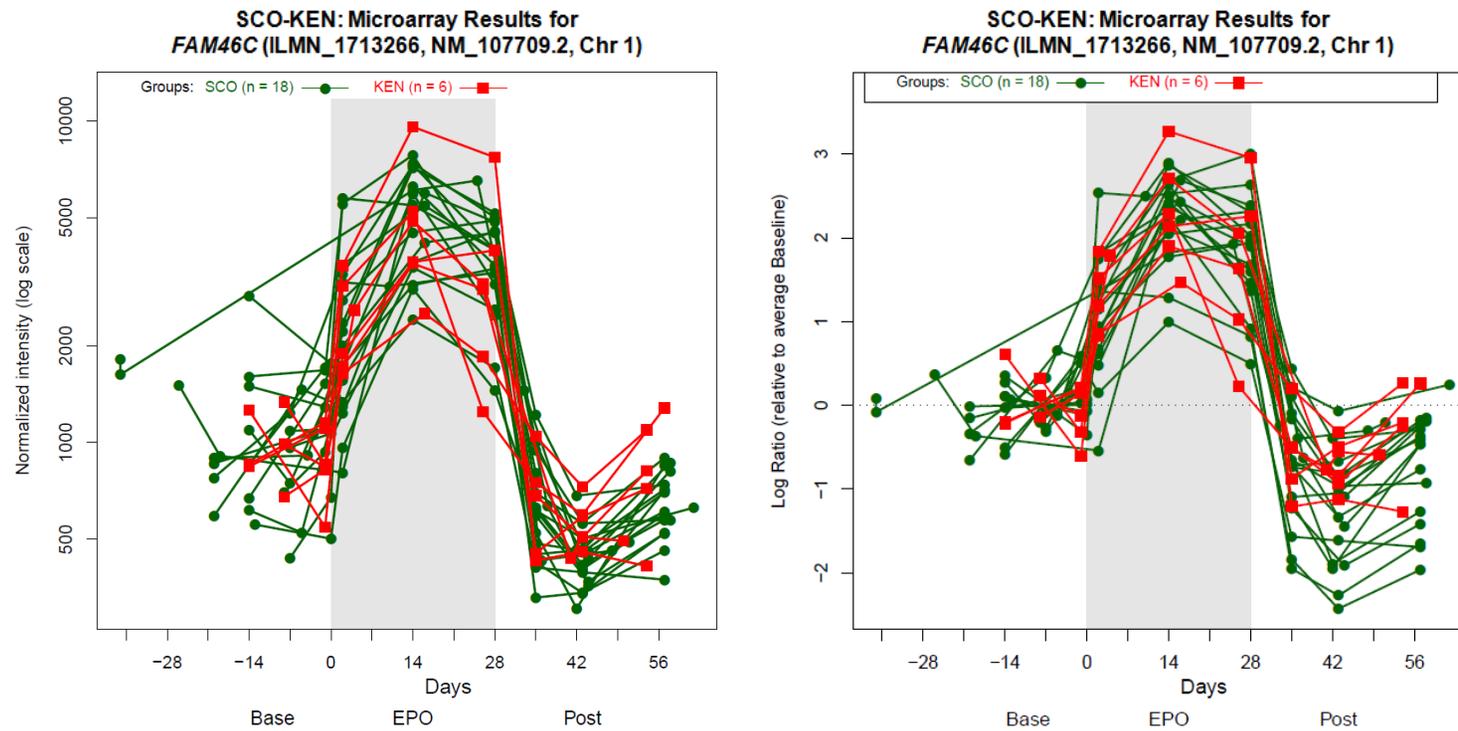
Figure 5.3B (continued): Family with sequence similarity 46 member C (*FAM46*): Changes are reported in normalised intensity (left panels) and log ratio compared to the average baseline values (right panels). Time point 0 represents the first injection. The grey zone represents the rHuEpo administration. Each line corresponds to one subject. SCO (n = 18) and KEN (n = 6) are represented in green and red, respectively.
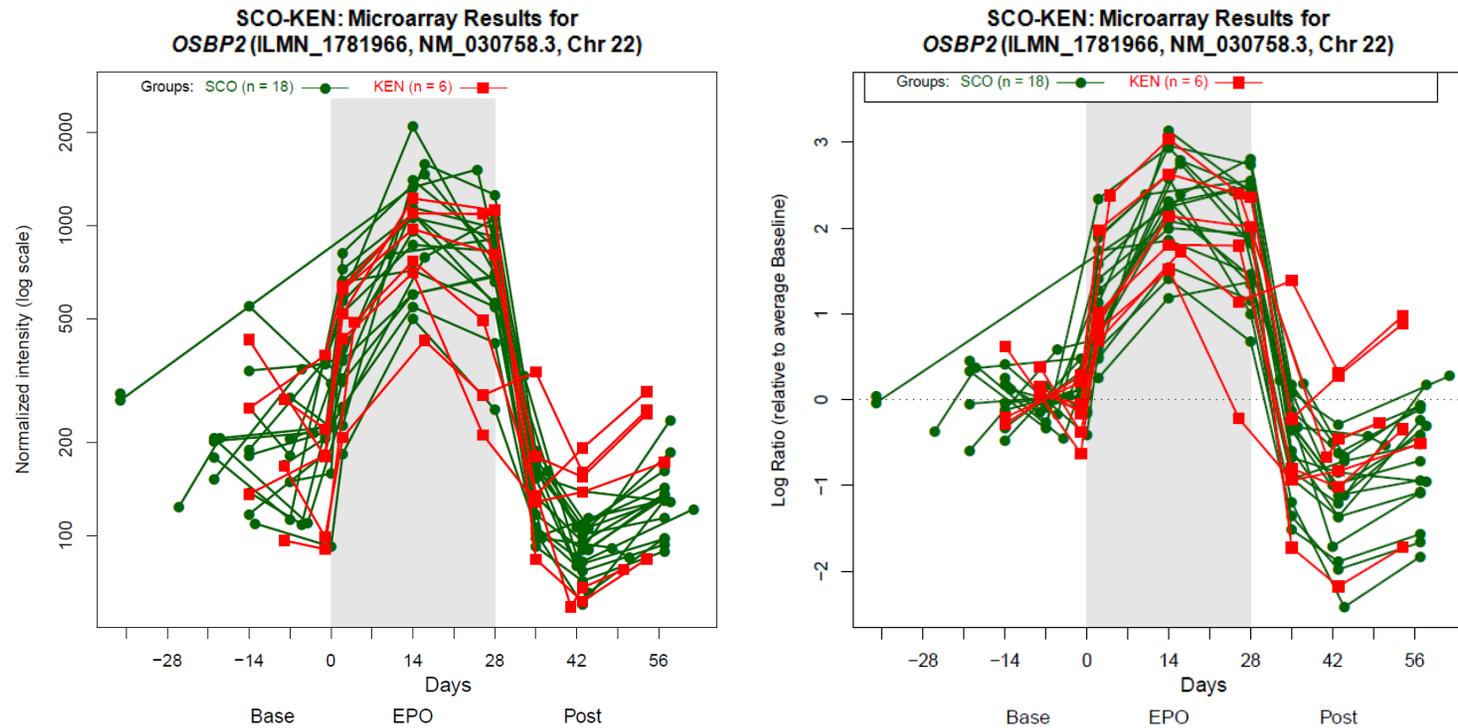
Figure 5.3C (continued): Oxysterol-binding protein 2 (*OSBP2*): Changes are reported in normalised intensity (left panels) and log ratio compared to the average baseline values (right panels). Time point 0 represents the first injection. The grey zone represents the rHuEpo administration. Each line corresponds to one subject. SCO (n = 18) and KEN (n = 6) are represented in green and red, respectively.
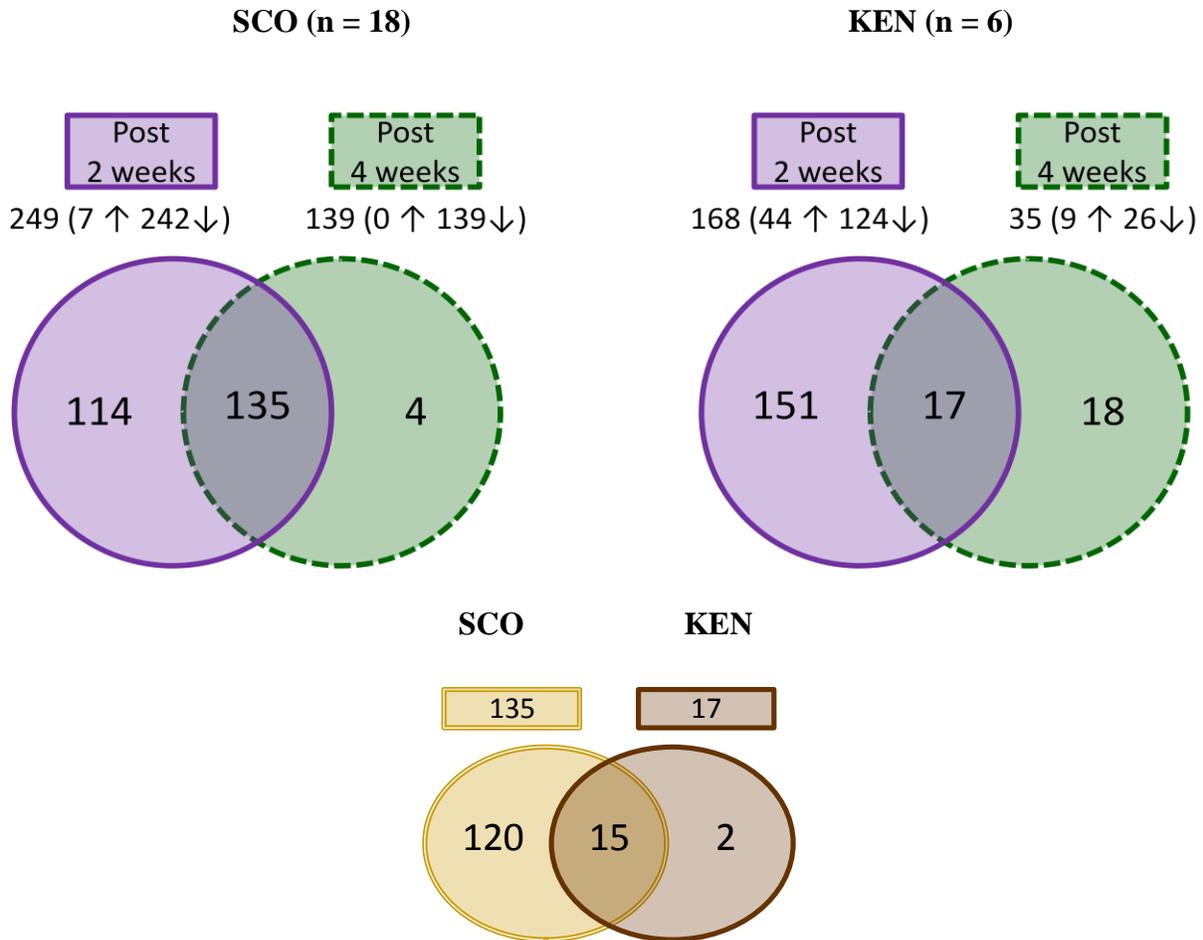
Figure 5.4: Venn diagrams of transcripts differentially expressed post rHuEpo compared to baseline in SCO and KEN.

The 2-way Venn diagrams in the top panels depict the transcripts differentially expressed (5% false discovery rate and 1.5 fold-change threshold) 2 weeks (purple, solid line circle) and 4 weeks (green, dashed line circle) after the rHuEpo administration compared to baseline, respectively. Values in brackets are number of transcripts up- (↑) and down-regulated (↓) compared to baseline. SCO (n = 18) and KEN (n = 6) are represented in the left and right top panels, respectively. The 2-way Venn diagram in the bottom panel depicts the intersection of the 2-way Venn diagrams in the top panels.
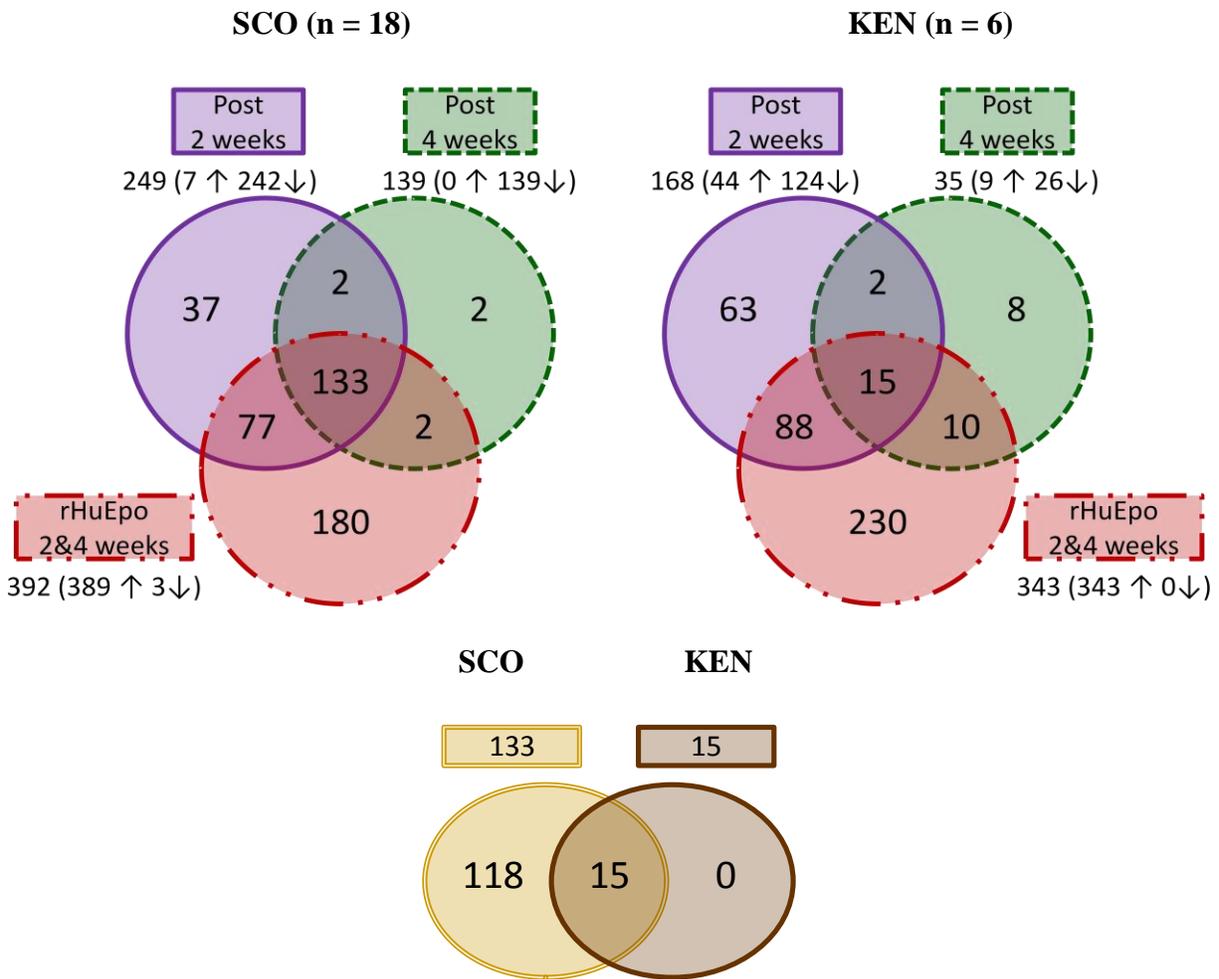
Figure 5.5: Venn diagrams of transcripts differentially expressed during and post rHuEpo compared to baseline in SCO and KEN.

The 3-way Venn diagrams (top panels) depicts the transcripts differentially expressed (5% false discovery rate and 1.5 fold-change threshold) during the last 2 weeks of rHuEpo (dark red, dashed and dotted line circle) as well as 2 weeks (purple, solid line circle) and 4 weeks (green, dashed line circle) post rHuEpo administration compared to baseline, respectively. Values in brackets are number of transcripts up- (↑) and down-regulated (↓) compared to baseline. SCO (n = 18) and KEN (n = 6) are represented in the left and right top panels, respectively. The 2-way Venn diagram (bottom panel) depicts the intersection of the 3-way Venn diagrams.

Figure 5.6A: Individual changes in gene expression in top three genes whose expression were down-regulated by a mean fold change higher than 4 in SCO and higher than 2 in KEN 2 weeks post rHuEpo administration compared to baseline.

Delta-aminolevulinate synthase 2 (*ALAS2*): Changes are reported in normalised intensity (left panels) and log ratio compared to the average baseline values (right panels). Time point 0 represents the first injection. The grey zone represents the rHuEpo administration. Each line corresponds to one subject. SCO (n = 18) and KEN (n = 6) are represented in green and red, respectively.
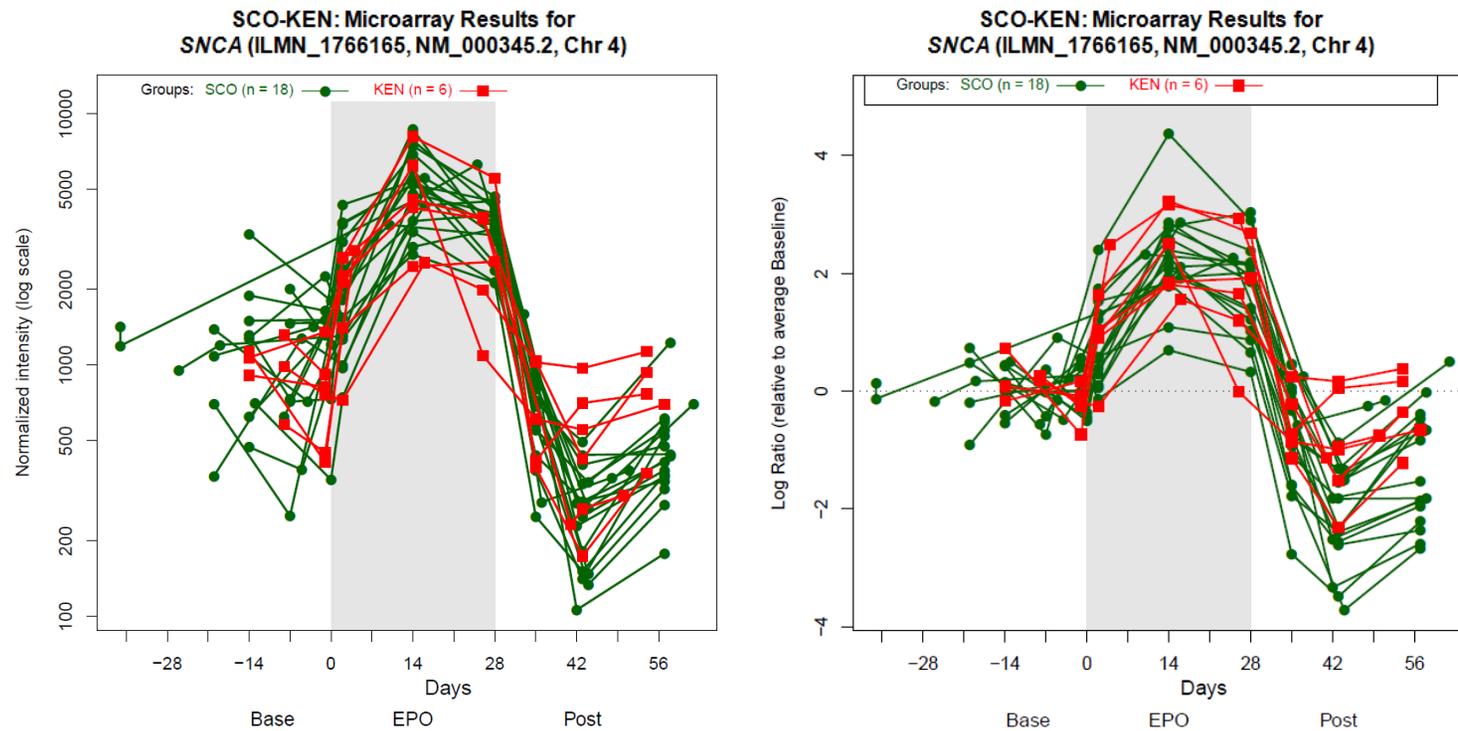
Figure 5.6B (continued): Alpha-synuclein (*SNCA* – 2 transcripts): Changes are reported in normalised intensity (left panels) and log ratio compared to the average baseline values (right panels). Time point 0 represents the first injection. The grey zone represents the rHuEpo administration. Each line corresponds to one subject. SCO (n = 18) and KEN (n = 6) are represented in green and red, respectively.
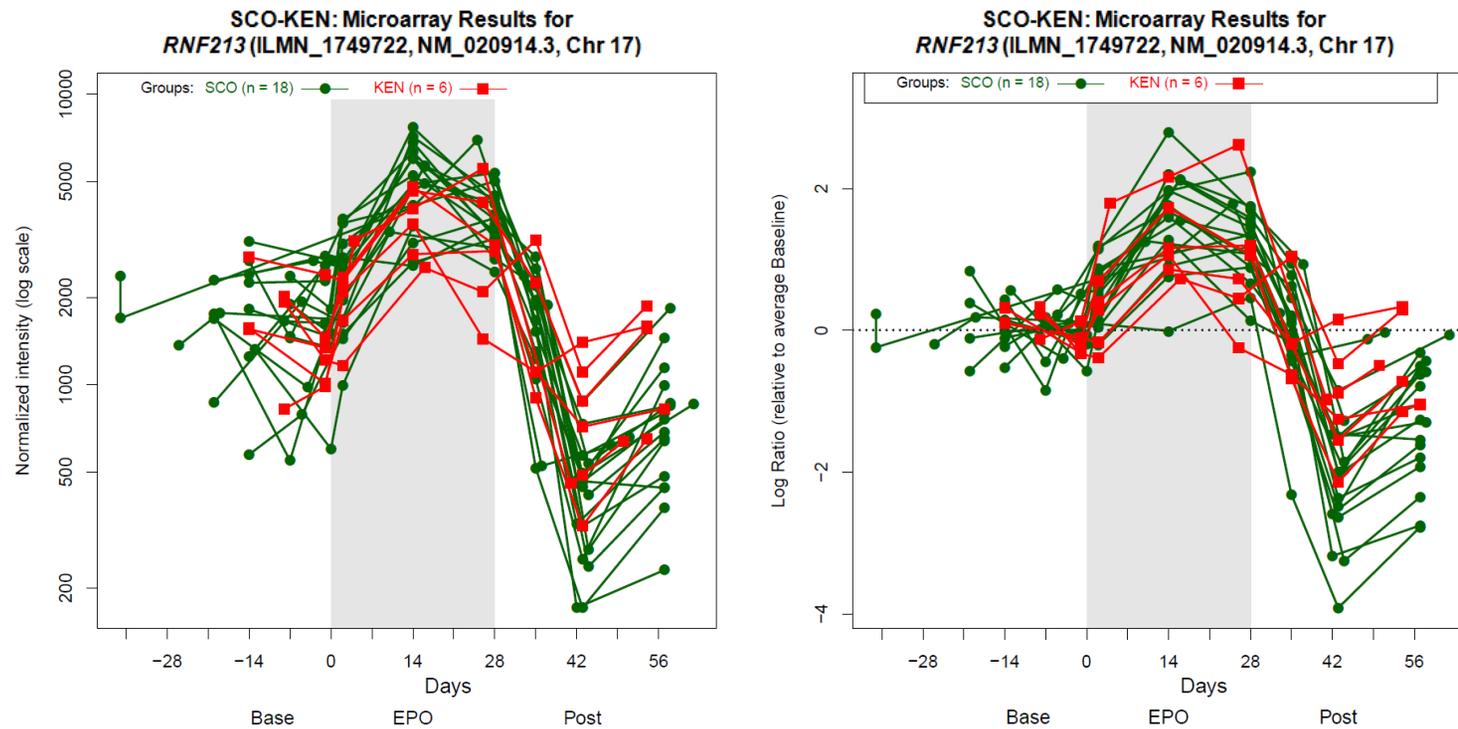
Figure 5.6C (continued): Ring finger protein 213 (*RNF213*): Changes are reported in normalised intensity (left panels) and log ratio compared to the average baseline values (right panels). Time point 0 represents the first injection. The grey zone represents the rHuEpo administration. Each line corresponds to one subject. SCO (n = 18) and KEN (n = 6) are represented in green and red, respectively.
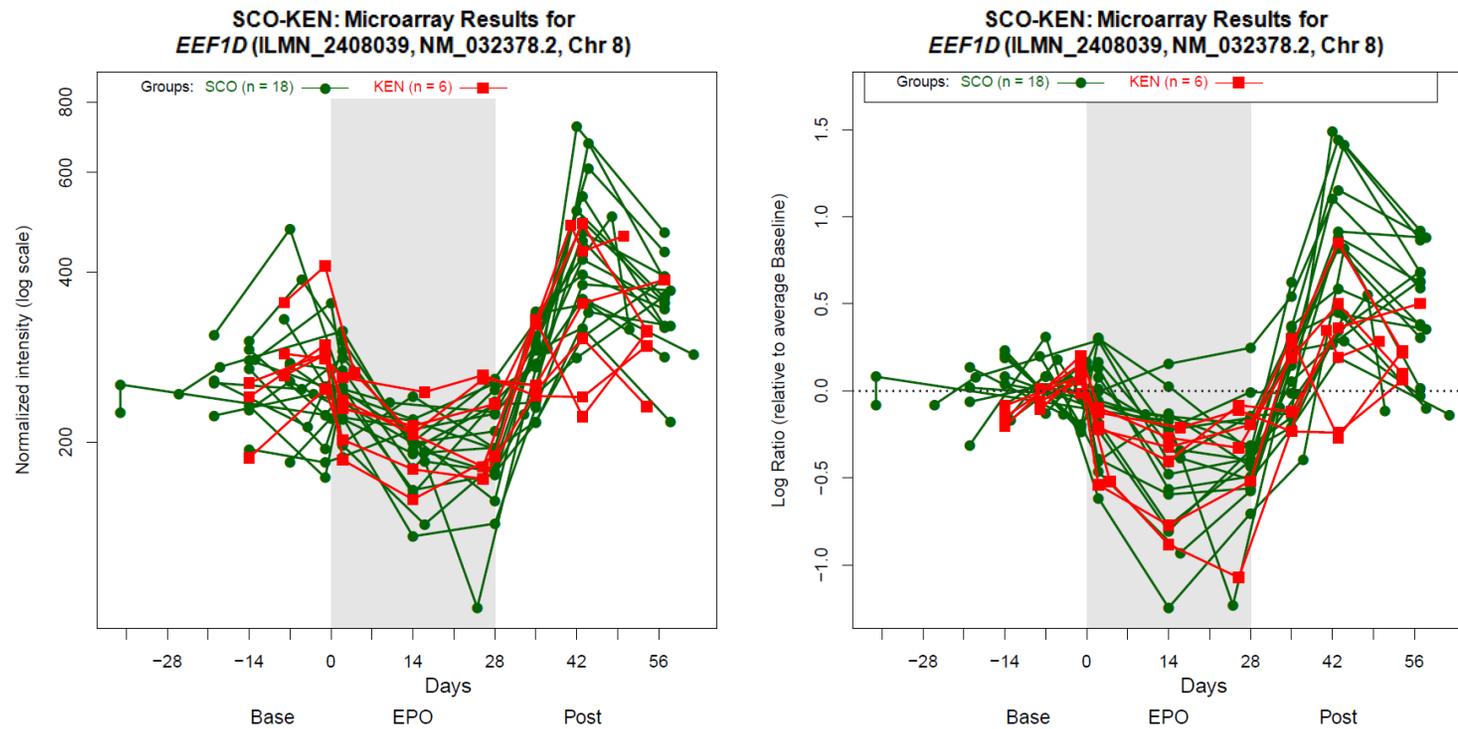
Figure 5.7A: Individual changes in gene expression in the genes which were down-regulated during rHuEpo and then up-regulated after administration.

Eukaryotic translation elongation factor 1 delta (*EEF1D*): Changes are reported in normalised intensity (left panels) and log ratio compared to the average baseline values (right panels). Time point 0 represents the first injection. The grey zone represents the rHuEpo administration. Each line corresponds to one subject. SCO (n = 18) and KEN (n = 6) are represented in green and red, respectively.
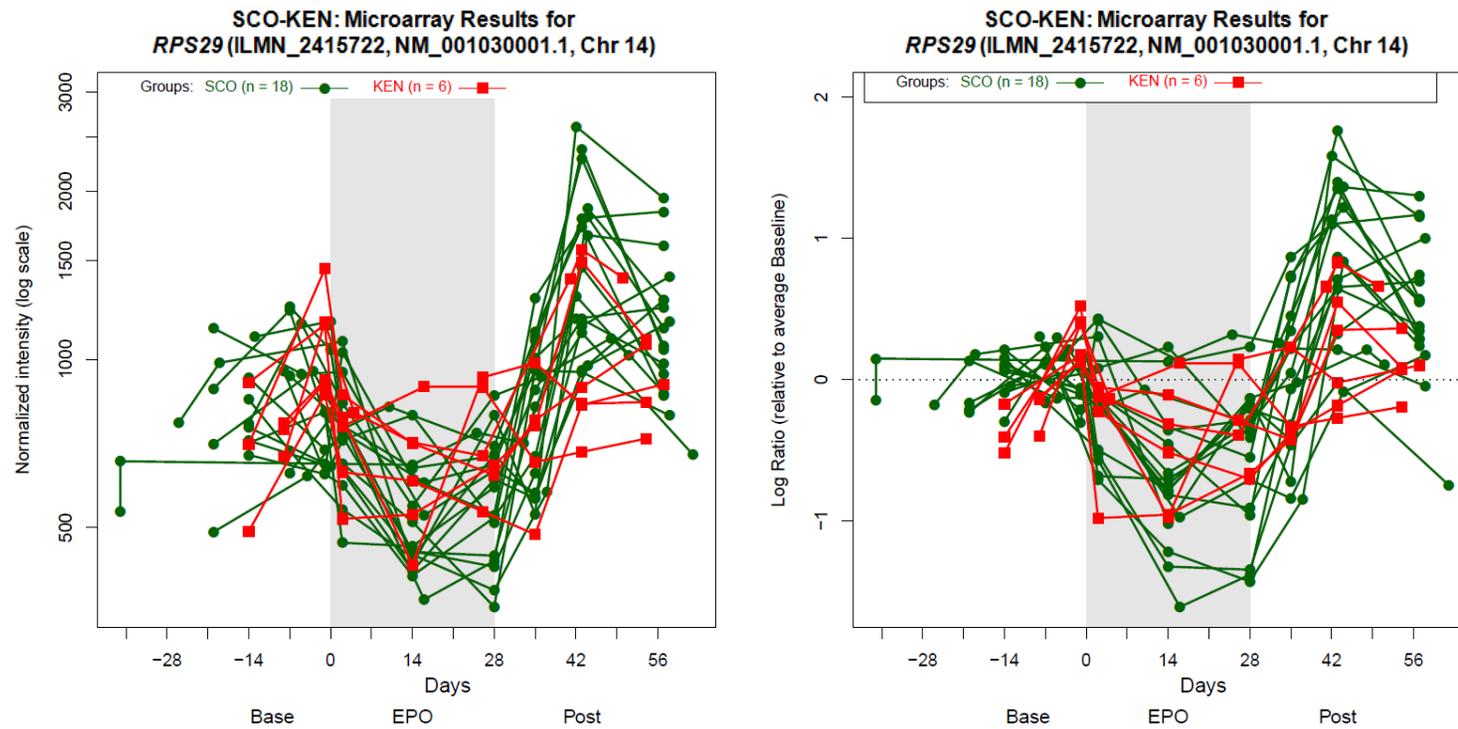
Figure 5.7B (continued): Ribosomal protein S29 (*RPS29*): Changes are reported in normalised intensity (left panels) and log ratio compared to the average baseline values (right panels). Time point 0 represents the first injection. The grey zone represents the rHuEpo administration. Each line corresponds to one subject. SCO (n = 18) and KEN (n = 6) are represented in green and red, respectively.
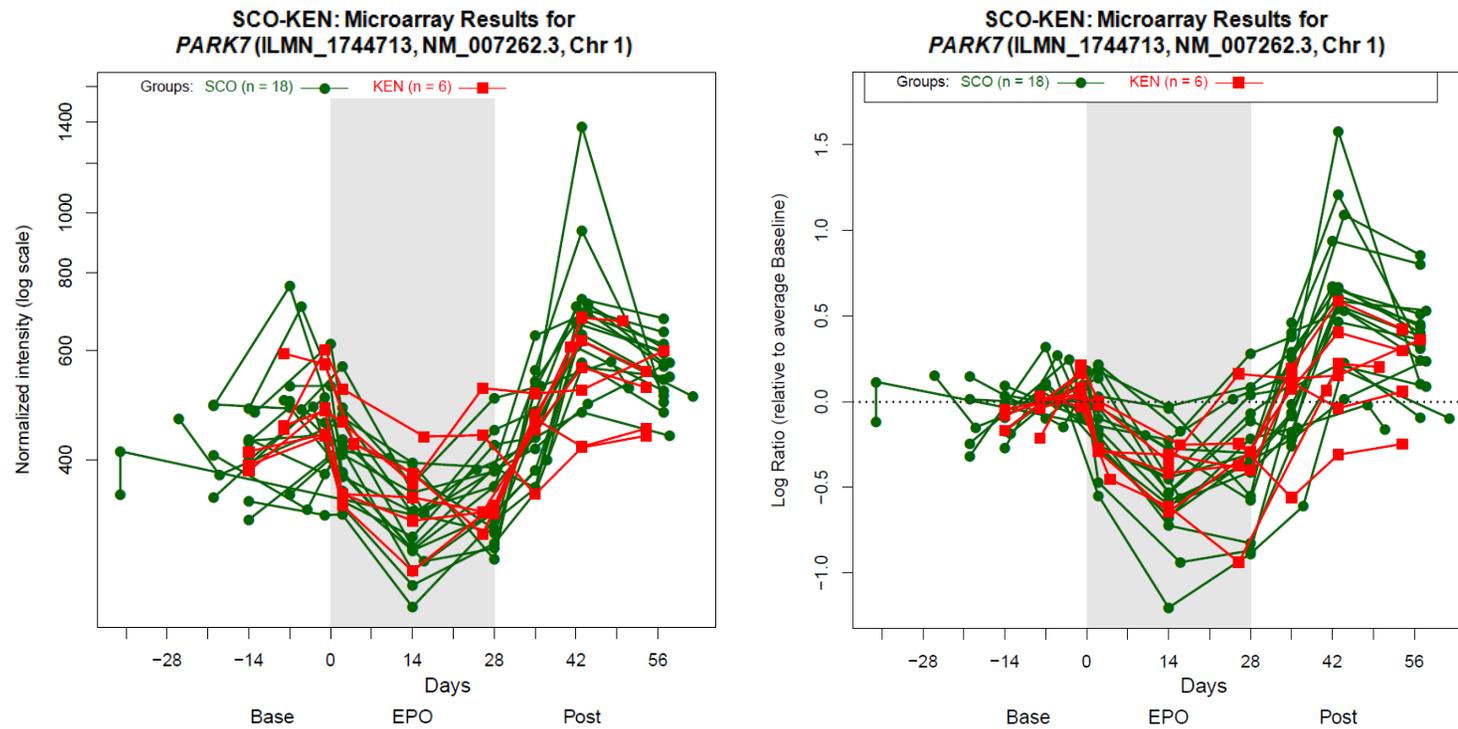
Figure 5.7C (continued): Parkinson protein 7 (*PARK7*): Changes are reported in normalised intensity (left panels) and log ratio compared to the average baseline values (right panels). Time point 0 represents the first injection. The grey zone represents the rHuEpo administration. Each line corresponds to one subject. SCO (n = 18) and KEN (n = 6) are represented in green and red, respectively.
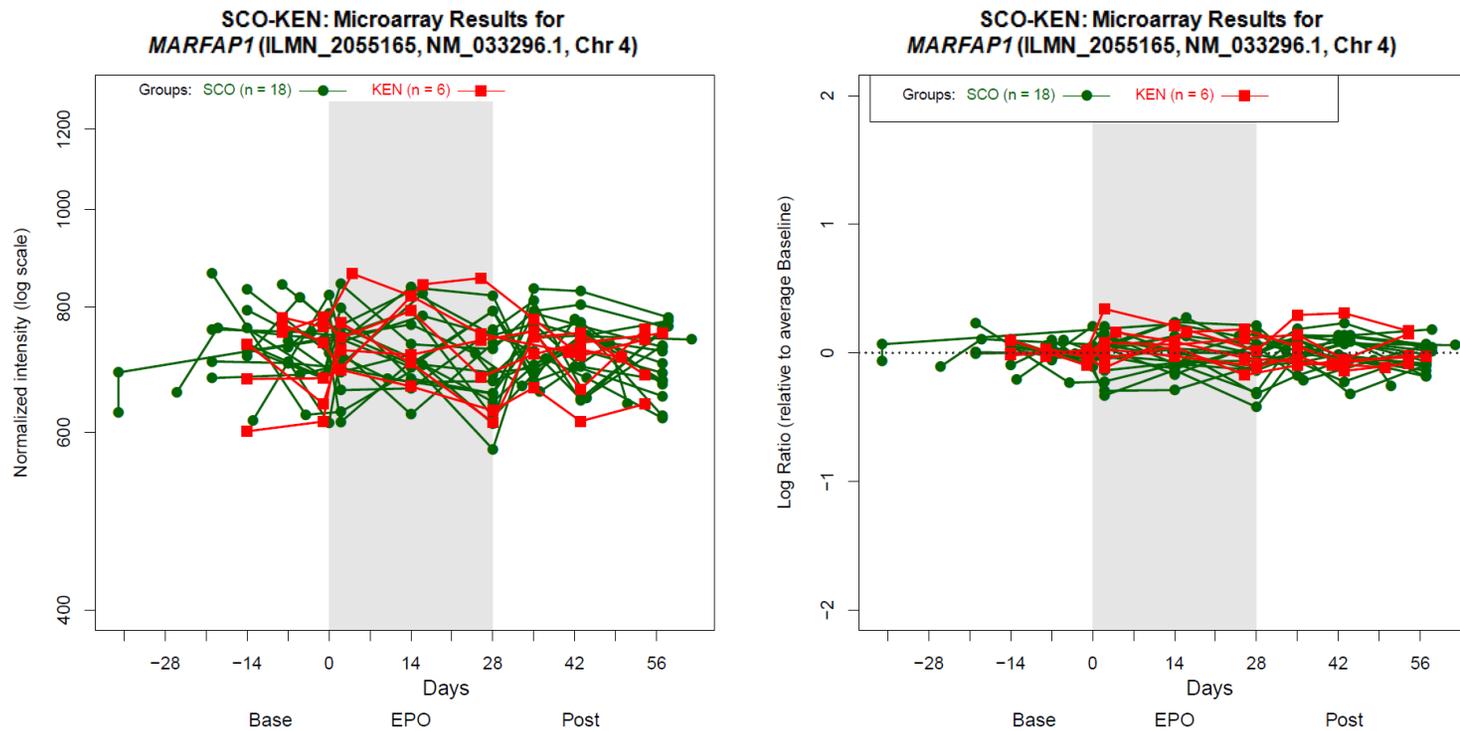
Figure 5.8: Individual changes in gene expression in the housekeeping gene MORF4 family-associated protein 1 (*MRFAP1*).

Changes are reported in normalised intensity (left panels) and log ratio compared to the average baseline values (right panels). Time point 0 represents the first injection. The grey zone represents the rHuEpo administration. Each line corresponds to one subject. SCO (n = 18) and KEN (n = 6) are represented in green and red, respectively.

Table 5.1. Common gene biomarkers in SCO and KEN differentially expressed during rHuEpo administration (including 2 days after the first rHuEpo injection).

Probe	Gene	SCO (n=18)	KEN (n=6)
ILMN_1776519	*RAP1GAP*	2.4 ± 1.8 (-1.1 ; 6.8)	2.2 ± 1.9 (1.3 ; 5.2)
ILMN_1713266	*FAM46C*	2.3 ± 1.8 (-1.9 ; 7.2)	2.4 ± 1.5 (1.5 ; 4.2)
ILMN_1781966	*OSBP2*	2.2 ± 1.5 (1.1 ; 4.1)	2.3 ± 1.9 (1.2 ; 4.9)
ILMN_1708323	*ALAS2*	1.9 ± 1.6 (-1.2 ; 4.0)	1.6 ± 2.1 (-1.7 ; 3.9)
ILMN_1652431	*CA1*	1.9 ± 1.6 (-1.1 ; 4.5)	2.2 ± 1.9 (-1.2 ; 5.0)
ILMN_3285762	*LOC100131164*	1.9 ± 1.6 (1.0 ; 3.7)	1.9 ± 2.0 (-1.3 ; 4.4)
ILMN_2352921	*BPGM*	1.8 ± 1.6 (-1.1 ; 4.6)	2.3 ± 1.8 (1.1 ; 6.1)
ILMN_1766165	*SNCA*	1.8 ± 1.6 (-1.1 ; 3.4)	1.9 ± 2.0 (-1.4 ; 5.0)
ILMN_1786328	*WDR40A*	1.8 ± 1.4 (1.1 ; 3.5)	1.9 ± 1.5 (1.2 ; 3.1)
ILMN_1683093	*GYPB*	1.8 ± 1.4 (-1.1 ; 3.1)	2.0 ± 1.7 (1.0 ; 3.7)
ILMN_1774091	*FECH*	1.8 ± 1.4 (1.0 ; 3.3)	1.9 ± 1.6 (1.0 ; 3.3)
ILMN_1654118	*BCL2L1*	1.7 ± 1.6 (-2.0 ; 3.5)	1.7 ± 1.5 (-1.0 ; 3.2)
ILMN_1680652	*SELENBP1*	1.7 ± 1.6 (-1.3 ; 3.7)	1.8 ± 1.9 (-1.3 ; 4.1)
ILMN_1701933	*SNCA*	1.7 ± 1.6 (-1.2 ; 4.2)	1.7 ± 1.8 (-1.4 ; 3.1)
ILMN_1814397	*EPB42*	1.7 ± 1.5 (-1.3 ; 3.0)	1.8 ± 1.7 (-1.0 ; 4.8)
ILMN_1678919	*YOD1*	1.7 ± 1.5 (1.0 ; 3.9)	1.7 ± 1.2 (1.4 ; 2.4)
ILMN_1735712	*KRT1*	1.7 ± 1.5 (1.0 ; 3.0)	1.8 ± 1.8 (-1.4 ; 4.8)
ILMN_1695187	*GYPE*	1.7 ± 1.4 (-1.2 ; 2.8)	1.9 ± 1.7 (1.0 ; 3.5)
ILMN_1801313	*SIAH2*	1.7 ± 1.4 (1.1 ; 3.5)	1.6 ± 1.3 (1.1 ; 2.5)
ILMN_1784207	*C1ORF128*	1.7 ± 1.4 (1.0 ; 3.0)	2.0 ± 1.5 (1.3 ; 4.0)
ILMN_2058782	*IFI27*	1.6 ± 1.9 (-3.7 ; 6.5)	2.1 ± 1.9 (-1.2 ; 5.0)
ILMN_1704079	*RBM38*	1.6 ± 1.5 (-1.4 ; 3.1)	1.6 ± 1.6 (-1.0 ; 3.2)
ILMN_1815527	*HBD*	1.6 ± 1.5 (-1.4 ; 3.0)	1.7 ± 1.8 (-1.2 ; 3.3)
ILMN_1704446	*SLC6A10P*	1.6 ± 1.5 (-1.4 ; 2.7)	1.5 ± 1.4 (-1.0 ; 2.5)
ILMN_1729487	*GMPR*	1.6 ± 1.5 (-1.3 ; 3.0)	1.7 ± 1.8 (-1.1 ; 3.8)
ILMN_3278170	*LOC441455*	1.6 ± 1.5 (-1.3 ; 2.9)	1.6 ± 1.7 (-1.4 ; 3.2)
ILMN_1759117	*XK*	1.6 ± 1.4 (1.1 ; 2.9)	1.8 ± 1.2 (1.3 ; 2.2)
ILMN_1708016	*C20ORF108*	1.5 ± 1.5 (-1.3 ; 2.6)	1.6 ± 1.7 (-1.3 ; 3.9)
ILMN_1736911	*TMOD1*	1.5 ± 1.5 (-1.2 ; 2.8)	1.7 ± 1.9 (-1.4 ; 3.9)
ILMN_2336335	*MARCH8*	1.5 ± 1.3 (-1.0 ; 2.4)	1.7 ± 1.4 (1.1 ; 3.1)

Values are fold-change at 2 days after the first rHuEpo injection compared to baseline. Values are means ± SD (minimum ; maximum).

Table 5.2. Common gene biomarkers in SCO and KEN differentially expressed during and post rHuEpo administration.

Probe	Gene	SCO (n=18)	KEN (n=6)
ILMN_2367126	ALAS2	-5.6 ± 1.9 (-18.7 ; -1.5)	-2.8 ± 1.3 (-3.6 ; -1.8)
ILMN_1701933	SNCA	-4.4 ± 1.9 (-12.5 ; -1.2)	-2.3 ± 1.7 (-5.2 ; -1.1)
ILMN_1766165	SNCA	-4.3 ± 1.9 (-14.1 ; -1.3)	-2.2 ± 1.7 (-5.2 ; -1.0)
ILMN_1749722	RNF213	-4.3 ± 1.9 (-13.9 ; 1.1)	-2.3 ± 1.7 (-4.9 ; -1.1)
ILMN_1651358	HBE1	-3.8 ± 1.8 (-10.6 ; -1.4)	-1.7 ± 1.3 (-2.6 ; -1.3)
ILMN_1701386	STRADB	-3.6 ± 1.8 (-9.4 ; -1.2)	-2.2 ± 1.4 (-3.5 ; -1.3)
ILMN_1804938	GPR175	-3.6 ± 1.8 (-8.5 ; 1.1)	-2.1 ± 1.5 (-3.8 ; -1.3)
ILMN_1815527	HBD	-3.5 ± 1.6 (-8.1 ; -1.5)	-2.0 ± 1.4 (-3.3 ; -1.1)
ILMN_1714765	LOC389599	-3.1 ± 1.7 (-8.1 ; -1.0)	-2.1 ± 1.3 (-2.7 ; -1.5)
ILMN_1715963	FBXO7	-3.0 ± 1.8 (-9.8 ; 1.2)	-1.8 ± 1.7 (-3.7 ; 1.1)
ILMN_1680652	SELENBP1	-3.0 ± 1.6 (-6.9 ; -1.3)	-2.2 ± 1.4 (-3.5 ; -1.4)
ILMN_2096322	ADIPOR1	-3.0 ± 1.6 (-6.8 ; -1.1)	-1.7 ± 1.4 (-2.6 ; -1.1)
ILMN_1786328	WDR40A	-2.9 ± 1.6 (-6.8 ; -1.2)	-1.9 ± 1.6 (-3.2 ; 1.1)
ILMN_1688322	ADIPOR1	-2.9 ± 1.6 (-6.1 ; 1.0)	-1.6 ± 1.4 (-2.4 ; -1.1)
ILMN_1652431	CA1	-2.4 ± 1.5 (-6.5 ; -1.3)	-1.8 ± 1.3 (-2.8 ; -1.3)

Values are fold-change at 2 weeks after the rHuEpo administration compared to baseline. Values are means ± SD (minimum ; maximum).

5.4 Discussion:

The major findings of the present study were that: firstly, that the discovered promising gene expression results found in Caucasian well trained males living and training at sea level (SCO) were successfully replicated in a cohort composed of Kenyan endurance runners living and training at moderate altitude (KEN) who underwent an identical rHuEpo administration regime; secondly, that only minimal differences in blood gene expression were found between the two groups. 15 transcripts were strongly up-regulated during rHuEpo and subsequently down-regulated up to 4 weeks after administration in both groups. In addition, 30 transcripts seemed to be very sensitive to rHuEpo injections as they were found to be already differentially expressed 2 days after only one single rHuEpo injection. As such, these latter transcripts are promising candidates to detect microdose rHuEpo doping strategies used by athletes (Ashenden *et al.* 2011; Ashenden *et al.* 2006). These results confirm that omics technologies such as gene expression have the potential to add a new dimension to the ABP to detect rHuEpo doping. However, the results of the present study still remain to be validated using another quantitative gene technology (see Chapter 6).

5.4.1 Transcripts altered by rHuEpo administration:

The major finding of the current project was that most of the transcripts that were found to be significantly altered by rHuEpo in SCO were also revealed in KEN. Thirty transcripts were sensitive to rHuEpo injections as they were found to be already differentially expressed 2 days after only one single rHuEpo injection. The top three genes were rap1 GTPase-activating protein (*RAP1GAP*), family with sequence similarity 46, member C (*FAM46C*) and oxysterol binding protein 2 (*OSBP2*). Rap1 is a small G protein of the Ras family involved in cell adhesion, cell proliferation and cell junction formation and which is regulated by guanine nucleotide exchange factors and GTPase-activating proteins including RAP1GAP (Gloerich and Bos 2011). Rap1 seems to play an important role in the cardiovasculature, such as platelet aggregation, angiogenesis, endothelial permeability, and myocyte growth (Jeyaraj *et al.* 2011). Unlike the other genes, where a similar pattern of changes in expression was observed in all subjects, the changes in *RAP1GAP* expression levels varied considerably between subjects. While *RAP1GAP* expression remained fairly constant throughout the study in four subjects, moderate and strong up- and subsequent down-regulation patterns were observed in the remaining subjects. It is,

nevertheless, difficult to speculate on the possible reasons for this finding due to the large diversity of mechanisms of rap1. The precise function of FAM46C remains unknown, however, mutation of FAM46C was found to be associated with multiple myeloma and was suggested to play a role in the regulation of translation as an mRNA stability factor (Chapman *et al.* 2011). OSBP2 was found to be mainly expressed in the retina but also in pineal, testis and fetal liver and it was suggested that OSBP2 may play a role in macular degeneration, however, despite 19 different tissues analyzed, whole blood was not included in this study (Moreira *et al.* 2001). It has been shown that expression of OSBP2 was increased in blood samples from patients with cholangiocarcinoma, a highly malignant tumor originating from bile duct epithelial cells (Loilome *et al.* 2012). In order to minimise the risk of being caught via current detection methods by inducing only minor fluctuations in the ABP blood markers and/or by "normalizing" these markers after blood manipulations such as autologous blood transfusion, athletes are now using the so-called "micro doses" of rHuEpo which allegedly range from 10 to a maximum of 40 IU·kg^{-1} body mass (Ashenden *et al.* 2011; Ashenden *et al.* 2006; Morkeberg *et al.* 2013). Indeed, Ashenden *et al.* have demonstrated that the ABP did not reveal any suspicious doping activities when a rHuEpo microdosing strategy was used (Ashenden *et al.* 2011). Except *RAP1GAP*, whose expression was highly variable across the subjects, the other genes are very promising candidates to detect microdose rHuEpo doping as a result of their high sensitivity to rHuEpo administration.

Fifteen transcripts were strongly up-regulated during rHuEpo and subsequently down-regulated up to 4 weeks after administration in both groups. The top three genes were delta-aminolevulinate synthase 2 (*ALAS2*), alpha-synuclein (*SNCA* – 2 transcripts) and ring finger protein 213 (*RNF213*). As previously described (Chapter 3), ALAS2 catalyzes the initial step in the haem pathway (Sadlon *et al.* 1999). The increase and subsequent decrease in the *ALAS2* expression was representative of the rHuEpo-induced changes in haemoglobin production illustrated by the changes in haematological parameters (Chapters 2 and 4). As previously discussed (Chapter 3), SNCA was initially thought to be "expressed only in nervous system tissue" (Maroteaux *et al.* 1988). However, as previously described, SNCA is highly present in the erythrocyte and is coexpressed with haem metabolism genes including *ALAS2* (Bartels *et al.* 2011; Fauvet *et al.* 2012; Scherzer *et al.* 2008). RNA genome-wide association study identified

RNF213 as the first gene associated to Moyamoya disease in which blood flow to the brain is restricted due to arterial stenosis (Kamada *et al.* 2011). It has been suggested that RNF213 is involved in novel signaling pathway in intracranial angiogenesis (Liu *et al.* 2011). Fewer transcripts were found to be significantly differentially expressed post rHuEpo administration compared to baseline in KEN relative to SCO. This finding can be explained either by a potential lack of power (*i.e.* 6 KEN were compared to 18 SCO[5]) or by a faster readjustment post rHuEpo towards baseline gene expression levels in KEN compared to SCO as illustrated, for instance, by the reticulocyte percentage and RNA yield (see Appendix A).

Unlike the majority of the dysregulated transcripts, a few genes such as eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein) (*EEF1D*), ribosomal protein S29 (*RPS29*) and parkinson protein 7 (*PARK7*) were found to be down-regulated during rHuEpo administration and then up-regulated post administration. Of note, PARK7 as well as SNCA are involved in Parkinson's disease. However, the usefulness of these genes and related proteins as biomarkers of Parkinson's disease depends on the tissue investigated such as human cerebrospinal fluid compared with plasma (Hong *et al.* 2010; Shi *et al.* 2010). In a similar manner, the biomarkers of rHuEpo administration discovered in whole blood in this research are not necessarily useful in other tissues such as monocytes (Jie *et al.* 2012) or saliva (see Appendix E). The same gene can have different function(s) in different tissue. As such, because the results of the present study are only based on whole blood, and although it has been demonstrated that rHuEpo had numerous positive effects in preclinical neurodegenerative disease models (Sargin *et al.* 2010), it would be adventurous to discuss in the present thesis the potential link between rHuEpo and neurodegenerative diseases such as Parkinson's disease. EEF1D is a subunit of the elongation factor 1, which catalyzes the transfer of aminoacyl-tRNA onto ribosomes. Elongation factor 1 plays a critical role in regulating gene expression and particularly during the cell cycle (Le Sourd *et al.* 2006; Sasikumar *et al.* 2012). Using a zebrafish model, it has been demonstrated that RPS29 is involved in the development of red blood cells and in the regulation of the cell cycle (Taylor *et al.* 2012). Using cells cultures model, it has been revealed that PARK7 plays an important role in protecting red blood cells against oxidative damage (Xu *et al.* 2010).

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[5] As stated in Chapter 7 and Appendix F, 12 more KEN subjects will be analysed to make a balanced microarray analysis.

5.4.2 Housekeeping genes:

Only one gene, MORF4 family-associated protein 1 (*MRFAP1*), was commonly in the top 100 best housekeeping genes in SCO and KEN. MRFAP1 protein was found to be only detectable in a small number of human tissues, in particular testis and brain but blood was not included in the analysis (Larance *et al.* 2012). Regardless of its exact function in whole blood, MRFAP1 was a relatively abundant transcript with low inter- and intra-subject variation over time and can therefore be used as a consistent housekeeping gene for both SCO and KEN samples.

5.4.3 Non-responders to rHuEpo administration:

As previously discussed in Chapter 4, based on the blunted response in the haematological parameters and exercise performance, one Kenyan subject (white triangle in Figure 4.2, Figure 4.4 and Figure 4.5) did not respond to rHuEpo administration and may therefore be representative of the 10% of the treated patients who are characterized by either no response or a suboptimal response to rHuEpo therapy (Macdougall 1995). There are several recognized causes of resistance or hyporesponsiveness to rHuEpo treatment such as iron deficiency and inflammatory conditions, however, exclusion of all known factors does not eliminate the marked variability in the sensitivity to rHuEpo (Kanbay *et al.* 2010; Macdougall and Cooper 2002). It has been shown in a small cohort of 10 patients with myelodysplastic syndromes that there were significant differences in gene expression between patients who are responsive or non-responsive to rHuEpo (Cortelezzi *et al.* 2008). Gene expression profile assessment may have the potential to provide a diagnostic test to predict the response to rHuEpo of anaemic patients. However, the gene response profile of the "non-responder" in our study did not seem to significantly differ from the other subjects. Although several genes which were found to be differentially expressed between responsive and non-responsive patients after rHuEpo administration were also found to be altered by rHuEpo in our study, such as Mitogen-activated protein kinase 3 (*MAP2K3*) and *RAP1GAP*, it is difficult to interpret the results further due to the difference in study design and methodology (Cortelezzi *et al.* 2008). In particular, RNA was extracted from bone marrow cells and response over time was not evaluated (Cortelezzi *et al.* 2008). Nevertheless, as this doctoral research provides valuable data on the "normal" blood gene expression response to rHuEpo in healthy volunteers, it would now be of interest, using the same methodology, to longitudinally investigate the blood gene expression response in anaemic patients receiving rHuEpo in order to

better understand as well as hopefully predict and improve the responsiveness to rHuEpo therapy.

5.4.4 Conclusion:

In conclusion, the blood "molecular signature" of rHuEpo doping discovered in Caucasian well trained males living and training at sea level (SCO) was successfully replicated in a cohort composed of Kenyan endurance runners living and training at moderate altitude (KEN) who underwent an identical rHuEpo administration regime. In addition to suggesting that ethnicity and residence at altitude have only minor influence on the effects of rHuEpo on blood gene expression profiles, these results confirm that omics technologies such as gene expression have the potential to add a new dimension to the ABP to detect rHuEpo doping. However, these results should be verified using another quantitative gene platform in order to provide further confidence in the validity of the findings (Allison *et al.* 2006; Chuaqui *et al.* 2002) (see Chapter 6).

Chapter 6 : Validation of the Newly Discovered Blood Gene Biomarkers of Recombinant Human Erythropoietin Administration

6.1 Introduction and aim:

In this doctoral research, gene microarray technology was successfully used to discover genes that are differentially regulated following rHuEpo administration in Caucasian trained males living at or near sea-level (Chapter 3). Thereafter, part of the operational validation process (Allison *et al.* 2006; Chuaqui *et al.* 2002), the same technology and methodology was successfully used to replicate the blood gene expression results in another cohort composed of Kenyan runners living at moderate altitude that underwent an identical rHuEpo administration regime (Chapter 5). In order to provide further confidence in the validity of the findings, a constructive validation which aims to replicate the results using another quantitative gene technology was required (Allison *et al.* 2006; Chuaqui *et al.* 2002).

The methodology used for the constructive validation varies depending on the research hypothesis of the study and is influenced by practical factors such as the quantity of sample material and the number of gene targets to be validated (Chuaqui *et al.* 2002). The QuantiGene Plex assay, which combines branched DNA and bead-based multiplexing technology, allows for the simultaneous analysis of a large number of target genes per sample (up to 500) and enables high-throughput screening (Flagella *et al.* 2006). In addition to being extremely reproducible, specific and sensitive, the QuantiGene Plex assay provides an independent method of measurement compared to the other technologies such as quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and microarray platforms by eliminating the need for RNA reverse transcription, labelling and amplification steps (Canales *et al.* 2006; Flagella *et al.* 2006). The aim of the present project was to validate the microarray-based results of a subset of promising target genes whose expression was altered following rHuEpo administration using the QuantiGene Plex 2.0 assay.

6.2 Methods:

6.2.1 Samples:

RNA samples of four subjects in each group of SCO and KEN (*i.e.* eight subjects in total including the "non-responder"), collected at the same 8 time points as previously described for the microarray analysis (*i.e.* two baseline samples, then one sample each at 2 days, 2 weeks and 4 weeks after the first rHuEpo injection and 1 week, 2 weeks and 4 weeks after rHuEpo administration) (Figure 2.1) and stored at -80°C after extraction from the Tempus Blood RNA tubes, were used for the QuantiGene Plex analysis (Panomics/Affymetrix, Vignate-Milano, Italy) (Figure 3.1).

6.2.2 QuantiGene Plex assay protocol:

The QuantiGene Plex 2.0 assay combines branched DNA (bDNA) signal amplification and xMAP profiling magnetic beads technologies. The bDNA enables target RNA quantification by amplifying the reporter signal rather than target sequences and the xMAP fluorescent microspheres or "beads" are used as a support to capture specific RNA molecules (Flagella *et al.* 2006). The signal amplification mediated by bDNA amplification technology consists of sequential hybridizations of preamplifiers, amplifiers and label probes. The RNA of target genes is first captured via hybridization using specific probe sets which are composed of capture extenders, labels extenders and blockers (Figure 6.1). The capture and label extenders contain oligonucleotides that are complementary to the target RNA as well as to the capture probes of the microsphere and the bDNA amplifier molecule, respectively, while the blockers contain oligonucleotides that are complementary to the target RNA where the two other type of probes cannot bind in order to maintain the RNA-DNA structure intact (Flagella *et al.* 2006) (Figure 6.1). By adding Strepatvidin-conjugated R-Phycoerythrin (SAPE) that binds to the label probes, the resulting fluorescence signal associated with the individual microspheres is read using a flow cytometer Luminex instrument. The fluorescence signal measured from each bead is proportional to the number of captured transcripts.

Step 1: Target transcripts are captured to their corresponding magnetic beads through the hybridization interactions of the capture probes (of the beads) and the capture extenders.
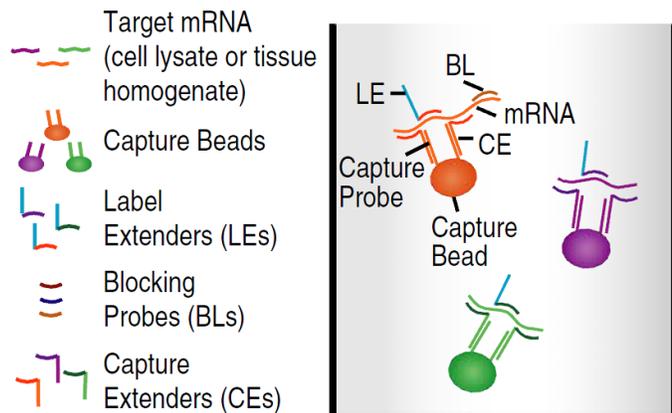
Step 2: The signal amplification is mediated by the bDNA amplifier and the label probe molecules that hybridize to the tails of the label extenders.
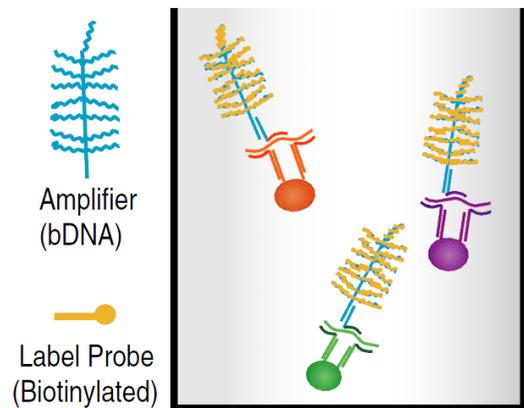
Step 3: Each amplification unit contains multiple hybridization sites for biotinalyted label probe that captures SAPE. The resulting fluorescence signal, which is proportional to the number of captured transcripts, is then read on a Luminex.
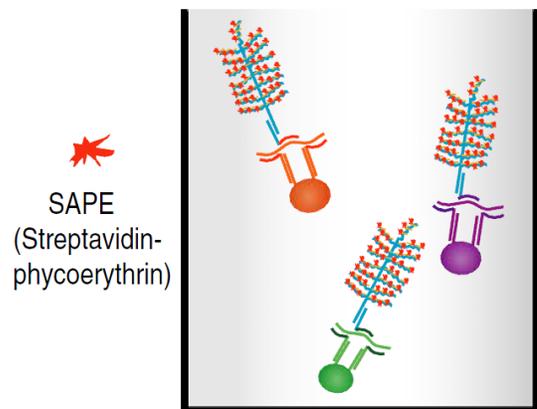
Figure 6.1: Overview of the workflow of the QuantiGene Plex assay adapted from (Flagella *et al.* 2006).

Each RNA sample was run in duplicate with two different sample RNA inputs. Sample RNA inputs of 500 ng and 50 ng (experiment 1 using 24 samples from 3 subjects) or 200 ng and 100 ng (experiment 2 using 40 samples from 5 subjects) were used. The fluorescence signal was read on the MAGPIX (Luminex, Oosterhout, The Netherlands) and median fluorescence intensity (MFI) values were saved using xPonent software.

6.2.3 QuantiGene data analysis:

Following background subtraction, the ratio of the target genes MFI to the geometric mean of the housekeeping genes MFI was calculated. Fold change compared to the average baseline values was then calculated and log2 transformed.

6.2.4 Selection of the target and housekeeping genes for QuantiGene Plex analysis:

Seven target genes were selected based on their fold change, their sensitivity to the first rHuEpo injection, their down-regulation post rHuEpo administration and the availability of the transcripts in the database of the company (Table 6.1). In addition, the common best housekeeping gene in SCO and KEN discovered using microarray (*MRFAP1*) as well two housekeeping genes from the database of the company were selected (Table 6.1).

Table 6.1. Selected genes for QuantiGene analysis

N°	Genes	Names	Type	ILMN_GENE	ACCESSION
1	*ACTB*	Actin, beta	Housekeeping gene	ILMN_2038777	NM_001101
2	*ALAS2*	Delta-aminolevulinate synthase 2	Target	ILMN_1708323	NM_000032
3	*BPGM*	2,3-bisphosphoglycerate mutase	Target	ILMN_2352921	NM_001724
4	*CA1*	Carbonic anhydrase 1	Target	ILMN_1652431	NM_001738
5	*FAM46C*	Family with sequence similarity 46, member C	Target	ILMN_1713266	NM_017709
6	*MRFAP1*	MORF4 family-associated protein 1	Housekeeping gene	ILMN_2055165	NM_033296
7	*OSBP2*	Oxysterol-binding protein 2	Target	ILMN_1781966	NM_030758
8	*PPIB*	Peptidylpropyl isomerase B (cyclophilin B)	Housekeeping gene	ILMN_1703622	NM_000942
9	*SELENBP1*	Selenium binding protein 1	Target	ILMN_1680652	NM_003944
10	*SNCA*	Alpha-synuclein	Target	ILMN_1766165	NM_000345

6.3 Results:

6.3.1 Housekeeping genes:

Beta actin (ACTB) was the most abundant and the most stable of the three housekeeping genes (Table 6.2 and Figure 6.2). However, overall, all three housekeeping genes were relatively consistently expressed over time in all subjects and at levels relatively similar to the target genes (Table 6.2 and Figure 6.2).

6.3.2 Sample RNA input:

The reproducibility of the results from duplicate samples using different RNA sample input was good (Figure 6.3). However, Luminex instruments exhibit saturation over ~20,000 MFI and highly abundant genes such as *ALAS2* and *FAM46C* often reached this threshold with RNA sample input ≥ 200 ng and even with 100 ng in some cases for *ALAS2* (Table 6.2 and Figure 6.3). On the other hand, RNA sample input lower than 100 ng may not be sufficient for the low expressed transcript such as *OSBP2*, whose signal may be confounded with the background noise (Table 6.2, Figure 6.3).

6.3.3 Target genes:

The correlation between QuantiGene Plex and microarray data was good and the QuantiGene Plex results overall replicated the Illumina microarray results (Figure 6.4 and Figure 6.5). The down-regulation in gene expression post rHuEpo administration was slightly more profound using the QuantiGene Plex compared to the Illumina microarray analysis (Figure 6.5). The relative expression of the target genes varied from very high, such as *ALAS2*, to very low, such as *OSBP2* (Table 6.2). Although the relative expression of the latter gene was close to the background noise, especially with low RNA sample inputs, the up- and subsequent down-regulation profile observed during and post rHuEpo administration using the Illumina microarray technology was nevertheless replicated (Table 6.2, Figure 6.3 and Figure 6.4E). Log ratios of relative expression target to housekeeping genes are presented in Figure 6.6.
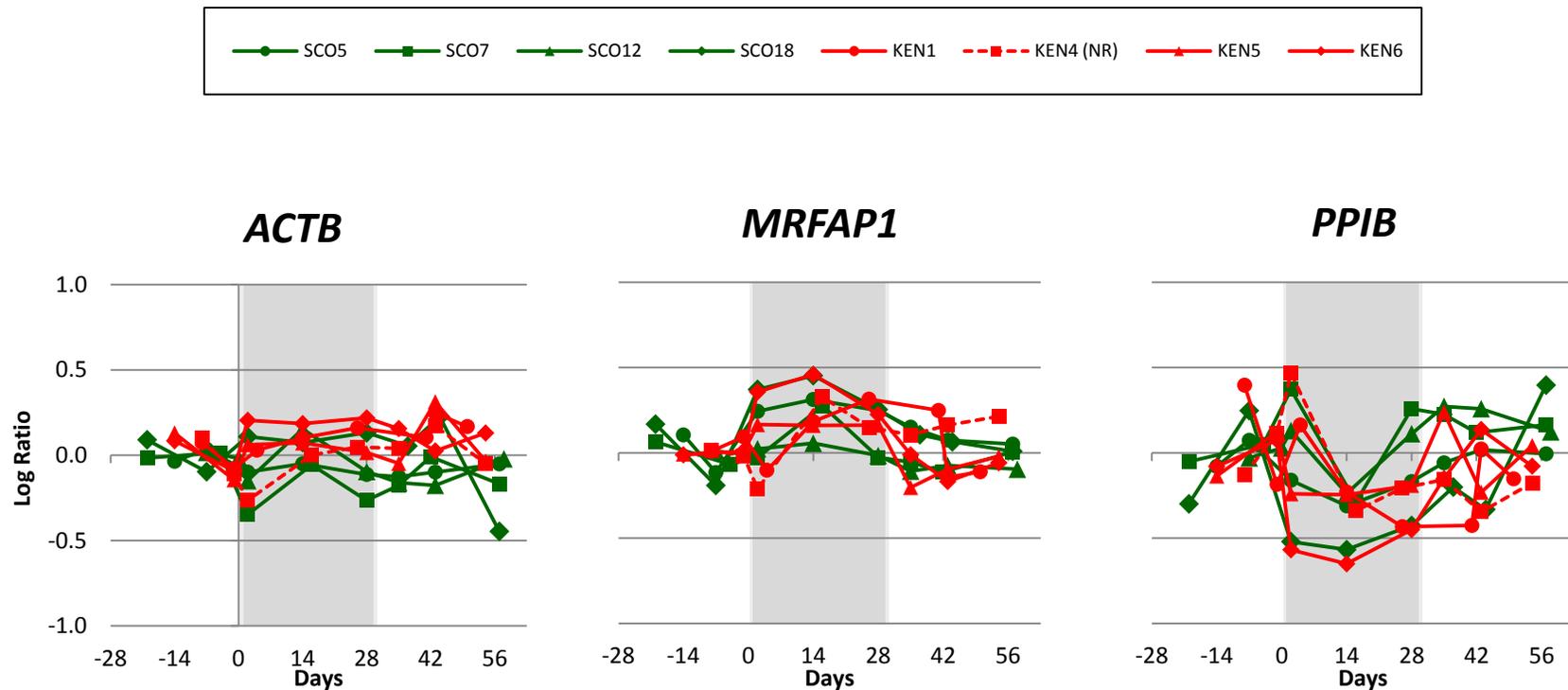
Figure 6.2: Individual changes in gene expression in the three housekeeping genes using the QuantiGene Plex assay.

Actin, beta (*ACTB*), MORF4 family-associated protein 1 (*MRFAP1*) and peptidylpropyl isomerase B (cyclophilin B) (*PPIB*): Changes are reported in log ratio compared to the average baseline values. Time point 0 represents the first injection. The grey zone represents the administration. Each line corresponds to one subject. SCO (n = 4) and KEN (n = 4) are represented in green and red, respectively. The dashed line corresponds to the "non-responder" (NR).
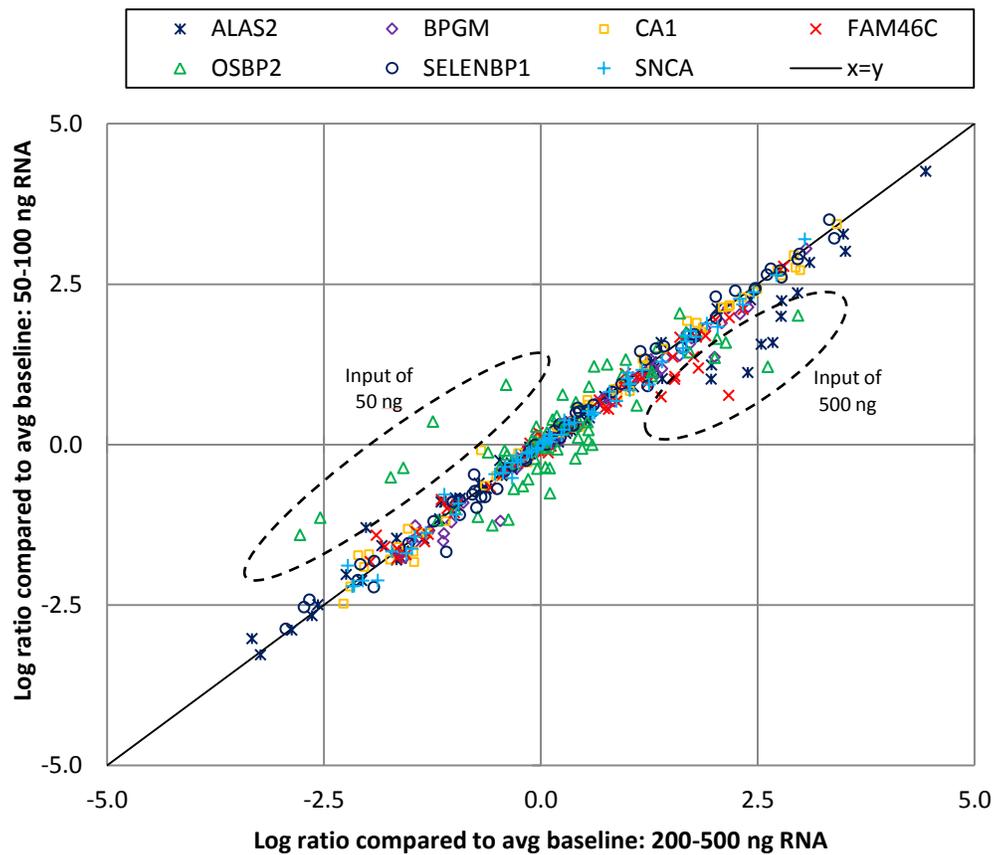
Figure 6.3: Relationship between dilutions of the sample input using QuantiGene Plex assay.

Results are reported in log ratio compared to the average baseline values. Sample inputs of 500 and 50 ng as well as 200 and 100 ng were used for the experiments 1 (24 samples from 3 subjects) and 2 (n = 40 samples from 5 subjects), respectively.
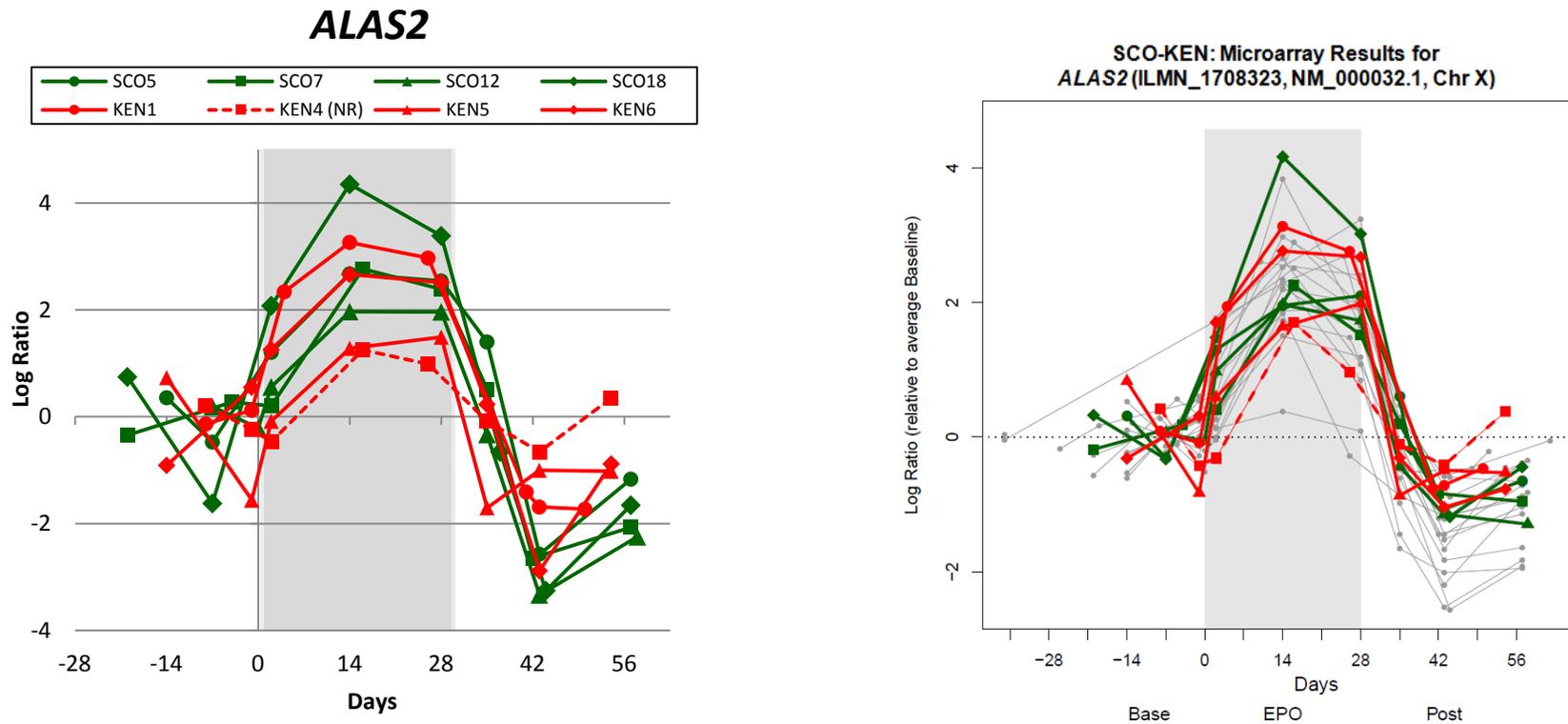
Figure 6.4A: Individual changes in gene expression in the seven target genes.

Delta-aminolevulinate synthase 2 (*ALAS2*): Results of the QuantiGene Plex assay and the Illumina microarray are presented in the left and right panel, respectively. Changes are reported in log ratio compared to the average baseline values. Time point 0 represents the first injection. The grey zone represents the administration. Each line corresponds to one subject. SCO (n = 4) and KEN (n = 4) are represented in green and red, respectively. The dashed line corresponds to the "non-responder" (NR).
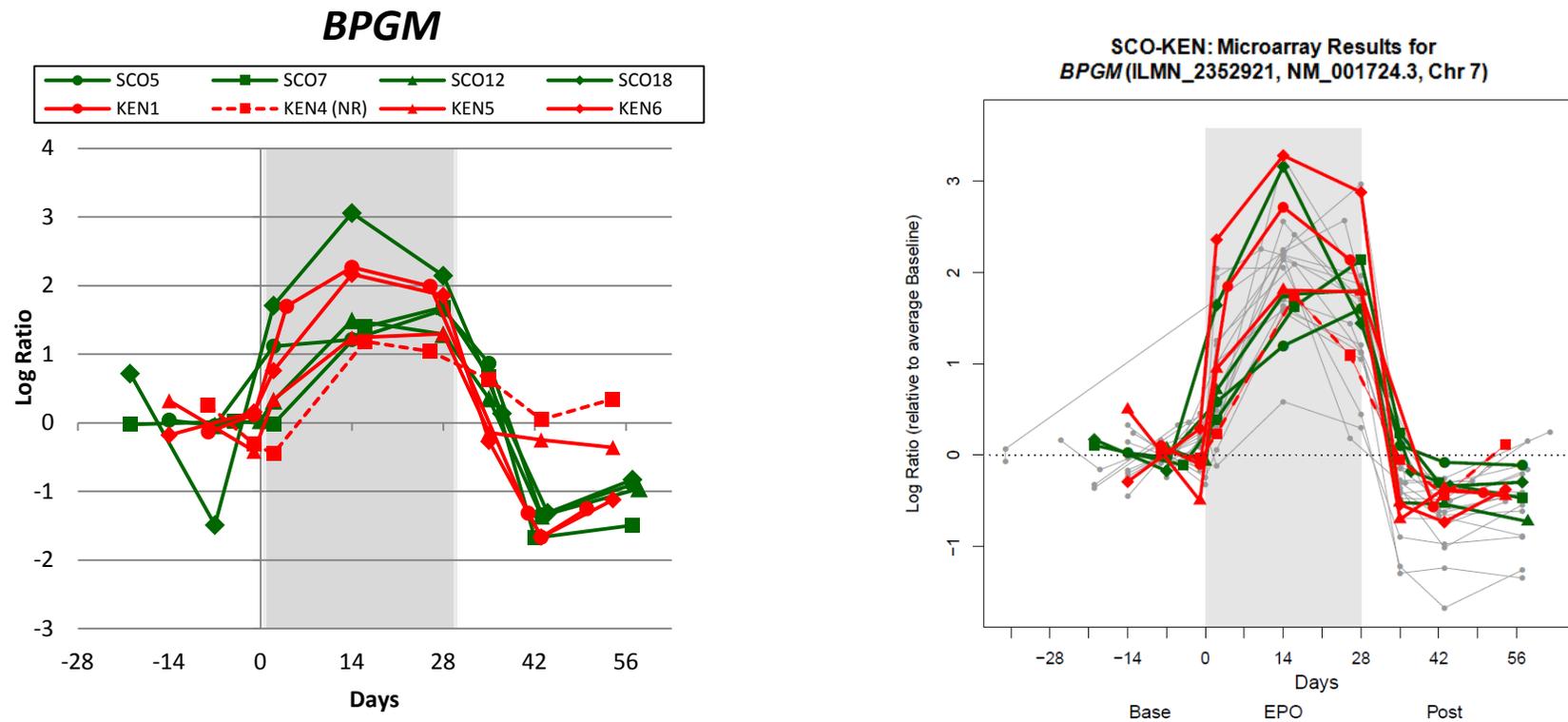
Figure 6.4B (continued): 2,3-bisphosphoglycerate mutase (*BPGM*): Results of the QuantiGene Plex assay and the Illumina microarray are presented in the left and right panel, respectively. Changes are reported in log ratio compared to the average baseline values. Time point 0 represents the first injection. The grey zone represents the administration. Each line corresponds to one subject. SCO (n = 4) and KEN (n = 4) are represented in green and red, respectively. The dashed line corresponds to the "non-responder" (NR).
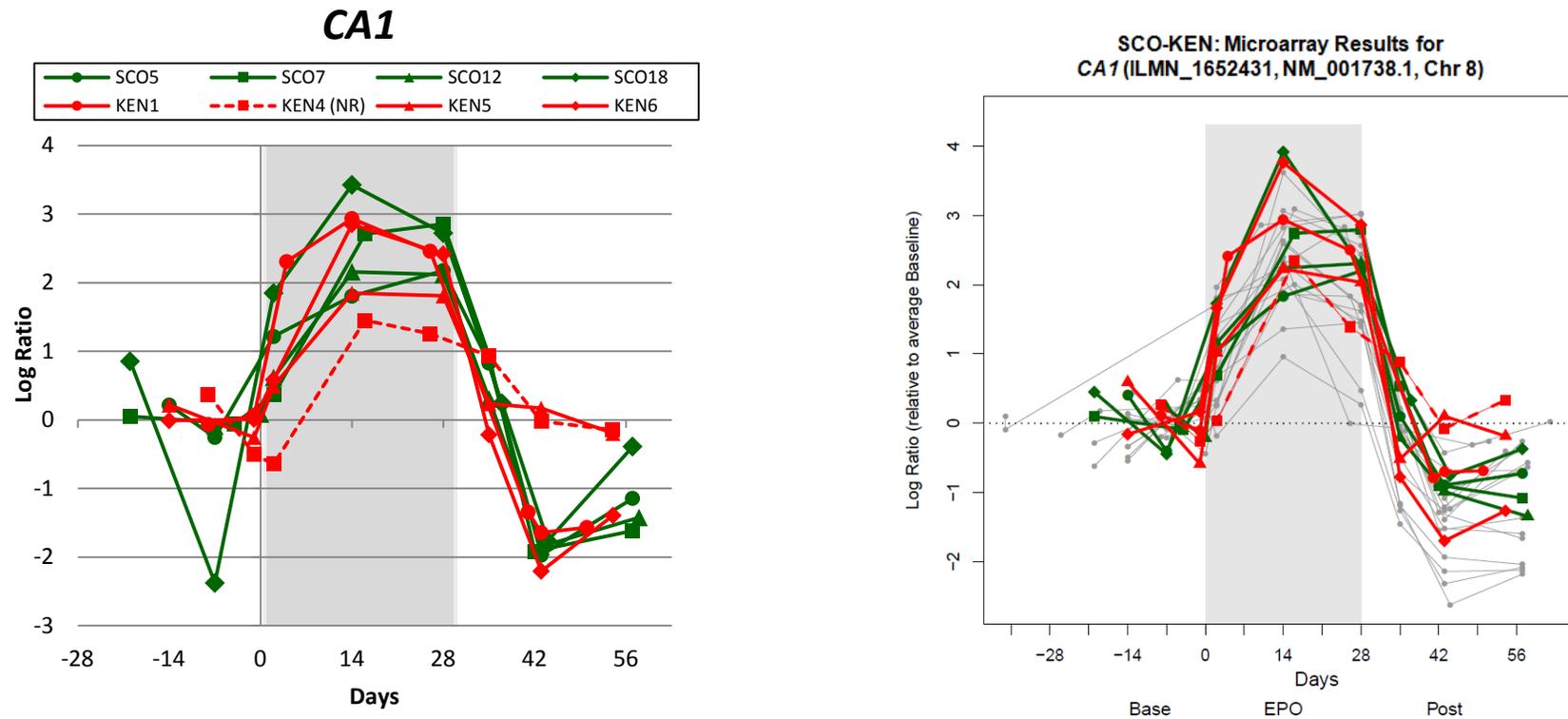
Figure 6.4C (continued): Carbonic anhydrase 1 (*CA1*): Results of the QuantiGene Plex assay and the Illumina microarray are presented in the left and right panel, respectively. Changes are reported in log ratio compared to the average baseline values. Time point 0 represents the first injection. The grey zone represents the administration. Each line corresponds to one subject. SCO (n = 4) and KEN (n = 4) are represented in green and red, respectively. The dashed line corresponds to the "non-responder" (NR).
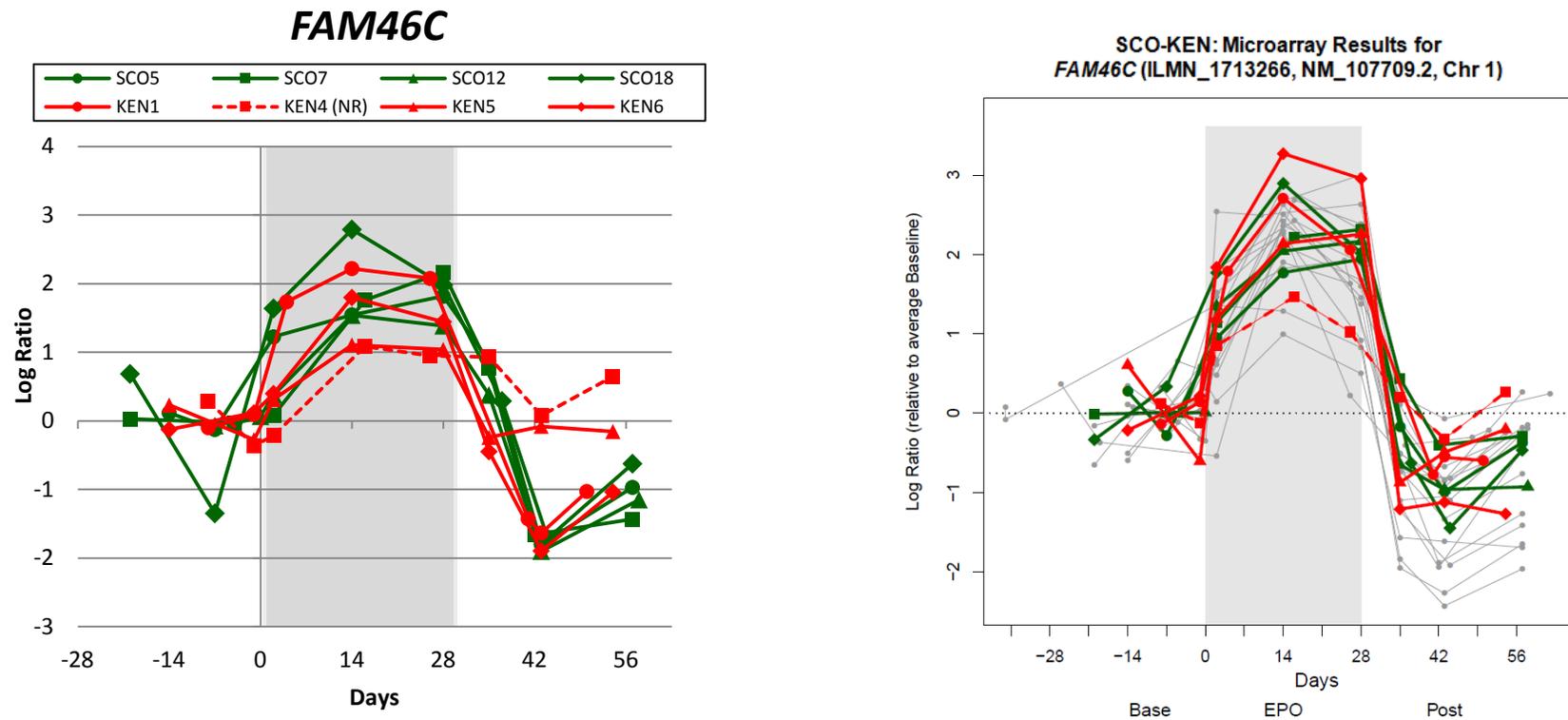
Figure 6.4D (continued): Family with sequence similarity 46, member C (*FAM46C*): Results of the QuantiGene Plex assay and the Illumina microarray are presented in the left and right panel, respectively. Changes are reported in log ratio compared to the average baseline values. Time point 0 represents the first injection. The grey zone represents the administration. Each line corresponds to one subject. SCO (n = 4) and KEN (n = 4) are represented in green and red, respectively. The dashed line corresponds to the "non-responder" (NR).
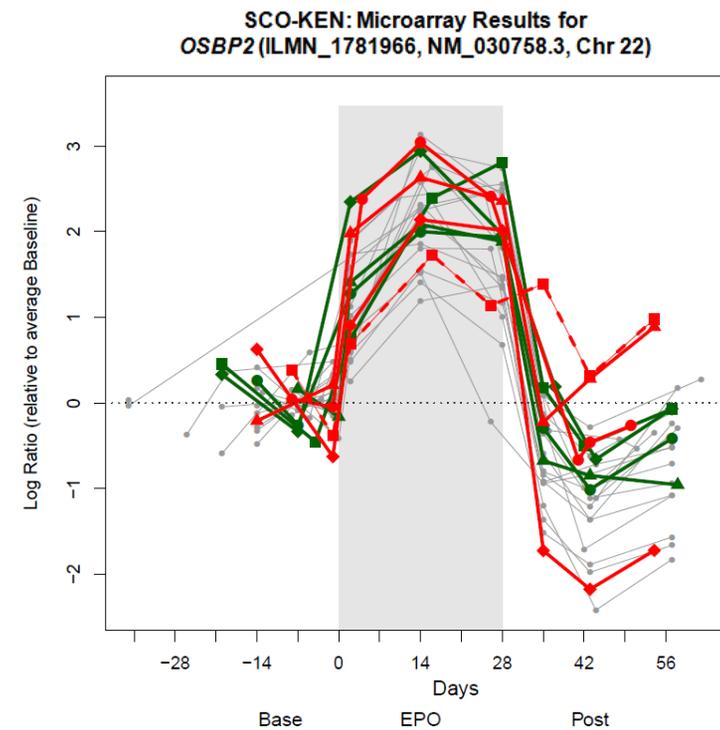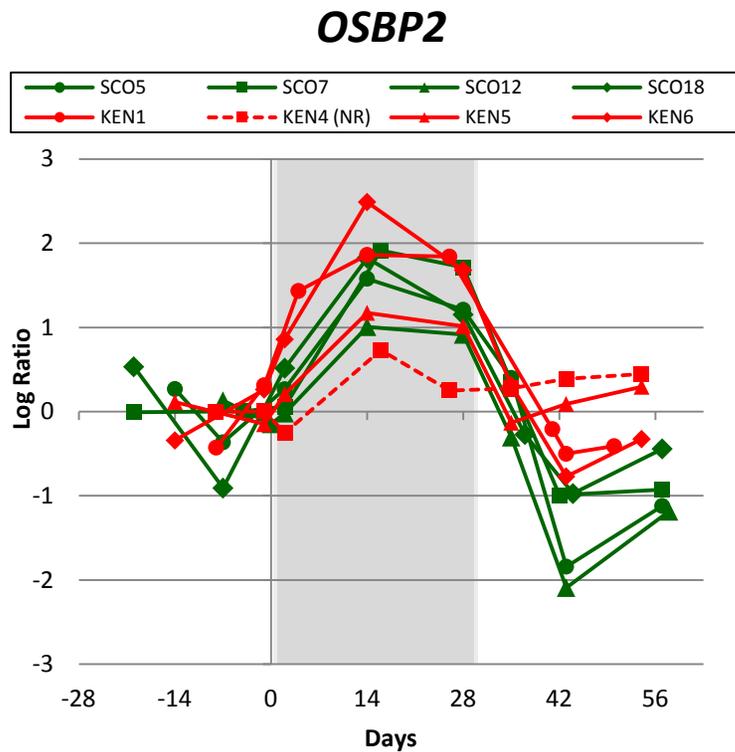
Figure 6.4E (continued): Oxysterol-binding protein 2 (*OSBP2*): Results of the QuantiGene Plex assay and the Illumina microarray are presented in the left and right panel, respectively. Changes are reported in log ratio compared to the average baseline values. Time point 0 represents the first injection. The grey zone represents the administration. Each line corresponds to one subject. SCO (n = 4) and KEN (n = 4) are represented in green and red, respectively. The dashed line corresponds to the "non-responder" (NR).
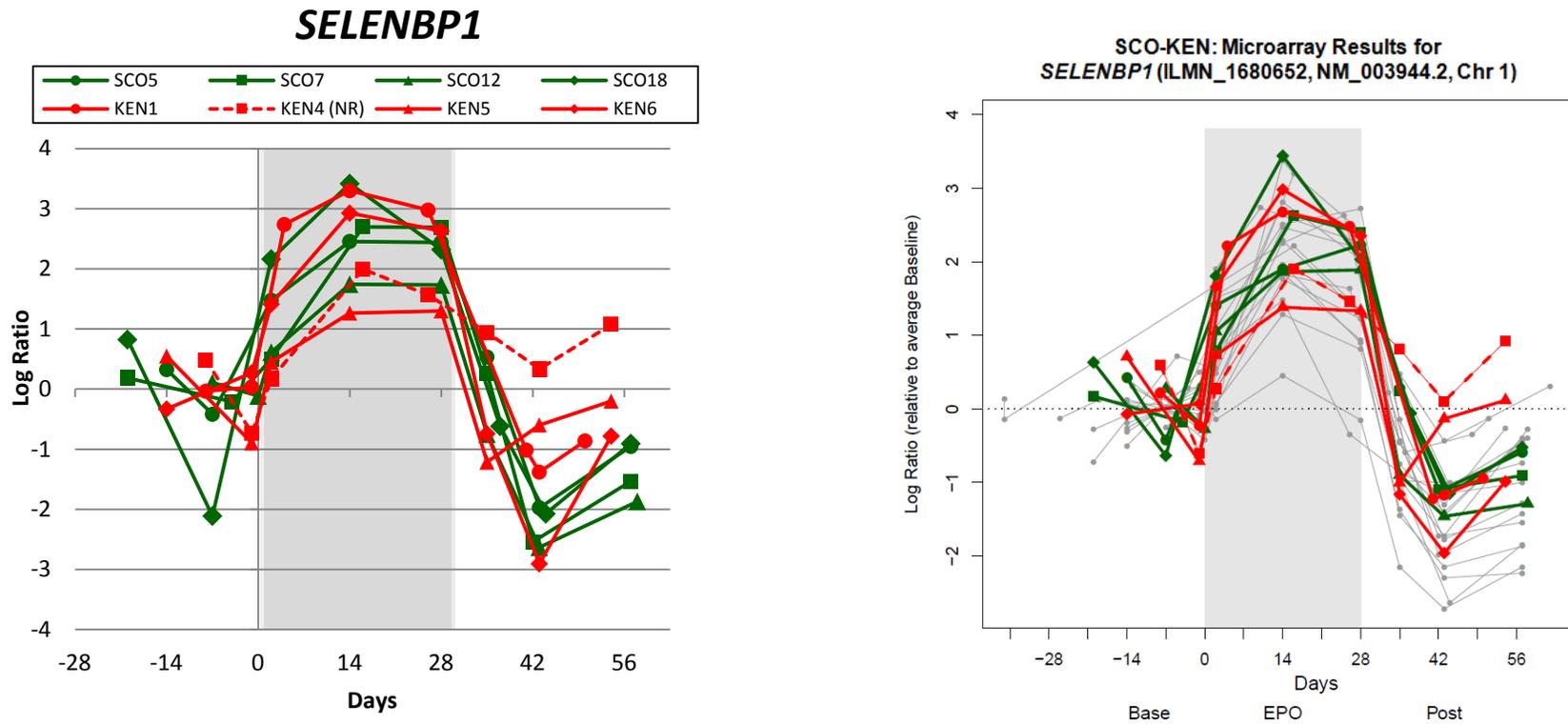
Figure 6.4F (continued): Selenium binding protein 1 (*SELENBP1*): Results of the QuantiGene Plex assay and the Illumina microarray are presented in the left and right panel, respectively. Changes are reported in log ratio compared to the average baseline values. Time point 0 represents the first injection. The grey zone represents the administration. Each line corresponds to one subject. SCO (n = 4) and KEN (n = 4) are represented in green and red, respectively. The dashed line corresponds to the "non-responder" (NR).
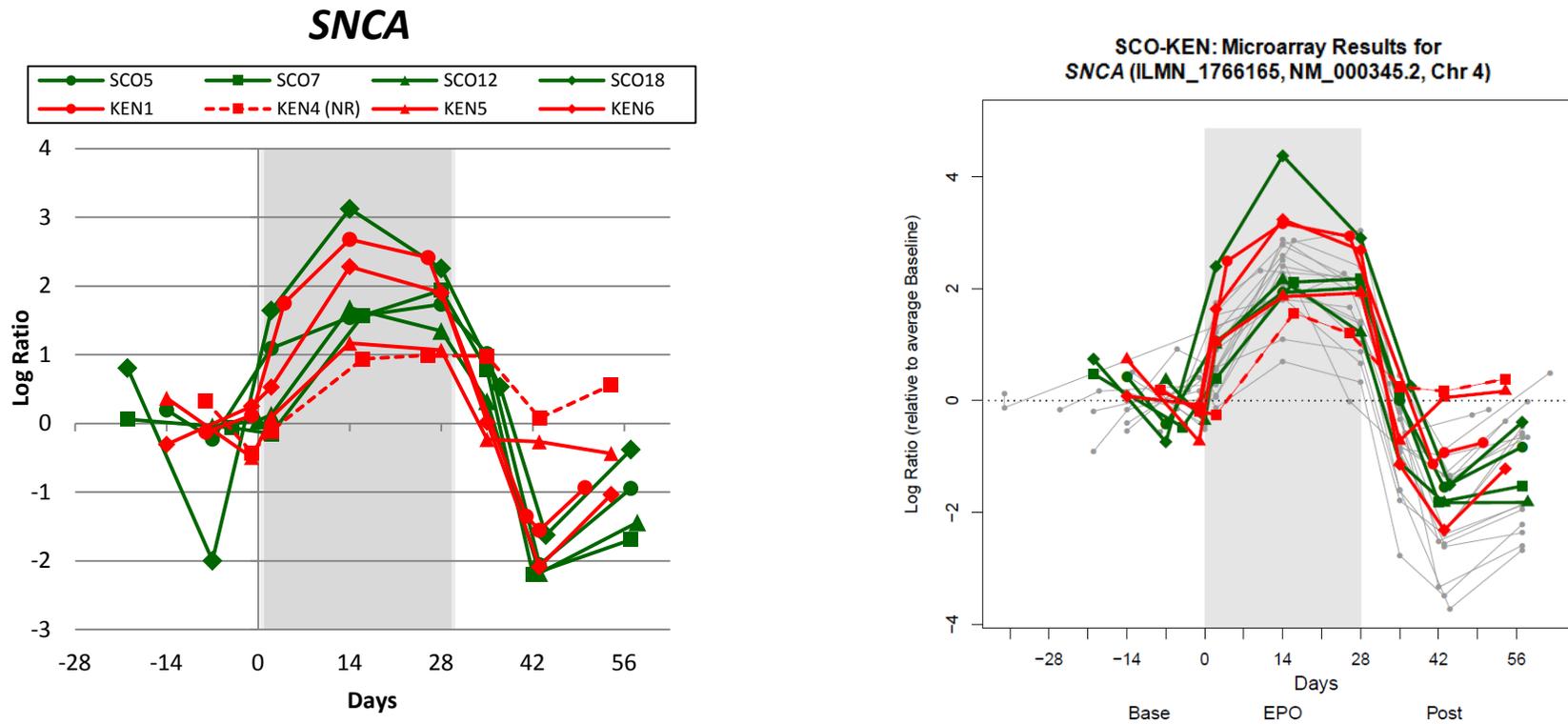
Figure 6.4G (continued): Alpha-synuclein (*SNCA*): Results of the QuantiGene Plex assay and the Illumina microarray are presented in the left and right panel, respectively. Changes are reported in log ratio compared to the average baseline values. Time point 0 represents the first injection. The grey zone represents the administration. Each line corresponds to one subject. SCO (n = 4) and KEN (n = 4) are represented in green and red, respectively. The dashed line corresponds to the "non-responder" (NR).

Figure 6.5: Relationship between results from QuantiGene Plex assay and Illumina microarray.

Results are reported in log ratio compared to the average baseline values.

Base1

	SELENBP1	CA1	OSBP2	BPGM	ALAS2	FAM46C	SNCA
SCO5	-0.6	-0.6	-6.0	1.3	2.4	2.5	-0.7
SCO7	-1.1	-1.5	-6.5	0.8	1.1	2.0	-1.0
SCO12	-0.2	-0.2	-5.8	1.4	2.3	2.6	-0.4
SCO18	-1.4	-1.3	-6.1	0.4	1.2	1.6	-1.0
KEN1	-1.8	-1.7	-7.9	0.8	1.1	1.3	-1.7
KEN4	-1.0	-0.4	-6.9	1.2	3.2	1.7	-0.9
KEN5	0.5	0.6	-6.7	1.7	3.2	2.7	-0.3
KEN6	-0.5	0.5	-6.7	1.3	1.3	2.4	-0.8

Base2

|	SELENBP1	CA1	OSBP2	BPGM	ALAS2	FAM46C	SNCA
SCO5	-1.3	-1.1	-6.5	1.2	1.6	2.3	-1.2
SCO7	-1.5	-1.6	-6.5	0.9	1.8	2.0	-1.1
SCO12	-0.4	-0.1	-6.1	1.5	1.9	2.7	-0.3
SCO18	-4.4	-4.5	-7.6	-1.8	-1.1	-0.4	-3.8
KEN1	-1.7	-1.6	-7.2	1.0	1.4	1.4	-1.5
KEN4	-2.2	-1.2	-6.9	0.6	2.7	1.0	-1.6
KEN5	-0.9	0.2	-6.9	0.9	0.8	2.2	-1.1
KEN6	0.1	0.6	-6.1	1.6	2.8	2.7	-0.3

EPO3 (2 days)

	SELENBP1	CA1	OSBP2	BPGM	ALAS2	FAM46C	SNCA
SCO5	0.6	0.4	-5.8	2.4	3.2	3.6	0.2
SCO7	-0.8	-1.2	-6.5	0.8	1.7	2.1	-1.2
SCO12	0.3	0.5	-6.0	1.8	2.7	3.1	-0.2
SCO18	-0.1	-0.3	-6.1	1.4	2.5	2.5	-0.1
KEN1	1.0	0.6	-6.0	2.6	3.7	3.1	0.1
KEN4	-1.3	-1.4	-7.1	0.5	2.5	1.2	-1.3
KEN5	0.4	0.9	-6.6	1.7	2.3	2.8	-0.6
KEN6	1.3	1.1	-5.5	2.2	3.6	3.0	0.0

EPO4 (2 weeks)

	SELENBP1	CA1	OSBP2	BPGM	ALAS2	FAM46C	SNCA
SCO5	1.5	1.0	-4.7	2.5	4.7	3.9	0.6
SCO7	1.4	1.2	-4.7	2.3	4.2	3.7	0.5
SCO12	1.4	2.0	-4.9	3.0	4.1	4.2	1.3
SCO18	1.2	1.3	-4.8	2.7	4.9	3.7	1.3
KEN1	1.5	1.3	-5.6	3.2	4.8	3.7	1.1
KEN4	0.5	0.7	-6.2	2.1	4.2	2.5	-0.3
KEN5	1.2	2.3	-5.6	2.6	3.7	3.6	0.5
KEN6	2.8	3.4	-3.9	3.6	5.2	4.5	1.8

EPO5 (4 weeks)

|	SELENBP1	CA1	OSBP2	BPGM	ALAS2	FAM46C	SNCA
SCO5	1.5	1.3	-5.0	2.9	4.6	4.2	0.8
SCO7	1.4	1.3	-4.8	2.5	3.9	4.1	0.9
SCO12	1.4	2.0	-5.1	2.8	4.1	4.1	1.0
SCO18	0.1	0.6	-5.5	1.8	3.9	2.9	0.5
KEN1	1.2	0.8	-5.6	2.9	4.3	3.5	0.8
KEN4	0.1	0.5	-6.6	2.0	4.0	2.3	-0.2
KEN5	1.3	2.2	-5.8	2.6	3.8	3.4	0.4
KEN6	2.5	3.0	-4.7	3.3	5.0	4.1	1.4

POST6 (1 week)

	SELENBP1	CA1	OSBP2	BPGM	ALAS2	FAM46C	SNCA
SCO5	-0.4	0.0	-5.7	2.1	3.4	3.3	0.1
SCO7	-1.1	-0.6	-6.2	1.5	2.0	2.8	-0.3
SCO12	-1.1	0.2	-6.2	1.8	1.8	3.1	-0.1
SCO18	-2.9	-1.9	-6.9	-0.2	-0.2	1.2	-1.3
KEN1	-2.8	-3.0	-7.7	-0.4	-0.1	0.0	-3.0
KEN4	-0.5	0.2	-6.6	1.6	2.8	2.4	-0.2
KEN5	-1.2	0.6	-6.9	1.2	0.6	2.2	-0.9
KEN6	-0.9	0.3	-6.1	1.2	2.6	2.1	-0.5

POST7 (2 weeks)

	SELENBP1	CA1	OSBP2	BPGM	ALAS2	FAM46C	SNCA
SCO5	-2.9	-2.8	-7.5	-0.1	-0.5	0.6	-3.0
SCO7	-3.8	-3.4	-7.3	-0.8	-1.2	0.3	-3.2
SCO12	-2.9	-2.0	-7.4	0.1	-1.2	0.8	-2.5
SCO18	-4.3	-3.9	-7.6	-1.6	-2.8	-0.8	-3.4
KEN1	-3.1	-3.3	-8.0	-0.8	-0.4	-0.2	-3.2
KEN4	-1.1	-0.8	-6.5	1.0	2.2	1.4	-1.1
KEN5	-0.6	0.6	-6.7	1.1	1.3	2.4	-0.9
KEN6	-3.0	-1.6	-7.2	-0.2	-0.6	0.6	-2.6

POST8 (4 weeks)

	SELENBP1	CA1	OSBP2	BPGM	ALAS2	FAM46C	SNCA
SCO5	-1.9	-2.0	-7.1	0.4	0.9	1.4	-1.9
SCO7	-2.9	-3.1	-7.3	-0.6	-0.6	0.6	-2.7
SCO12	-2.2	-1.6	-7.0	0.5	-0.1	1.5	-1.8
SCO18	-3.1	-2.5	-7.1	-1.1	-1.6	0.3	-2.2
KEN1	-2.6	-3.2	-7.9	-0.4	-0.4	0.3	-2.6
KEN4	-0.4	-0.9	-6.4	1.3	3.3	2.1	-0.6
KEN5	-0.2	0.2	-6.5	1.0	1.3	2.3	-1.1
KEN6	-0.9	-0.8	-6.7	0.3	1.3	1.5	-1.5

Figure 6.6: Log ratios of relative expression target to housekeeping genes.

Results are grouped according to the 8 analysed time points *i.e.* two baseline samples, then one sample each at 2 days, 2 weeks and 4 weeks after the first rHuEpo injection and 1 week, 2 weeks and 4 weeks after rHuEpo administration. Each line per box represents one subject. Each column per box represents one target gene. Relative to extreme values for each target genes, red, yellow and blue colours indicate high, medium and low ratio results, respectively.

Table 6.2. Median fluorescence intensity (MFI) signals of the selected genes for the different RNA inputs

Genes	Type	50 ng N = 24 (3 x 8)	100 ng N = 40 (5 x 8)	200 ng N = 40 (5 x 8)	500 ng N = 24 (3 x 8)
ACTB	Housekeeping gene	974 ± 375 (271 ; 1437)	5855 ± 1501 (2653 ; 10272)	10126 ± 2903 (1315 ; 15951)	7389 ± 1864 (3752 ; 10160)
MRFAP1	Housekeeping gene	253 ± 94 (74 ; 382)	617 ± 102 (347 ; 870)	1107 ± 237 (280 ; 1556)	2739 ± 505 (1678 ; 3521)
PPIB	Housekeeping gene	217 ± 97 (46 ; 401)	531 ± 215 (122 ; 1268)	978 ± 407 (300 ; 2310)	2330 ± 764 (1308 ; 4221)
ALAS2	Target	1879 ± 1418 (221 ; 6206)	8877 ± 8038 (229 ; 29659)	14318 ± 11149 (363 ; 37083)	15860 ± 9091 (2324 ; 29342)
BPGM	Target	955 ± 504 (252 ; 2532)	3363 ± 2257 (431 ; 9709)	6124 ± 3737 (227 ; 15571)	10662 ± 4960 (2887 ; 21479)
CA1	Target	315 ± 291 (39 ; 1429)	1520 ± 1453 (73 ; 5576)	2786 ± 2614 (116 ; 11670)	3573 ± 3069 (526 ; 12621)
FAM46C	Target	2293 ± 1258 (617 ; 6165)	5985 ± 3591 (935 ; 13925)	10669 ± 5717 (786 ; 21747)	19268 ± 7277 (6297 ; 31763)
OSBP2	Target	7 ± 2 (4 ; 11)	14 ± 6 (5 ; 36)	26 ± 12 (7 ; 68)	40 ± 18 (17 ; 87)
SELENBP1	Target	271 ± 207 (32 ; 964)	1135 ± 995 (80 ; 3950)	2106 ± 1828 (126 ; 7601)	3261 ± 2529 (355 ; 8681)
SNCA	Target	259 ± 153 (56 ; 752)	836 ± 525 (119 ; 2219)	1533 ± 963 (147 ; 3934)	2602 ± 1559 (492 ; 6904)

Values are reported as median fluorescence intensity (MFI). Values are means ± SD (minimum ; maximum) across the 24 (*i.e.* 3 subjects x 8 time points; experiment 1 in green) samples analysed with RNA input of 50 and 500 ng as well as the 40 (*i.e.* 5 subjects x 8 time points; experiment 2 in red) samples analysed with RNA input of 100 and 200 ng, respectively.

6.4 Discussion:

The main results of the present study were that the microarray-based gene expression profiles of a subset of promising target genes whose expression was profoundly altered following rHuEpo administration were successfully replicated and validated using QuantiGene Plex 2.0 assay. The present results offer the exciting prospect of the possibility of setting up in the near future a new diagnostic test which can potentially reveal the blood molecular signal signature of rHuEpo doping although thorough validation experiments are required. In addition, while current anti-doping blood samples have important logistic and cost implications since blood samples must be shipped refrigerated (2-12ºC) and analysed within 36 h of collection (Sottas *et al.* 2010), RNA stabilised in Tempus tubes is easy to handle and is stable for 5-7 days at room temperature and for months when kept frozen (Matheson *et al.* 2008; Prezeau *et al.* 2006; Shou *et al.* 2005). These findings have important implications for current and future anti-doping practices.

6.4.1 Housekeeping genes:

Beta actin (*ACTB*), MORF4 family-associated protein 1 (*MRFAP1*) and peptidylpropyl isomerase B (cyclophilin B) (*PPIB*) were selected as housekeeping genes. All three housekeeping genes were relatively consistently expressed over time in all subjects and at levels relatively similar to the target genes, although *ACTB* was the most abundant and seemed to be the most stable. The three housekeeping genes were included in the geometric mean, which was used to normalise the target gene signals. However, additional experiments that further define intra- and inter-individual variability of the housekeeping genes are required in order to establish the optimal relative expression normalization method.

6.4.2 Sample RNA input:

In order to determine the optimal RNA input, 50 and 500 ng were initially used in the first experiment (24 samples in duplicate corresponding to 3 subjects x 8 time points) and 100 and 200 ng were then used in the second experiment (40 samples in duplicate corresponding to 5 subjects x 8 time points). As Luminex instruments exhibit saturation over ~20,000 MFI and the relative abundance of our target genes extends over a wide range, at least duplicates with two different RNA inputs will be necessary to ensure valid signal detection, *i.e.* over background

noise and lower than the saturation threshold, in future studies. Further experiments are needed to define the optimal RNA inputs but it would probably range from approximately 50 to 200 ng.

6.4.3 Target genes:

In agreement with previous work which evaluated the performance characteristics of three quantitative gene expression technologies including QuantiGene assay and correlated their expression measurements to those of commercial microarray platforms including Illumina, our results showed a good correlation between QuantiGene Plex and microarray results (Canales *et al.* 2006). Overall, the QuantiGene Plex results replicated and validated the Illumina microarray results for the target genes, whose expression was profoundly altered following rHuEpo administration. In agreement with previous reports, the down-regulation in gene expression post rHuEpo administration was slightly more profound when measured using the QuantiGene Plex compared to the Illumina microarray analysis (Canales *et al.* 2006; Raymond *et al.* 2010). This latter feature may make the blood molecular signature of rHuEpo doping even more defined post administration.

Testosterone over epitestosterone (T/E) ratio is used to indirectly detect testosterone doping (Aguilera *et al.* 2001; Sottas *et al.* 2008b). Following this example, ratios of relative expression target to housekeeping genes were determined in order to investigate whether this approach can potentially be applied to rHuEpo detection. Based on our initial results from a limited number of subjects and target genes, it seems that population-based thresholds, despite being potentially informative, are probably not the best anti-doping strategy to detect rHuEpo doping. It has been demonstrated that the performance of the population-based T/E limit (*i.e.* greater than 4) to detect testosterone doping is poor while individual longitudinal profile monitoring can be much more efficient (Sottas *et al.* 2007; Sottas *et al.* 2008b). Further studies are necessary to evaluate the intra- and inter- variations of the blood gene expression profiles and their respective gene expression ratios.

In conclusion, although further thorough validation experiments are required, particularly in order to define the intra- and inter-individual variability of the housekeeping as well as target genes, the results of the present study offer the exciting prospect of the possibility of setting up in

the near future a new, specific, sensitive, practical and cost-effective diagnostic test which can potentially reveal the blood molecular signature of rHuEpo doping and can add another dimension to the ABP in terms of robustness and performance to detect rHuEpo doping.

Chapter 7 : General Discussion and Conclusion

7.1 General discussion:

Through this doctoral thesis, several research investigations have been conducted to assess the effects of rHuEpo administration on blood gene expression profiles in endurance trained males living at or near sea-level and at moderate altitude (~2,150 m) in order to identify a molecular signature of rHuEpo doping and thus provide a basis for the development of novel and robust testing models to detect blood doping. First, the time course of changes in Hb_{mass} and related blood parameters as well as time trial performance were defined in endurance trained males living and training at or near sea-level following four weeks of rHuEpo administration; second, the ergogenic effects of rHuEpo were investigated via the assessment of changes in haematological parameters and laboratory- and field-based endurance performance tests in endurance runners living and training at moderate altitude in Kenya and then compared with the sea-level cohort that underwent an identical rHuEpo administration regime; third, the effects of rHuEpo administration on blood gene expression profiles were longitudinally evaluated using whole transcriptome microarray technology in trained males living and training at or near sea-level; fourth, the same molecular methodology was applied in order to replicate and compare the gene expression profiles in another cohort living and training at moderate-altitude in Kenya who underwent an identical rHuEpo administration regime; fifth, the gene microarray based findings were validated using a specific and sensitive quantitative gene expression technology.

The main findings described in this doctoral thesis were: first, the improvements in the laboratory based measurements after rHuEpo administration in endurance trained males translated into improvement in exercise performance in the field. More particularly, 3,000 time trial running performance was significantly improved following 4 weeks of rHuEpo administration and remained significantly elevated 4 weeks after administration. These performance effects coincided with significantly rHuEpo-induced elevated $\dot{V}O_{2max}$ and Hb_{mass}. Second, 4 weeks of rHuEpo administration was able to significantly increase the already relatively high haematological parameters as well as $\dot{V}O_{2max}$ of Kenyan endurance runners living and training at moderate altitude (~2,150 m). Third, although the rHuEpo-induced increase in the key haematological parameters was not as high in the Kenyan group compared to the Scottish group, the relative improvements of approximately 5% and 3% in running performance

immediately after the rHuEpo administration and 4 weeks after the last injection were similar between both groups. Third, using whole transcriptome microarray technology, blood gene expression profiles were found to be profoundly and significantly altered from 2 days after the first rHuEpo injection and up to 4 weeks after the rHuEpo administration, leading to an initial blood molecular signature of rHuEpo doping. A large number of the identified genes were related to the functional or structural properties of the erythrocyte. Fourth, using the same gene microarray methodology, the molecular signature of rHuEpo doping discovered in the Scottish group was successfully replicated in the Kenyan cohort composed of endurance runners living and training at moderate altitude and who underwent an identical rHuEpo administration regime. Fifth, the microarray-based gene expression profiles of a subset of the promising genes whose expression was profoundly altered following rHuEpo administration were successfully validated using a different specific and sensitive quantitative gene expression platform. In addition to providing the strongest evidence to date that omics biomarkers such as gene expression have the potential to add another dimension to the ABP to detect rHuEpo doping, the main findings described in this doctoral thesis offer the exciting prospect of the possibility of setting up in the near future a new, specific, sensitive, practical and cost-effective diagnostic test which can potentially reveal the blood molecular signature of rHuEpo doping.

7.1.1 Limitations of the research:

Like all research this doctoral research is not without limitations. As there were only minimal data regarding the assessment of the effects of erythropoiesis stimulating drugs using whole transcriptome analysis from whole blood, this research can be characterized as an extensive and key proof-of-principle study. The main limitation of the design stems from the fact that our study was not blinded and there was no control group. Nevertheless, in addition to having established adequate molecular methods and molecular workflow to enable valid and optimal assessment of blood gene expression for follow-up and future studies (see below), this doctoral research provided convincing and very promising findings in the development of a robust test to detect rHuEpo doping. Another limitation was that elite competitive athletes could not be recruited for such studies for obvious ethical reasons (Howman 2013; Wagner 2013). Factors such as genetic traits, training history and training load almost certainly differ from those of the subelite yet endurance trained population investigated in this research (Hopkins *et al.* 1999). In addition, the

rHuEpo dose regimen used in this research (*i.e.* 50 IU·kg^{-1} body mass every two days for four weeks) did not closely reproduce the strategy of "micro doses" rHuEpo doping allegedly currently used by athletes (see below). As such, caution should be taken in the interpretations of the applications of the findings and further thorough and careful validation is necessary. For instance, it is of paramount importance to evaluate the intra- and inter- variations of the blood gene expression profiles in elite competitive athletes including potential confounding effects such as intense exercise and altitude training.

7.1.2 Bayesian approach to the interpretation of blood doping gene markers:

The ABP was introduced as a new tool to indirectly detect blood manipulation, such as rHuEpo doping, and which can lead to disciplinary sanctions imposed by a sports organization or its tribunal, as well as to intelligently target athletes for additional testing (Schumacher and d'Onofrio 2012; Schumacher *et al.* 2012; Zorzoli and Rossi 2012). The ABP approach relies on identifying intra-individual abnormal variability over time of selected haematological parameters (Figure 1.2) (Callaway 2011; Sottas *et al.* 2010). More precisely, the ABP approach uses Bayesian networks for the evaluation of the likelihood of doping based on several variables and/or factors which can then be used as evidence for disciplinary sanction. Bayesian networks are interpretable and flexible models for representing the probabilities of causal relationships between multiple interacting variables (Sottas *et al.* 2008a; Sottas *et al.* 2010). In the anti-doping field, the Bayesian model directly estimates the probability of blood doping based on previous individual test history and heterogeneous factors known to influence blood parameters. This feature allows the model to remove the variance due to inter-subject differences and heterogeneous factors such as gender, ethnicity, altitude exposure, age and sport discipline (Sottas *et al.* 2008a). Based on the promising findings of this doctoral research, blood gene biomarkers can potentially be integrated into the ABP in order to further improve its performance. Therefore, the inter- and intra-subject variability as well as factors which can affect blood gene expression profiles must be precisely determined. The present research demonstrated that ethnicity and residence at altitude only minimally influence the blood gene expression signature of rHuEpo doping. However, further research is required to measure reference ranges and the impact of external factors on blood gene expression profiles.

7.2 Future work:

By identifying the initial blood molecular signature of rHuEpo doping, this doctoral research provides a strong and necessary basis for the development of novel and robust testing models to detect rHuEpo doping. Given these promising results, it is of paramount importance to precisely determine the reference values of the blood molecular signature of rHuEpo doping using different dose regimens as well as to thoroughly assess the effects of external factors on blood gene expression profiles, such as altitude training including different hypoxic "dose" and protocols, sport discipline, level of competition, gender, ethnicity and age. These investigations are necessary before potentially including the promising blood gene biomarkers in the ABP.

7.2.1 Microarray and QuantiGene experiments:

Following the success of the QuantiGene Plex pilot experiment and with the prospect of setting up a potential future test which can reveal the molecular signature of rHuEpo doping, our research group has recently been granted additional short notice funding (see Appendix F). This additional fund will allow us to: 1) finalise the microarray experiment, *i.e.* 12 more Kenyan subjects to be analysed to make a balanced design, although this is unlikely to significantly change the overall results and the transcripts of interests (see Appendix A); 2) increase the number of target genes to 50 which will allow for a more defined and precise molecular signature of rHuEpo doping. Fifty target genes in all subjects (*i.e.* 39 in total) will be analysed using the specific, sensitive, time and cost-effective QuanitiGene Plex technology. This study will enable the validation of the molecular signature of rHuEpo doping as well as the establishment of the bases for a testing procedure.

7.2.2 Microdose rHuEpo:

The gene expression results generated by this doctoral research are very promising. For example, using a 1.5 fold-change threshold, 30 genes were found to be already up-regulated 2 days after only one single rHuEpo injection, such as *FAM46C* (family with sequence similarity 46 member C) gene (Figure 5.3). These data provide the strongest evidence to date that omics technologies such as gene expression have the potential to improve the ABP approach for rHuEpo detection. However, in order to induce only minor fluctuations in the ABP blood markers and/or to "normalize" these markers after blood manipulations such as autologous blood transfusion and

by doing so minimising the risk of being caught, athletes are now using "micro doses" of rHuEpo which allegedly range from 10 to a maximum of 40 IU·kg^{-1} body mass and remained undetectable by the ABP (Ashenden *et al.* 2011; Ashenden *et al.* 2006). It has been recently shown that the so-called MAIIA test has the potential to identify trace amounts of exogenous rHuEpo with altered glycosylation in blood and urine samples and can therefore potentially be used to detect very small doses of rHuEpo (Ashenden *et al.* 2012; Lonnberg *et al.* 2012; Morkeberg *et al.* 2013). The next question is whether the biomarkers of the molecular signature of rHuEpo doping discovered in this doctoral research using a fairly high regimen of rHuEpo injections of 50 IU·kg^{-1} body mass are able to reveal microdose rHuEpo doping as a stand-alone test or in combination with new developing methods such as the MAIIA test. Our research group successfully applied for a new WADA research grant (12C09YP) to answer this question. Ten healthy endurance trained subjects not involved in competition will participate in a randomized, double-blind, placebo-controlled crossover microdose rHuEpo regimen (Figure 7.1). This new study entitled "A systems biology biomarker based approach to the detection of microdose recombinant human erythropoietin doping" will start in September 2013.
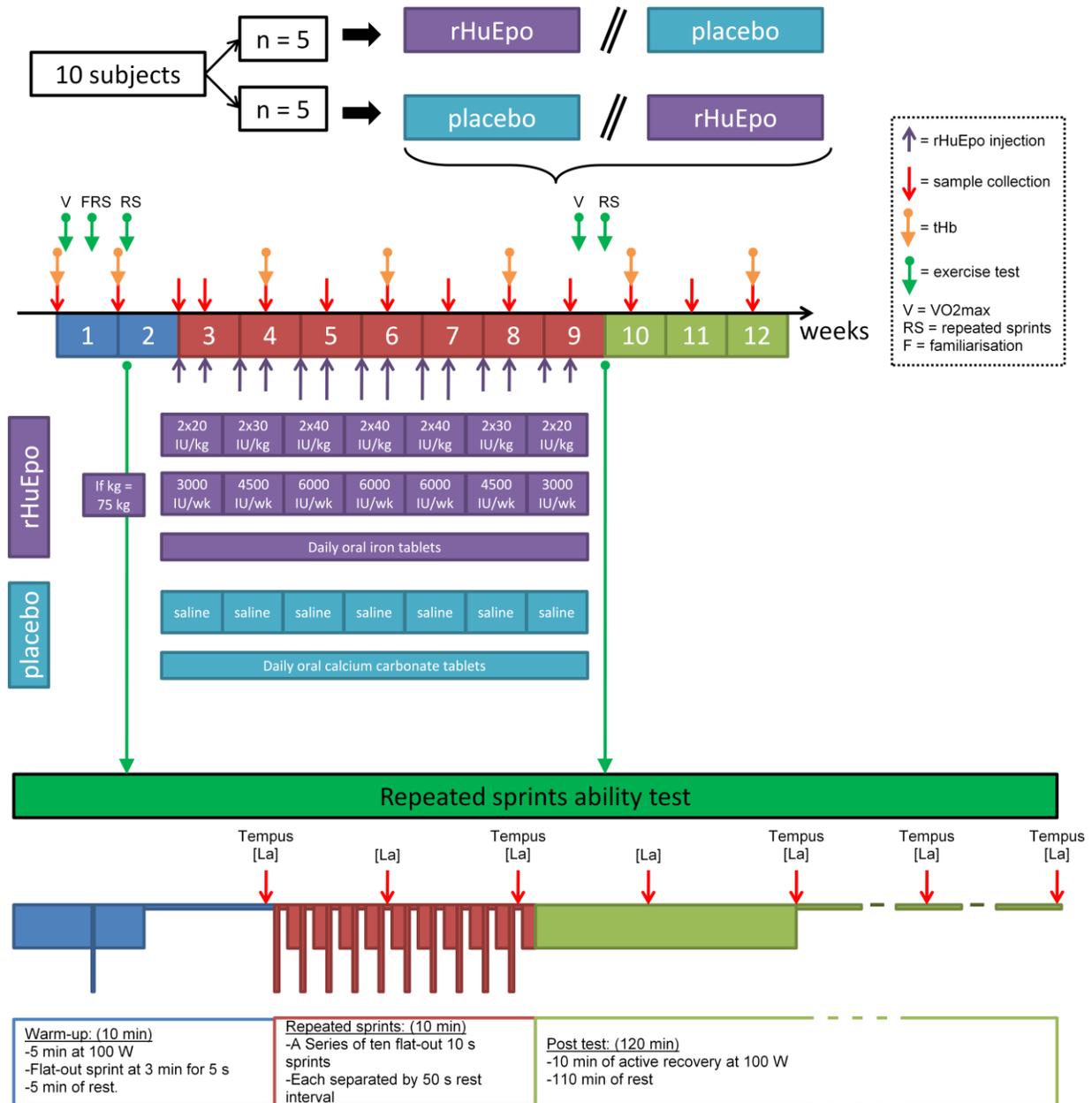
Figure 7.1: Experimental design of the microdose rHuEpo study.

7.2.3 Assessment of the confounding effects of exercise:

Samples for anti-doping purposes are often collected at sporting or training venues after intense exercise (Rupert 2009). However, it has been previously demonstrated that exercise can significantly affect gene expression profiles of peripheral blood mononuclear cells and white blood cells (Buttner *et al.* 2007; Connolly *et al.* 2004). It is therefore essential to define the effects of exercise on blood gene expression profiles and particularly on the "molecular signature" of rHuEpo doping. To do so, we will use the follow-up project entitled "A systems biology biomarker based approach to the detection of microdose recombinant human erythropoietin doping" (12C09YP) to address our research question. Briefly, in order to provide a set of robust candidate genes that can be used for the detection of rHuEpo doping, blood gene expression assessment will be performed during and post intense exercise after microdose rHuEpo or placebo in the double-blinded trial (Figure 7.1). The intense exercise will consist of a series of 10 s Wingate cycle ergometer bouts to test not only the effects of exercise on blood gene profiles but also the effects of rHuEpo on progressive anaerobic fatigue and to assess repeated sprint ability. The ability to reproduce maximal performance in subsequent short-duration sprints separated by brief recoveries is a key fitness requirement in most team and racket sports (Girard *et al.* 2011). Of note, the International Tennis Federation recently adopted the ABP program (BBC 2013b), while there are personalities in football, such as the Arsenal manager Arsène Wenger, who publicly said that he feared doping was rife in football (The Telegraph 2013). The research study outlined above will also be used to provide a basis for the development of recommendations on the collection of samples in order to ensure "valid" blood gene expression profiles results. In particular, given the potential robustness of specific gene expression profiles, it is envisaged that athletes will no longer need to wait for two hours after training or competition before a blood sample is taken for the ABP (Operating Guidelines V3.1, WADA, 2012).

7.2.4 Assessment of the confounding effects of altitude exposure:

It is known that natural or simulated altitude training is used by athletes for performance enhancement (e.g Millet *et al.* 2010), although the scientific evidence on the real effects of altitude training on elite performance when athletes return to sea level is not clear-cut. Only the live high-train low (LHTL) method developed by Levin and Stray-Gundersen (Levine and Stray-

Gundersen 1997) using natural altitude seems to be consensually accepted to be effective (Bonetti and Hopkins 2009; Siebenmann *et al.* 2012). In addition, it remains unclear to what extent these effects on performance are due to the mechanism of erythropoiesis (Bonetti and Hopkins 2009). Regardless of these conflicting results, it has been shown that altitude exposure has the potential to influence haematological indices of rHuEpo doping (Ashenden *et al.* 2003; Ashenden *et al.* 2001). As a result, there are concerns not only about the risk of false positives but also about the misuse of altitude exposure by some athletes in order to mask blood doping practices such the administration of rHuEpo (Lundby *et al.* 2012b; Sanchis-Gomar *et al.* 2009). There is, therefore, an urgent need for developing specific and robust testing models which can differentiate altitude training from blood doping combined (or not) with altitude exposure. Omics technologies such as gene expression have the potential to provide a "molecular signature" specific to rHuEpo and altitude exposure. The aim of this follow-up project is to compare blood gene expression profiles altered by rHuEpo (fairly high and microdose regimens) with altitude exposure in order to provide a set of candidate genes that can be used to differentiate rHuEpo from altitude training.

7.2.5 Other external factors to be investigated:

It has been previously demonstrated that other external factors can influence blood profiles. For instance, strenuous endurance exercise can induce changes in some blood parameters not only by the well-described increase in plasma volume known as the athlete's pseudoanaemia (Weight *et al.* 1991) but also by exercise-induced haemolysis due to metabolic modifications, oxidative stress and especially repeated foot impact (foot strike) (Lombardi *et al.* 2013; Telford *et al.* 2003; Yusof *et al.* 2007). The effects of strenuous long distance exercise such as cycling stage race and ultra marathons on blood gene expression profiles should consequently be investigated in elite endurance athletes. As most blood parameter values differ substantially between males and females, the currently used reference ranges in the ABP were determined separately for each sex (Sharpe *et al.* 2002; Sottas *et al.* 2008a). A similar differentiation approach should be applied to blood gene expression profiles in order to evaluate gender differences and similarities. Last but not least, injury-related immobility has the potential to affect blood gene profiles, as it has been reported that immobility for a period of 4 weeks reduced Hb_{mass} by ~19% in a female athlete competing at international level (Schumacher *et al.* 2008). The knowledge of the influence of

these external factors on blood gene expression profiles will lead to higher sensitivity and specificity of the ABP.

7.3 General conclusion:

In conclusion, the main findings of the research presented in this thesis are as follows:

❖ The improvements in the laboratory based measurements of exercise capacity after rHuEpo administration in endurance trained males translated into improvement in exercise performance in the field.

❖ rHuEpo administration increased the already relatively high haematological parameters as well as $\dot{V}O_{2max}$ of Kenyan endurance runners living and training at moderate altitude (~2,150 m).

❖ Although the rHuEpo-induced changes in haematological parameters were not as high in KEN as in SCO, the improvement in exercise performance was similar.

❖ Using gene microarray analysis, blood gene expression profiles were profoundly altered by rHuEpo administration.

❖ Living and training at moderate-altitude (~2,150 m) together with ethnicity did not significantly influence the blood gene expression response induced by rHuEpo administration as the findings were replicated and only minimal differences were found between the two groups.

❖ Further to the gene microarray based analysis, a subset of blood gene biomarkers which defines the molecular signature of rHuEpo doping was validated using another sensitive and specific quantitative gene technology.

❖ The results generated by this research provide the strongest evidence to date that blood gene expression profiles have the potential to improve and add a new dimension to the ABP for rHuEpo detection.

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VIII Appendices:

Appendix A: Illumina microarray experiments carried out in batches

Appendix B: Molecular laboratory work issues and troubleshooting

Appendix C: Lists of genes from Venn diagrams

Appendix D: Electropherograms of cRNA from two subjects as typical examples

Appendix E: Transcriptomic analysis from saliva samples

Appendix F: Approved proposal for emergency funding

Appendix G: One page summary to include in WADA's website

Appendix A: Illumina microarray experiments carried out in batches:

The Illumina microarray experiments were carried out in batches with 3 subjects (April 2011), 6 subjects (February 2012) and 9 subjects (September 2012) for SCO. Interestingly, increasing the number of subjects only marginally changed the findings, *i.e.* transcripts whose expression was significantly altered by rHuEpo administration (Figure VIII.1).
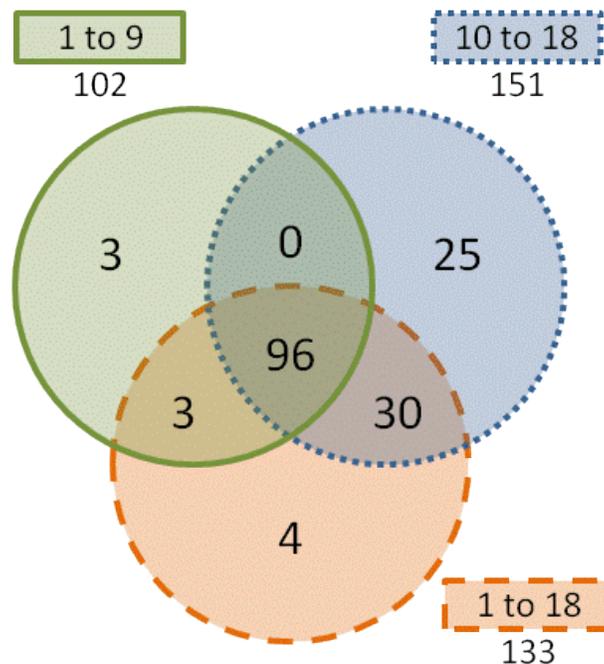
Figure VIII.1: Venn diagrams of transcripts differentially expressed during rHuEpo compared to baseline in the different microarray experiments in SCO.

The 3-way Venn diagrams depicts the transcripts differentially expressed (5% false discovery rate and 1.5 fold-change threshold) during and after rHuEpo administration revealed by including subjects 1 to 9 (green, solid line circle), 10 to 18 (blue, dotted line circle) and 1 to 18 (orange, dashed line circle) in the analysis.

Although it will likely not significantly change the overall results and the transcripts of interests, 12 more KEN subjects will be analysed to make a balanced microarray analysis (*i.e.* 18 subjects in each group).

Figure VIII.2 depicts the latest results of the changes in total RNA yield for 18 subjects in each group. Except slightly higher values during rHuEpo especially due to one subject, no major differences were observed compared to Figure 5.1 (KEN = 6).
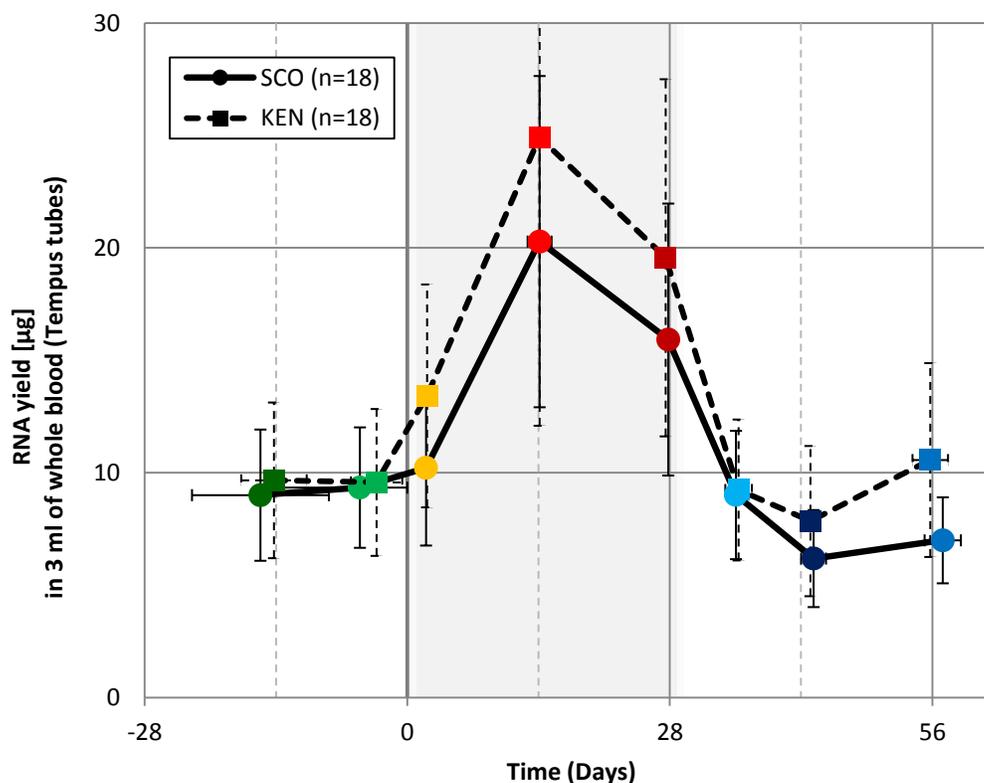
Figure VIII.2: Changes in total RNA yield extracted from 3 ml of whole blood stabilized in Tempus tubes in SCO (n = 18) and KEN (n = 18).

Values are means ± SD. Time point 0 represents the first rHuEpo injection. The grey zone represents the 4 weeks rHuEpo administration. SCO and KEN are represented by the circle and the square, respectively.

Appendix B: Molecular laboratory work issues and troubleshooting:

B.1 Issue with Illumina microarrays in December 2011:

Due to the constraints of the study protocol (Figure 2.2), a maximum of five subjects could take part in the trial at the same time and therefore the collection of samples was carried out over a period of two years. During this period, the microarray experiments were performed in batches. The initial microarray experiment including three subjects was successfully performed in April 2011. The second batch including six subjects was performed in December 2011 but surprisingly failed. This was completely unexpected since all the intermediate results and quality controls were similar between both batches *i.e.* good quantity and quality of RNA and cRNA assessed by the NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and the Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, USA). After verifying the lab tracking form to make sure that no mistake had been made during the laboratory manipulations, the microarray results were analysed and discussed (Table VIII.1 and Figure VIII.3). The initial conclusion was that the problem came from either a faulty batch of BeadChips or the streptavidin-Cy3, although the latter was stored in a frozen state and no problem had been reported during previous experiments. A decision was made to perform a troubleshooting experiment using an unused BeadChip which was a year past its expiry date, a newly ordered batch of streptavidin-Cy3 and six cRNA samples (two cRNA from the successful batch in April 2011, two cRNA from the failed batch in December 2011 and two newly made cRNA from RNA of the December 2011 batch). This troubleshooting experiment was performed in January 2012 (Table VIII.1 and Figure VIII.3). The conclusions were that the troubleshooting experiment was successful but the results were not as good as the April 2011 experiment because of the expired BeadChip. The samples were therefore not the problem, and we re-contacted the company and provided them with our results and conclusions. Illumina sent new microarray BeadChips free of charge. We were relieved when the microarray experiment using the new BeadChips was successful and confirmed our initial results obtained in April 2011.

A. Successful microarray "April 2011"   **B. Failed microarray "December 2011"**
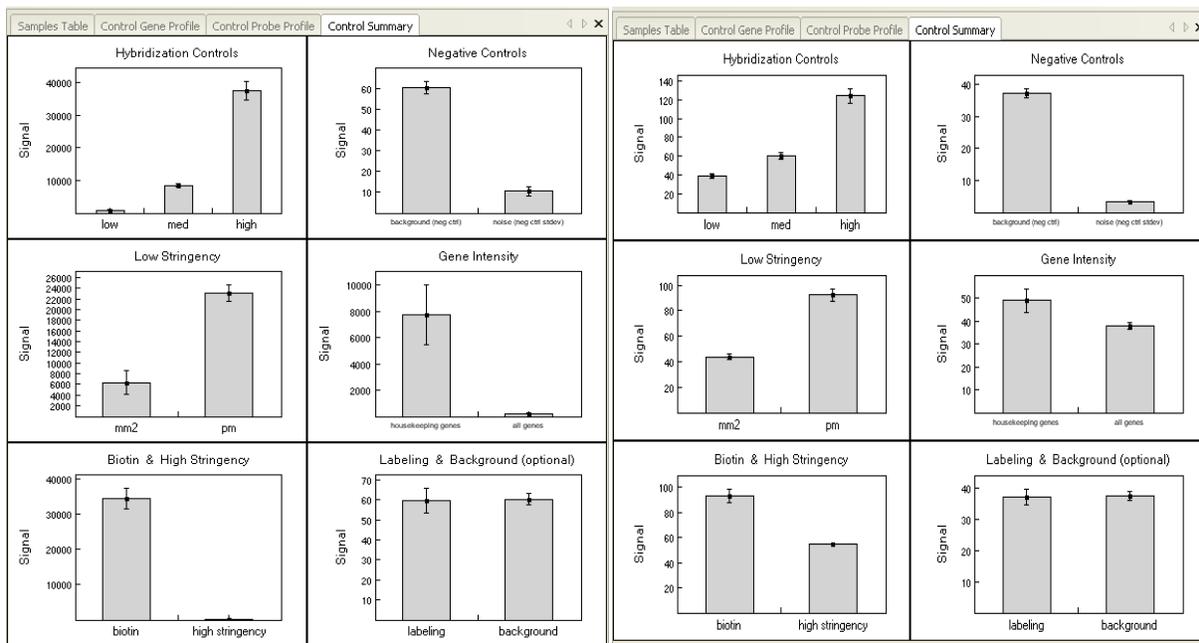
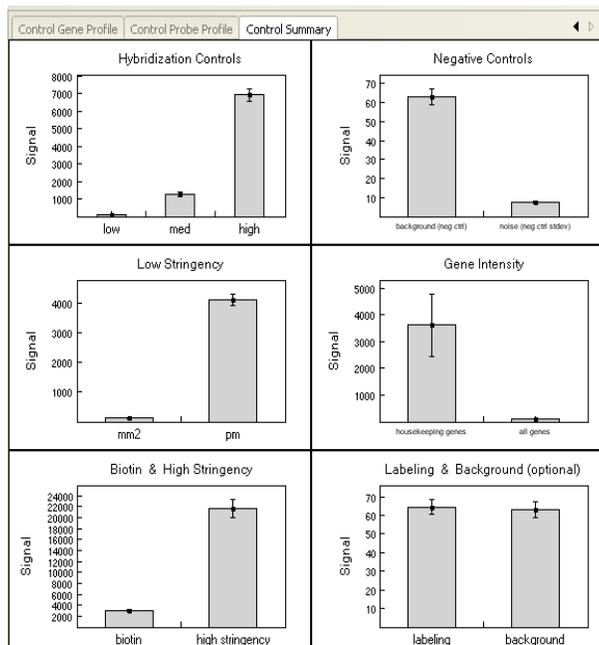**C. Troubleshooting expired microarray "January 2012"**

Figure VIII.3: Summary of the microarray experiments results which were successful in April 2011 (A) but failed in December 2011 (B). The experiment performed in January was a troubleshooting experiment using an Illumina BeadChip over a year past its expiry date.

Table VIII.1. Comparison of the control summary microarray experiments results after performing the troubleshooting experiment.

	Successful microarray "April 2011"	Failed microarray "December 2011"	Troubleshooting expired microarray "January 2012"
Gene detected (0.01)	~ 9,000	~ 700	~ 5,000
Gene detected (0.05)	~ 12,000	~ 2,500	~ 8,000
Signal Average	222	38	94
Signal P95	540	43	142
Biotin	34,000	90	3,000
CY3 HYB	15,000	75	2,500

B.2 Issue with MagMAX kit in October/November 2012:

The MagMAX for stabilized blood tubes RNA isolation kit (Ambion, Austin, USA) was used to extract RNA from the stored frozen Tempus Blood RNA tubes (Applied Biosystems, Foster City, USA) instead of the Tempus Spin RNA isolation kit (Life Technologies, Paisley, UK) because, according to the company, the latter removes most of the small RNAs including microRNA (miRNA) while the former purifies miRNA with total RNA. In addition to the gene expression analysis using messenger RNA, our research group wanted to include miRNA in the analysis and the samples were consequently processed accordingly. It has been shown that miRNAs, a class of recently discovered small RNA molecules, play a major role in post-transcriptional regulation, thought to directly regulate thousands of genes (Bartel 2004; Filipowicz *et al.* 2008; Selbach *et al.* 2008). Interestingly enough, although mature erythrocytes (rHuEpo stimulates their production) are generally thought to lack RNA, it has recently been demonstrated that they do contain both RNA and especially miRNA, whose presence, however, has not yet been explained (Chen *et al.* 2008; Hamilton 2010). Therefore, the assessment of miRNAs, providing an insight into the interaction between messenger RNA and miRNA and consequently the impact of gene expression on protein synthesis seemed to be an important factor to be included in a gene expression profile approach to detect rHuEpo.

The MagMAX for stabilized blood tubes RNA isolation kit worked well for all the SCO samples with a good quantity and quality of RNA assessed by the NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and the Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, USA). After processing more than 150 samples from 18 different subjects (SCO), the average RNA yield was 10.9 ± 6.0 with an average RIN value of 8.3 ± 1.1 and the lowest RIN value was 7.0. However, KEN samples were processed in October 2012 using a new MagMAX for stabilized blood tubes RNA isolation kit and despite an expected good yield, the quality was poor. The RNA was highly degraded with RIN values below 5.0 (Figure VIII.4A).

In order to determine whether the problem came from the KEN samples, two samples which had not been extracted yet from SCO, as well as two new samples freshly collected were processed. In addition to these four samples, two other freshly collected samples with an old Tempus Spin RNA isolation kit were also processed. The results were clear (Figure VIII.4). Regardless of the origin of the samples, the quality was poor with the MagMAX for stabilized blood tubes RNA

isolation kit and good with the Tempus Spin RNA isolation kit, respectively. As a result, the problem did not come from the samples but from the extraction kit. The company wanted to replace our MagMAX for stabilized blood tubes RNA isolation kit free of charge, however, they were out of stock at that time, which in turn would had significantly delayed the KEN microarray experiment. Meanwhile, the samples used for miRNA analysis were not whole blood anymore (*i.e.* Tempus Blood RNA tubes) but plasma. In addition, we realized that more samples can be processed in less time with a better quality using the Tempus Spin RNA isolation kit compared to the MagMAX for stabilized blood tubes RNA isolation kit. As a result, due to these practical reasons, the Tempus Blood RNA tubes from KEN were extracted using the Tempus Spin RNA isolation kit. The average RIN value for KEN was 9.1 ± 0.2 and the lowest RIN value was 8.5.

Electropherogram of a RNA sample extracted using the faulty MagMAX kit

Overall Results for sample 1 :   __MD3__

RNA Area:	80.3
RNA Concentration:	80 ng/µl
rRNA Ratio [28s / 18s]:	1.0
RNA Integrity Number (RIN):	3.3 (B.02.07)
Result Flagging Color:	
Result Flagging Label:	RIN: 3.30

Fragment table for sample 1 :   __MD3__

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41.06	42.04	1.7	2.1
28S	46.70	47.77	1.7	2.1
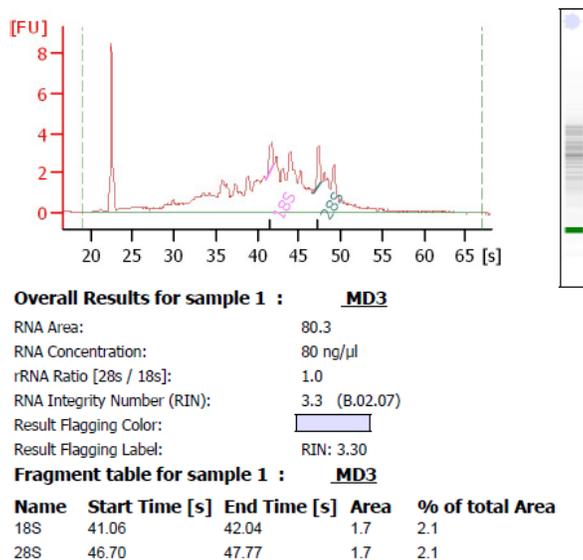
A. Electropherogram of a duplicate of the above sample extracted using the Tempus Spin kit

Overall Results for sample 3 :   __MD5__

RNA Area:	74.5
RNA Concentration:	75 ng/µl
rRNA Ratio [28s / 18s]:	1.6
RNA Integrity Number (RIN):	9.1 (B.02.07)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.10

Fragment table for sample 3 :   __MD5__

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.88	42.91	16.6	22.3
28S	46.70	50.58	27.1	36.4
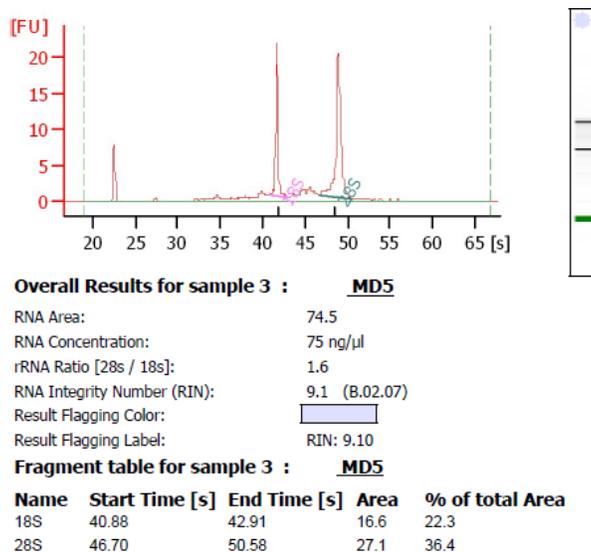
Figure VIII.4: Typical electropherograms (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, USA) observed during the troubleshooting RNA quality experiments in October/November 2012.

Appendix C: Lists of genes from Venn diagrams:
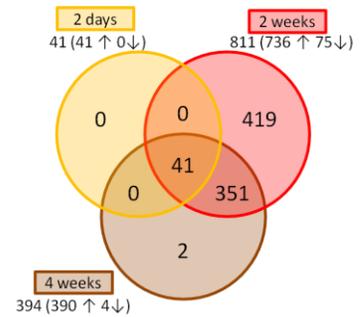
Figure 3.7:

41 transcripts:

ALAS2	E2F2	IFI27	RBM38	TMOD1
ALAS2	EPB42	KRT1	RNF10	TRIM58
BCL2L1	FAM46C	LOC100131164	SELENBP1	WDR40A
BPGM	FECH	LOC441455	SIAH2	XK
C16ORF35	GLRX5	MARCH8	SLC4A1	YOD1
C1ORF128	GMPR	OSBP2	SLC6A10P	
C20ORF108	GYPB	PIP5K2A	SNCA	
C5ORF4	GYPE	RAP1GAP	SNCA	
CA1	HBD	RBM38	TMEM158	

351 transcripts:

ABCC13	BCR	CHD8	EPB49	GMPPB
ACPT	BDNF	CHPT1	EPHA8	GNA12
ACTRT1	BLVRB	CHPT1	FAM104A	GPR109B
ADIPOR1	BMP2K	CISD2	FAM116B	GPR146
ADIPOR1	BNIP3L	CLK2	FAM117A	GPR175
ADORA1	BSG	CMTM3	FAM134A	GPX1
ADRA2C	C14ORF45	COL8A2	FAM83F	GRINA
AHSP	C16ORF35	CREG1	FBXO7	GRINA
ALAS2	C18ORF10	CSDA	FBXO7	GSPT1
ALDH5A1	C18ORF10	CTNNAL1	FBXO9	GUK1
AMAC1L3	C19ORF22	CTSB	FCGR3A	GYPC
ANKRD9	C19ORF62	DARC	FGF23	GYPC
AP2A1	C19ORF62	DCAF6	FHL2	HAGH
AP2A1	C20ORF132	DDR1	FIS1	HAGH
AP2M1	C22ORF13	DNA2	FKBP8	HBE1
AP2M1	C22ORF25	DNAJA4	FLJ20489	HBM
ARL4A	C22ORF30	DNAJB2	FOXO3	HBQ1
ARL4A	C2ORF24	DPM2	FOXO3	HDAC6
ARTN	C9ORF62	DPM2	FOXO4	HDAC7A
ASCC2	C9ORF78	DPYSL5	FRMPD2L1	HDGF
ATP6AP1L	CAMK1G	DSCAM	FURIN	HEBP1
ATP6V0C	CARM1	EIF1B	FXR2	HEMGN
ATP6V0C	CCDC23	EIF2AK1	FZD7	HEMGN
ATXN10	CCDC52	EIF2C2	G6PC	HIVEP3
BAT3	CDC34	ELOF1	GDF1	HMBS
BBC3	CES3	EPB41	GJC2	HMBS
BCL2L1	CFHR2	EPB41	GLUL	HOXD3

HPS1	LOC642377	MED1	RAB2B	STRADB
HPS1	LOC642469	MED22	RAB3IL1	TBC1D10B
HS.105618	LOC643665	MEGF10	RAD23A	TESC
HS.149244	LOC643933	MGC13057	RANBP10	TFDP1
HS.187290	LOC644039	MICAL2	RGS10	TFDP1
HS.291319	LOC644852	MICALCL	RIOK3	TGM2
HS.412918	LOC645173	MIR1976	RIOK3	TGM2
HS.537603	LOC645284	MIR877	RNF11	TMCC2
HS.539888	LOC646463	MKRN1	RNF213	TMEM111
HS.544069	LOC646508	MPP1	RNF40	TMEM63B
HS.544245	LOC647742	MUC6	RNU6-1	TMEM86B
HS.564949	LOC648003	MXI1	ROPN1B	TNS1
HS.569831	LOC648390	MXI1	RP11-529I10.4	TRAK2
HS.57079	LOC648526	MXI1	RPIA	TRIM10
HS.571622	LOC648796	MYL4	RTF1	TRIM10
IFIT1L	LOC650832	NFE2	RUNDC3A	TSPAN5
IGF2BP2	LOC652045	NFIX	SEMA6B	TSTA3
IGFBP1	LOC652140	NINJ2	SENP7	TTC25
IRX1	LOC652968	NOC4L	SERPINA13	TUBB2A
ISCA1L	LOC653073	NOXO1	SESN3	TYW1
ITLN1	LOC653498	NR1D1	SIPA1L3	UBE2F
KAT2B	LOC653635	NTAN1	SLC14A1	UBE2H
KCNJ10	LOC653778	OPTN	SLC1A5	UBE2O
KDM2B	LOC653907	OR2A42	SLC25A23	UBE2W
KIAA1539	LOC654103	OR2W3	SLC25A37	UBXN6
KLC3	LOC654155	PDZK1IP1	SLC25A39	VTI1B
KLF1	LOC729870	PHLDB1	SLC25A45	VWCE
LGALS3	LOC730226	PHOSPHO1	SLC30A3	WASF2
LMNA	LYL1	PINK1	SLC38A5	WDR26
LOC100130332	LYPD3	PIP4K2A	SLC6A8	WDR45
LOC100130623	MAF1	PLEK2	SLC6A9	WDR89
LOC100130905	MAGEL2	PNPLA2	SLC7A5	WNK1
LOC100131391	MAP2K3	POLR1D	SLITRK1	WNK1
LOC100131726	MAP2K3	PPM1A	SMOX	WWP2
LOC100132266	MAP2K3	PPP2R5B	SMOX	XPO7
LOC148430	MAP2K3	PRDM9	SNORD8	YBX1
LOC284422	MAP4K4	PRDX2	SNX3	YIPF6
LOC387686	MARCH2	PRDX2	SPRYD3	YPEL3
LOC388588	MARCH2	PRR5	SPTB	ZNF653
LOC389599	MARCH8	PRR5	SRRD	ZRANB1
LOC440313	MARCH8	PSME4	SRXN1	
LOC440359	MBNL3	PSMF1	ST6GALNAC4	
LOC441081	MBNL3	PTMS	ST6GALNAC4	
LOC441481	MCOLN1	PTPN6	STOM	
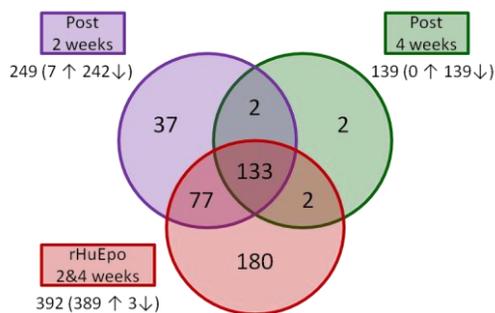
Figure 3.9:

133 transcripts:

ALAS2	MUC6	MIR1976	G6PC	FOXO3
SNCA	HS.571622	UBXN6	FRMPD2L1	CAMK1G
SNCA	FCGR3A	GMPR	TMOD1	LOC388588
HBD	ROPN1B	LOC387686	GYPE	HS.539888
RNF213	ADIPOR1	LOC646508	CSDA	C5ORF4
DPYSL5	ADIPOR1	SESN3	GSPT1	RBM38
LOC440313	HBQ1	LOC730226	LOC648390	SEMA6B
HBE1	ARTN	LOC441455	LOC654155	HEMGN
HBM	SELENBP1	CMTM3	IGF2BP2	C20ORF132
SERPINA13	FBXO7	IGFBP1	C20ORF108	IRX1
MYL4	SLC25A39	ADRA2C	DNA2	HS.569831
LOC642377	GUK1	HS.544245	DPM2	FAM83F
LOC440359	MBNL3	SPRYD3	LYPD3	LOC284422
EPB42	SIPA1L3	PIP5K2A	SLC6A10P	MKRN1
STRADB	TESC	IFIT1L	ASCC2	ATP6V0C
LOC644852	WDR40A	TSPAN5	PDZK1IP1	BSG
LOC389599	LOC652968	TBC1D10B	TNS1	GLRX5
LOC642469	GYPC	FBXO7	MGC13057	OSBP2
LOC100131726	AHSP	SLC25A37	MAP2K3	RBM38
LOC100131164	HS.149244	RTF1	RIOK3	LOC650832
LOC100131391	ACTRT1	CLK2	LOC652045	BCL2L1
CA1	HAGH	EPB49	FAM116B	DSCAM
LMNA	LOC653635	GYPC	TMEM111	MARCH2
LOC645284	LOC652140	LOC647742	CCDC52	HS.564949
SLC4A1	TRIM58	BCL2L1	FZD7	LOC100130905
GPR175	KRT1	VWCE	C19ORF22	
GDF1	HAGH	ALAS2	GYPB	

Appendix D: Electropherograms of cRNA from two subjects as typical examples:
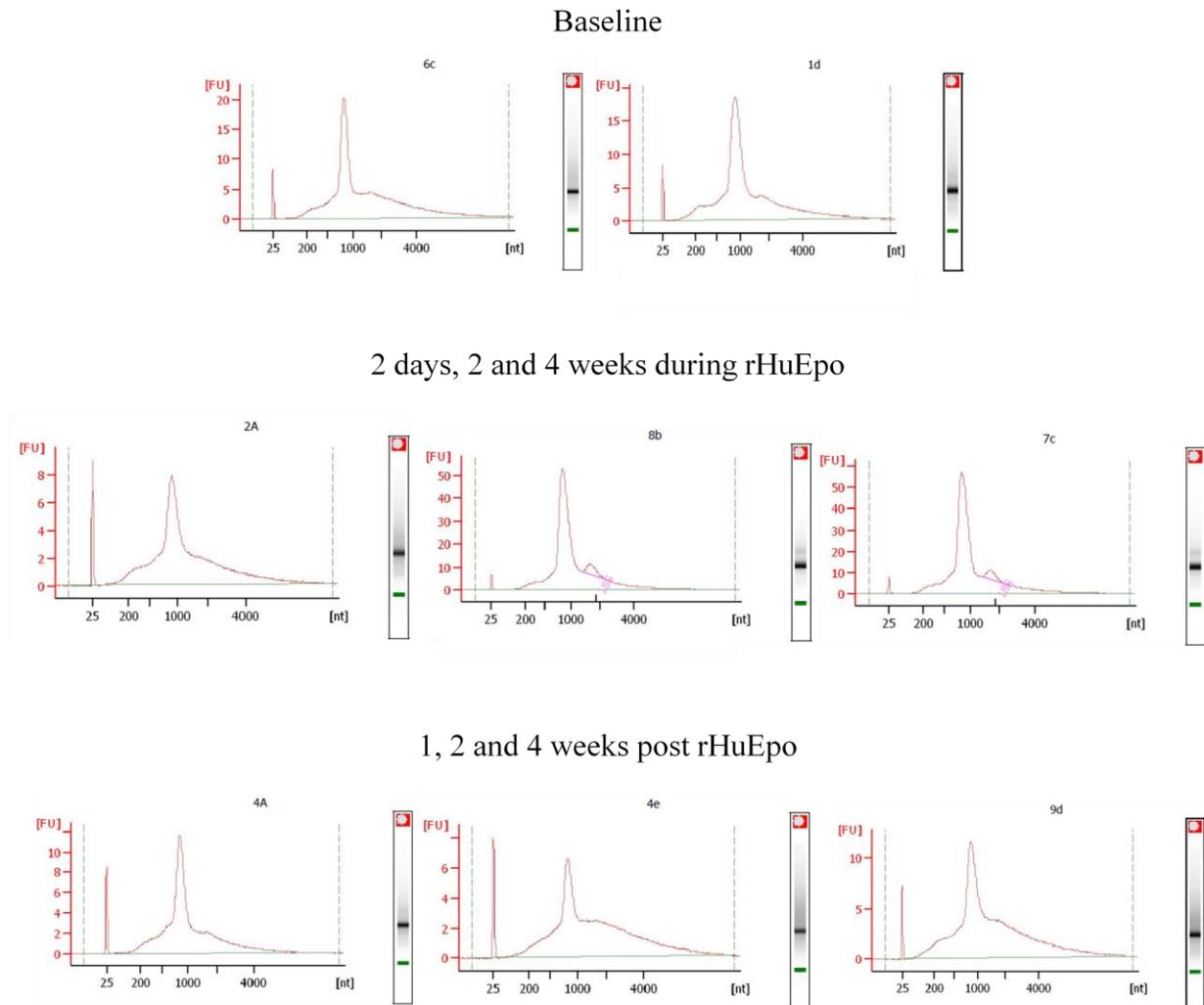
Subject A: as a first typical example

Baseline

2 days, 2 and 4 weeks during rHuEpo

1, 2 and 4 weeks post rHuEpo

Figure VIII.5A: Representative electropherograms (Agilent 2100 Bioanalyzer) of cRNA.

Subject B: as a second typical example

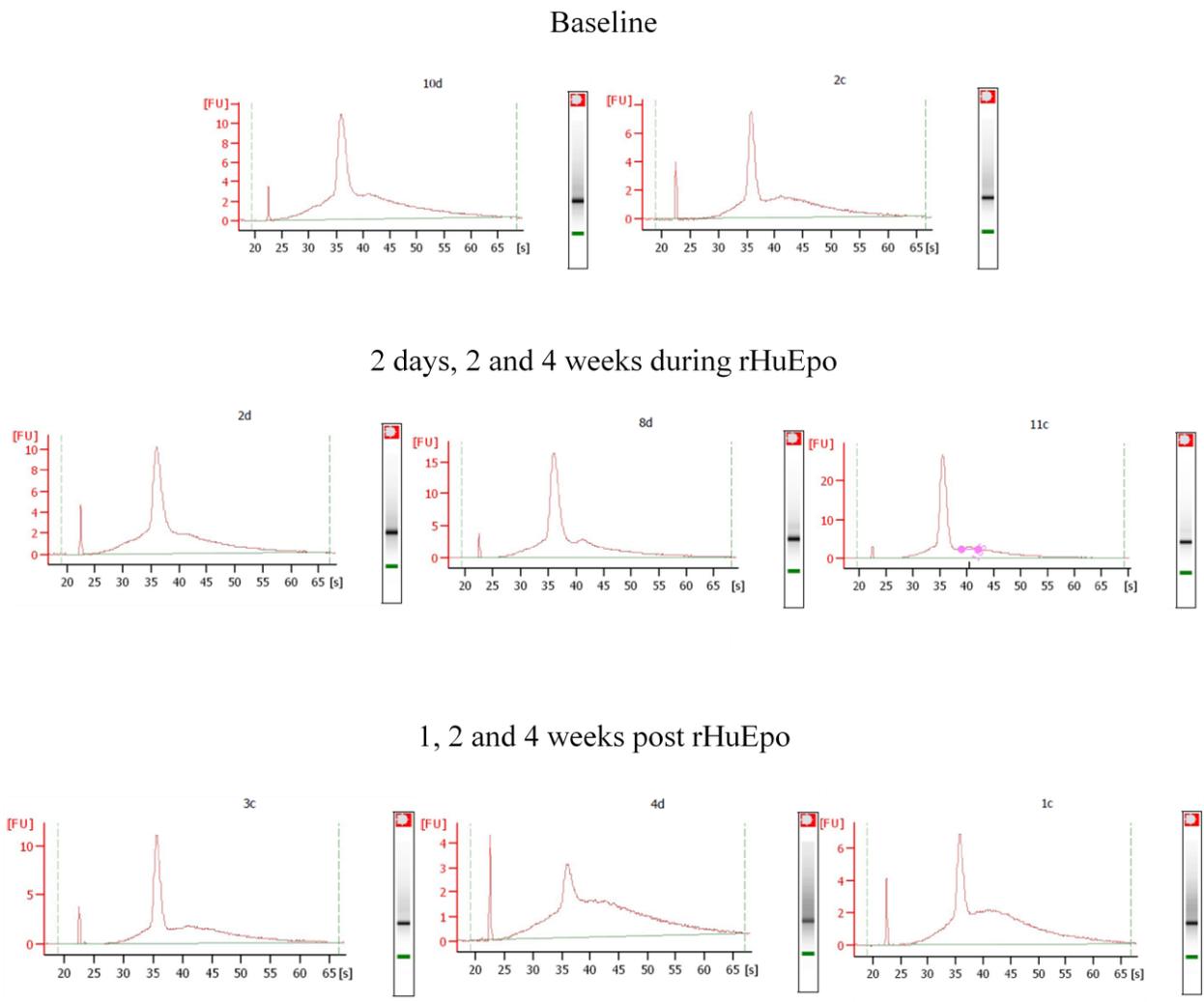## Baseline

2 days, 2 and 4 weeks during rHuEpo

1, 2 and 4 weeks post rHuEpo

Figure VIII.5B: (continued)

Appendix E: Transcriptomic analysis from saliva samples:

A minimally invasive source of potential biomarkers with advantages in collection convenience which may improve athlete compliance with anti-doping tests is saliva. Saliva samples were collected at similar time points as blood samples during the study (Figure 2.1) using two different methods, *i.e.* Oragene RNA collection kit (DNA Gentok Inc., Ottawa, Canada) and indicating FTA mini cards (Whatman, GE Healthcare, Little Chalfont, UK) according to their respective manufacturers' instructions. Saliva samples initially stored in the Oragene RNA containers at -20°C were then incubated at 50°C for 1 hour and aliquoted into 8 x 500 µl. Four aliquots were kept at -80°C and the remaining at -20°C. The FTA mini cards were stored at -20°C. RNA was extracted following the Oragene RNA purification protocol using the Qiagen RNeasy micro kit (Qiagen, Crawley, UK) and using the MagMAX viral RNA isolation kit (Ambion, Austin, USA) for the Oragene and FTA mini cards saliva samples, respectively

The RNA yields were inconsistent in terms of quantity and quality. Although further analyses are necessary, there was very high inter- and high intra-individual variability 1) in the RNA yield possibly due to the differences in the levels of oral bacterial RNA across individual donors (Figure VIII.6) (Patel *et al.* 2011) and 2) in the quality of the RNA samples (Figure VIII.7), respectively. Nevertheless, the degradation of the RNA extracted from the Oragene saliva samples was much lower compared to a previously published study which used microarray analysis and concluded that "the quality of the majority of the microarrays was acceptable" despite the poor quality of the saliva RNA samples (Kupfer *et al.* 2010).

Four Oragen saliva RNA samples were analysed alongside the blood RNA samples during the QuantiGene validation experiment. Samples collected at baseline and at the end of the rHuEpo administration in two subjects were selected. Not surprisingly, the genes were either not expressed in saliva or the results were not consistent (Figure VIII.8). The results confirmed that the biomarkers of rHuEpo administration discovered in whole blood in this research are not necessarily useful in other tissues such as saliva. Whole transcriptome analysis for each tissue of interest is required for biomarker discovery.
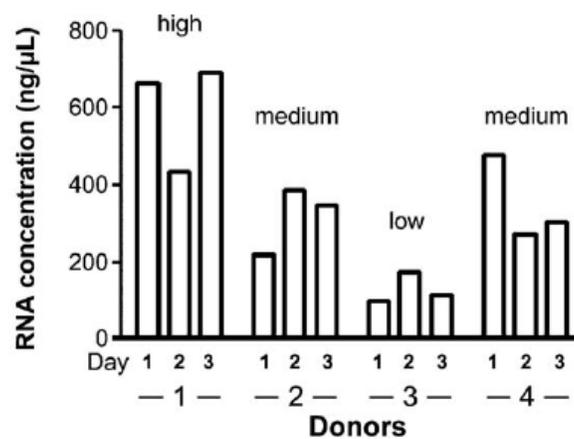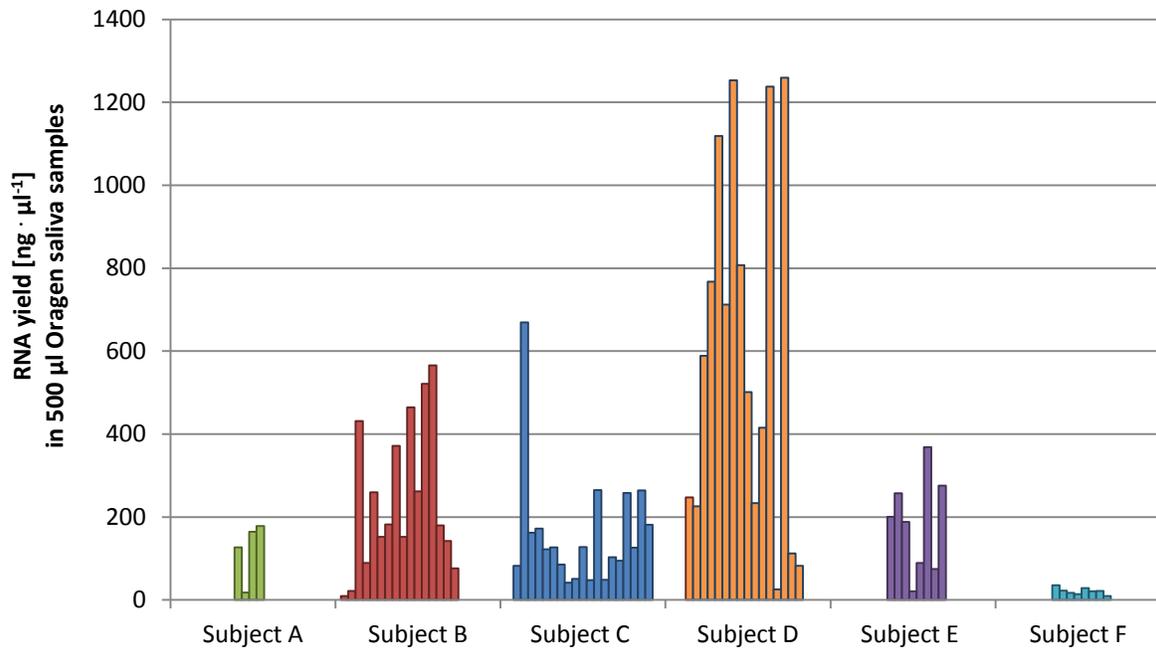
Figure VIII.6: Total RNA yield of Oragene RNA samples.

RNA yield concentration extracted from 500 μl of saliva stabilized in Oragene RNA collection kit in 6 subjects of our research study (top panel) and in a published study (bottom panel) (Patel *et al.* 2011). Bottom figure taken from Patel *et al.* (2011).
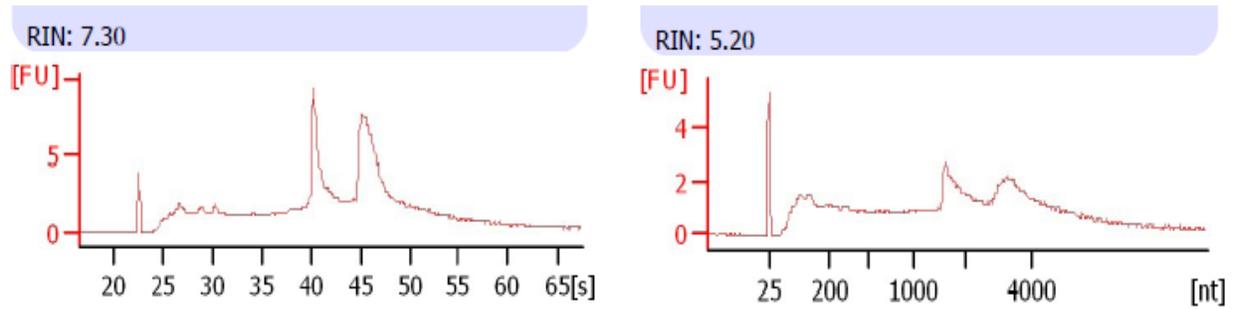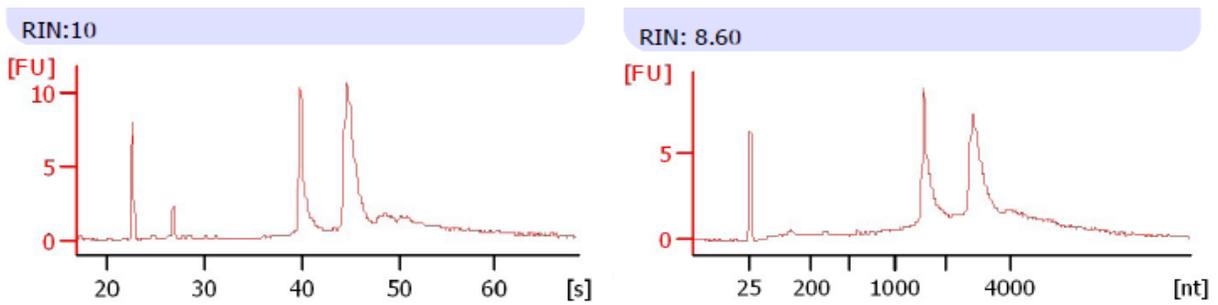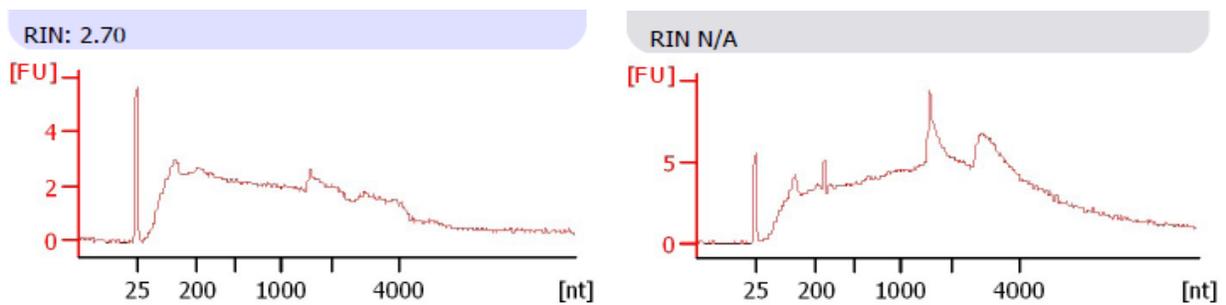
Subject B of Figure VIII.6: Typical examples

Subject C of Figure VIII.6: Typical examples

Subject D of Figure VIII.6: Typical examples

Figure VIII.7: Representative electropherograms (Agilent 2100 Bioanalyzer) of Oragene RNA saliva samples.
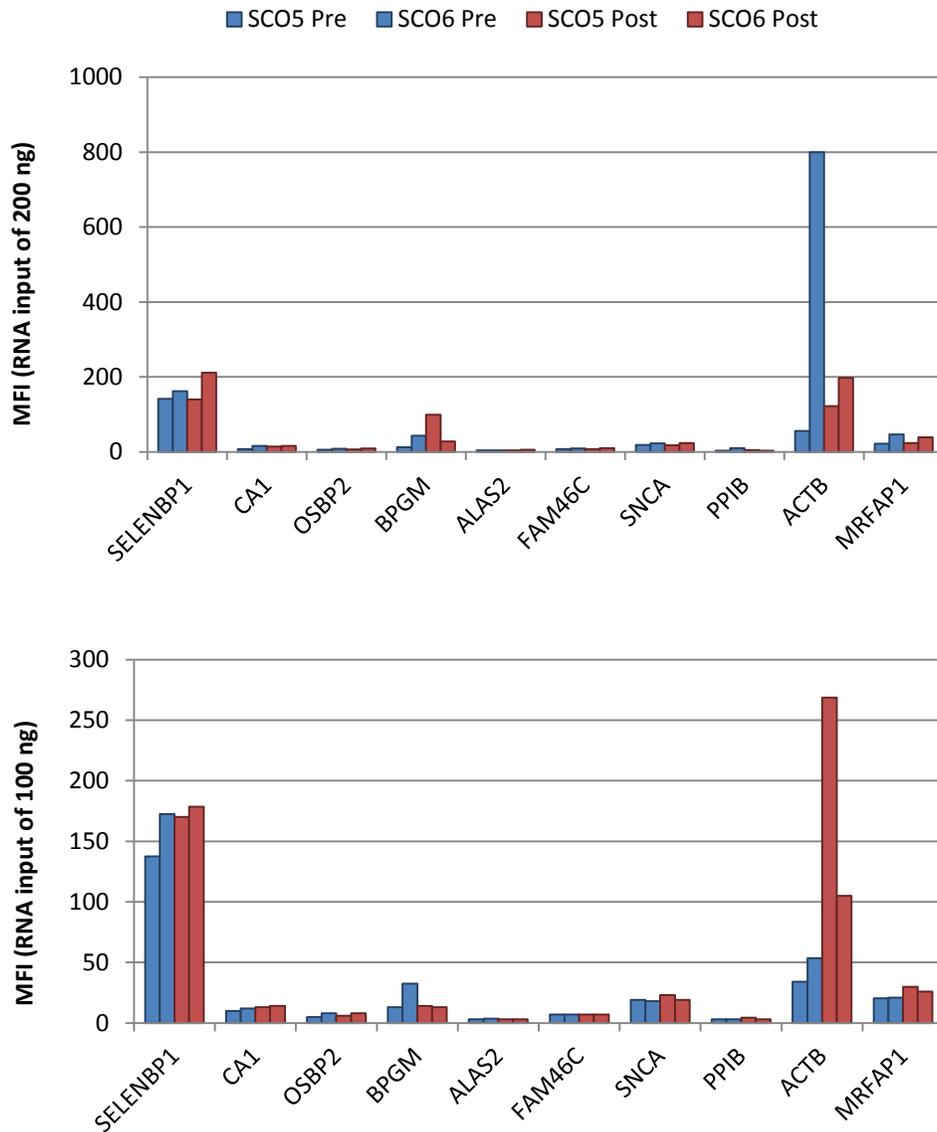
Figure VIII.8: Median fluorescence intensity (MFI) signals of four Oragene saliva RNA samples with the two different RNA inputs

Samples collected at baseline and at the end of the rHuEpo administration in two subjects were selected. Of note, RNA from whole blood produced MFI signals > 1,000 for most of the target and housekeeping genes (see Table 6.2).

Appendix F: Approved proposal for emergency funding:

A New Dimension to the Detection of Recombinant Human Erythropoietin Doping Using Blood Gene Expression Profiles: Development of Direct Anti-Doping Applications

INTRODUCTION:

The main purpose of the research project funded by the World Anti-Doping Agency (WADA) in 2008 entitled "A gene-microarray based approach to the detection of recombinant human erythropoietin (rHuEpo) doping in endurance athletes" (08C19YP) was to develop new improved methods based on gene expression profiles. This research project has generated most promising results which have the potential to add a new dimension to the current detection methods such as the Athlete Biological Passport (ABP). However, further works are required before establishing a robust diagnostic test. Please see below a summary of the research project in lay language as well as more technical information.

SUMMARY (lay language):

The use of rHuEpo is prohibited by WADA. Omics technologies can be used to reveal the specific molecular signature of a particular stimulus (rHuEpo doping for instance). Therefore, molecular markers, such as gene markers, characteristic of rHuEpo doping have the potential to improve further the discriminatory power of current detection methods such the ABP (Reichel 2011a). The aim of the study was to assess the effects of rHuEpo on blood gene expression profiles in endurance trained males in order to identify a molecular signature of rHuEpo doping and hence provide a basis for the development of novel testing models to detect doping.

39 endurance trained males, 19 based at sea-level (Glasgow, Scotland) and 20 based at moderate altitude (Eldoret, Kenya, 2100-2800m a.s.l), received rHuEpo for 4 weeks. Blood was obtained 2 weeks before, during and 4 weeks after administration (Figure 1). Blood gene expression profiles were profoundly altered during rHuEpo and for at least 4 weeks after administration leading to a "molecular signature" of rHuEpo doping. Importantly, as illustrated in the genes selected as typical example in Figure 3, the same pattern was observed in all subjects. These most promising data provide the strongest evidence to date that gene biomarkers will add a new dimension to the ABP in terms of sensitivity and specificity.

In order to set up a potential future test which can reveal the molecular signature of rHuEpo doping, a pilot study using a subset of subjects (n=8) and promising target genes (n=7) was carried out using a specific, sensitive, time and cost-effective technology (QuantiGene Plex assay, Panomics/Affymetrix, Italy). Following the success of this pilot study, we would like to increase the number of target genes to 50 which will allow a more defined and precise molecular signature of rHuEpo doping and we would like to analyse these 50 target genes in all subjects (*i.e.* 39 in total). This study will enable 1) the validation of the molecular signature of rHuEpo doping and 2) the development of the establishment of the testing procedure.

FURTHER TECHNICAL INFORMATION:

Methods: 39 endurance trained males, 19 based at sea-level (Glasgow, Scotland - SCO) and 20 based at moderate altitude (Eldoret, Kenya, 2100-2800m a.s.l. - KEN), received rHuEpo injections of 50 $IU \cdot kg^{-1}$ body mass every two days for 4 weeks. Blood was obtained 2 weeks before, during and 4 weeks after administration (Figure 1). Eight time points per subjects (SCO: n = 18, KEN: n = 6) were selected for gene expression analysis, *i.e.* two baseline samples, then one sample each at 2 days, 2 weeks and 4 weeks after the first rHuEpo injection and 1 week, 2 weeks and 4 weeks after rHuEpo administration (Figure 1). RNA was extracted from blood stabilized in Tempus RNA tubes and stored at -80°C.

Illumina experiment: RNA of 18 and 6 subjects in SCO and KEN, respectively, was amplified, labelled and hybridized to Illumina HumanHT-12v4 Expression BeadChips. Expression data was analysed using RankProducts (Breitling *et al.* 2004) with a 5% false discovery rate and an additional 1.5 fold-change threshold.

QuantiGene Plex pilot experiment: RNA of four subjects in SCO and KEN (*i.e.* 8 subjects in total) was hybridized, amplified and labelled using branched DNA and xMAP magnetic beads technologies. The signal was read using the Luminex MAGPIX. Following background subtraction, the ratio of the target genes signal to the geometric mean of the housekeeping gene signal was calculated.

More information on QuantiGene Plex assay: The QuantiGene Plex assay, which combines branched DNA and bead-based multiplexing technology, allows the simultaneous analysis of a large number of target genes per sample and enables high-throughput screening (Flagella *et al.* 2006). In addition to being extremely reproducible, specific and sensitive, the QuantiGene assay provides an independent method of measurement compared to the other technologies such as quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) and microarray platforms by eliminating the need for RNA reverse transcription, labelling and amplificat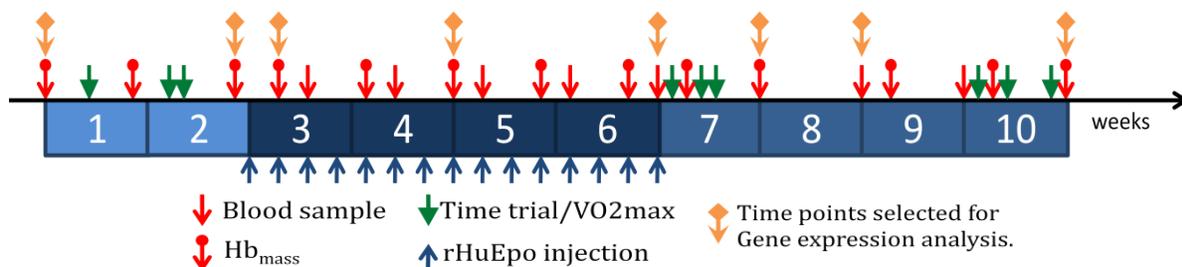ion steps (Canales *et al.* 2006; Flagella *et al.* 2006). (http://www.panomics.com/products/gene-expression/plex-2.0-assay/how-it-works)

Figure 1: Experimental design

Results of the Illumina experiment (summary):

Using the additional stringent >1.5 FC threshold, hundreds of genes were found to be differentially expressed following rHuEpo administration in both groups (Figure 2). Among these genes, 30 were already strongly up-regulated 2 days after only one single injection in common in both groups (Figure 2). 15 genes were strongly up-regulated during the last 2 weeks of rHuEpo and subsequently down-regulated at 2 and 4 weeks after administration (Figure 2). Importantly, as illustrated in the genes selected as a typical example in Figure 3, the same pattern was observed in all subjects.
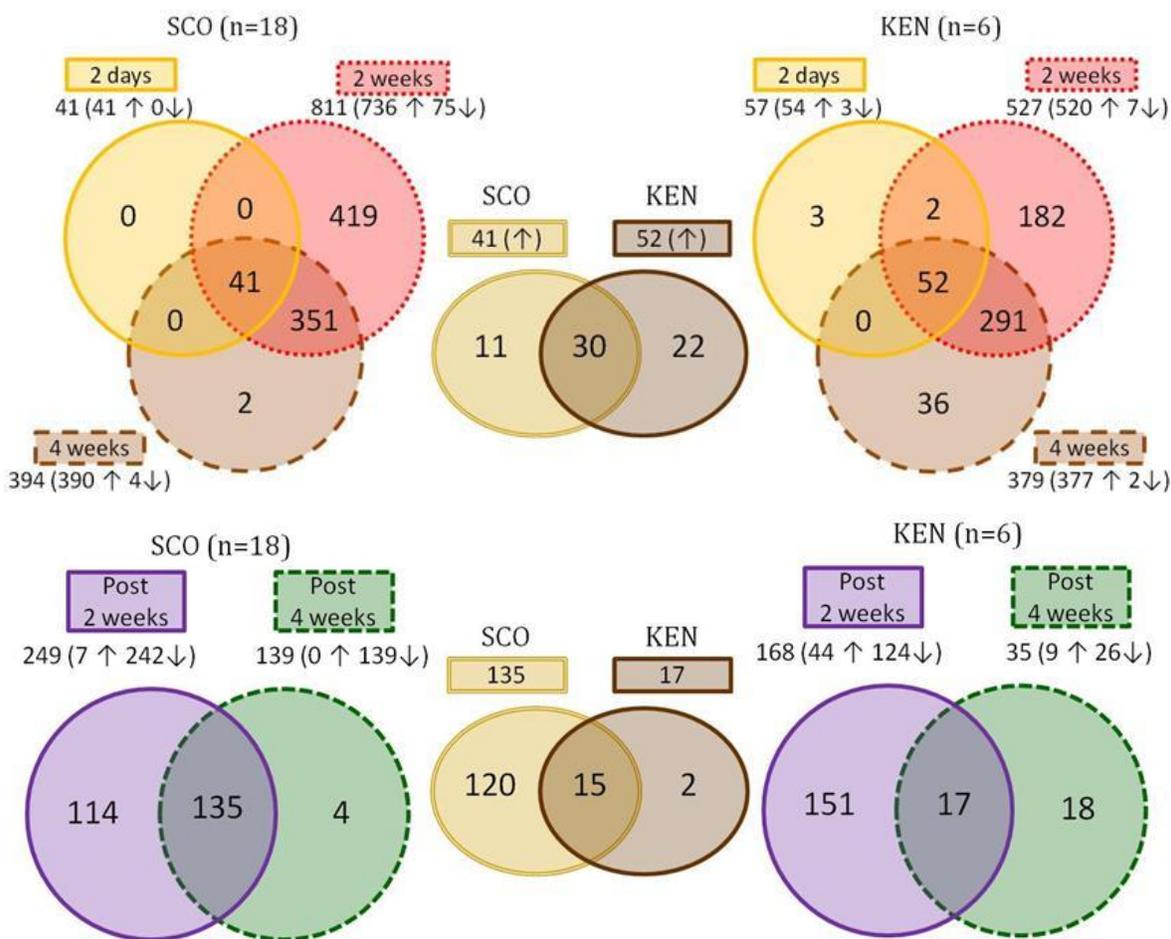

Figure 2: Venn diagrams of genes differentially expressed during (top panels) and post (bottom panels) rHuEpo compared to baseline in SCO (left panels) and KEN (right panels). Values in brackets are number of transcripts up- (↑) and down-regulated (↓) compared to baseline. The Venn diagram in the middle depicts the intersection of Venn diagrams in SCO and KEN.
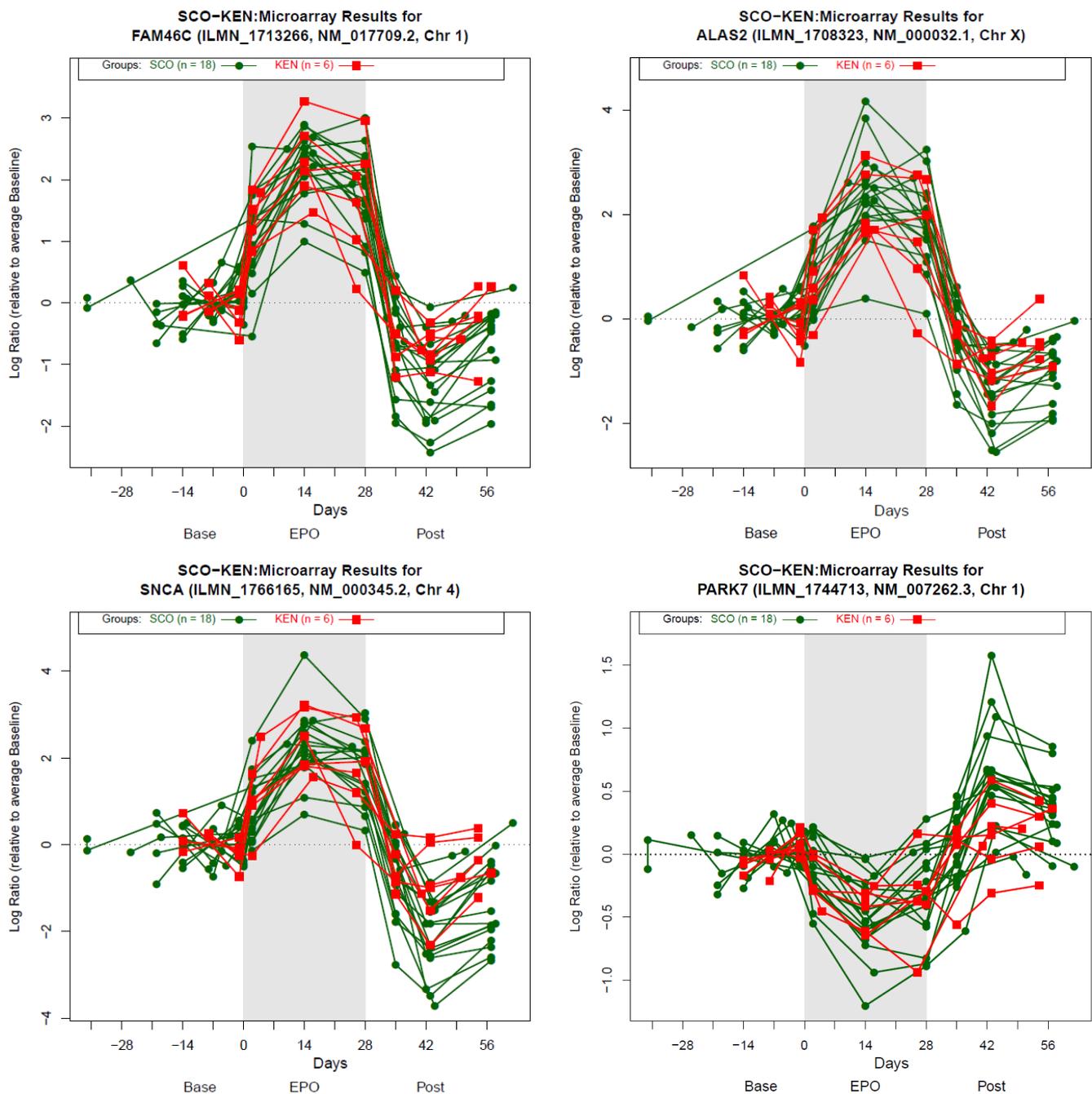
Figure 3: Individual changes in gene expression in four genes selected as typical example. Changes are reported in log ratio compared to the average baseline values. Time point 0 represents the first injection. The grey zone represents the administration. Each line corresponds to one subject. SCO (n = 18) and KEN (n = 6) are represented in green, respectively.

Results of the QuantiGene Plex pilot experiment (summary):

The three housekeeping genes were found to be fairly stable over time in all subjects (Figure 4). The QuantiGene Plex results successfully replicated the Illumina microarray results as illustrated in the two genes selected as typical example in Figure 5.
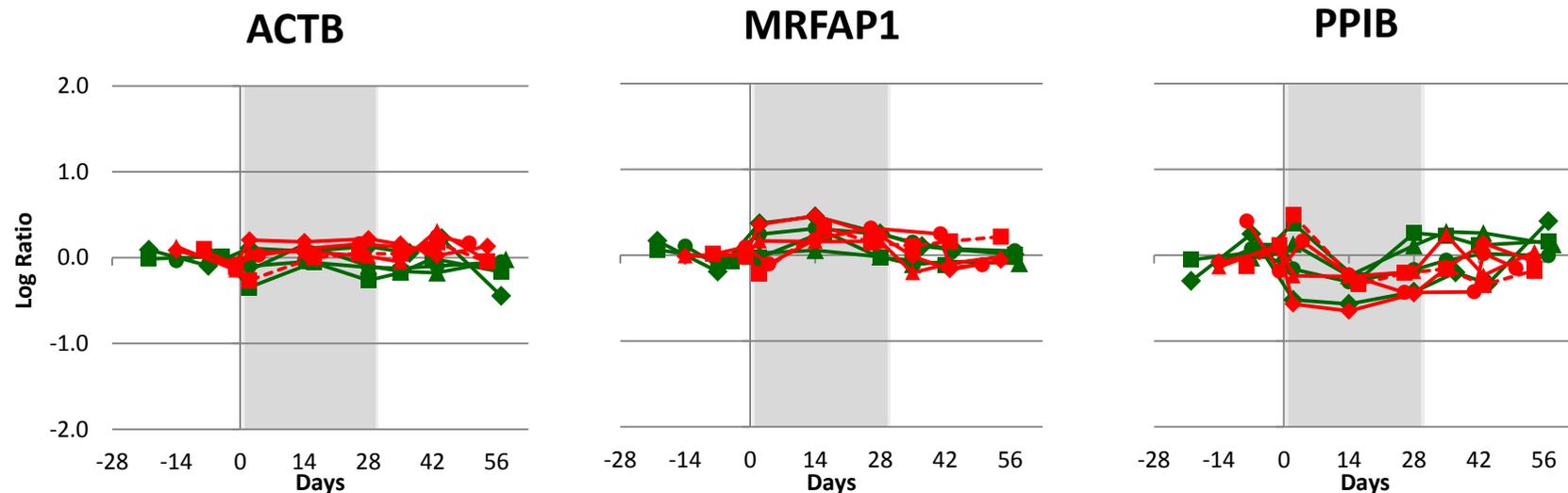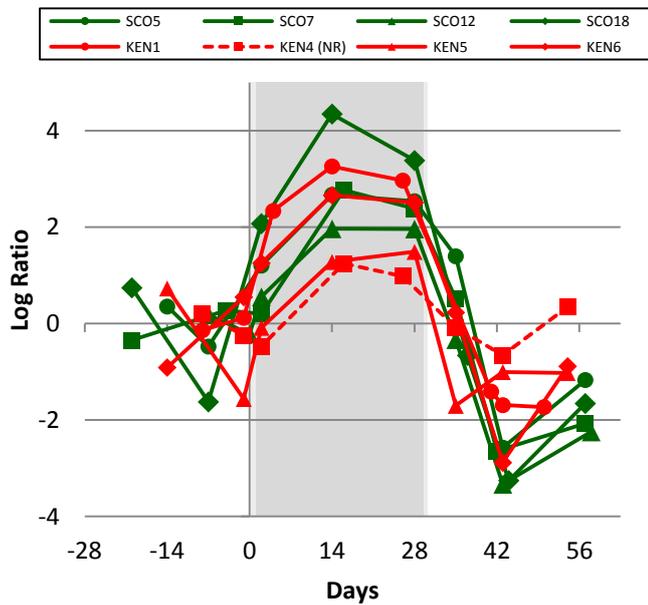

Figure 4: Individual changes in gene expression in the three housekeeping genes using the QuantiGene Plex assay. Actin, beta (ACTB), MORF4 family-associated protein 1 (MRFAP1) and Peptidylpropyl isomerase B (cyclophilin B) (PPIB): Changes are reported in log ratio compared to the average baseline values. Time point 0 represents the first injection. The grey zone represents the administration. Each line corresponds to one subject. SCO (n = 4) and KEN (n = 4) are represented in green and red, respectively.

QuantiGene Plex   **Illumina Microarray**
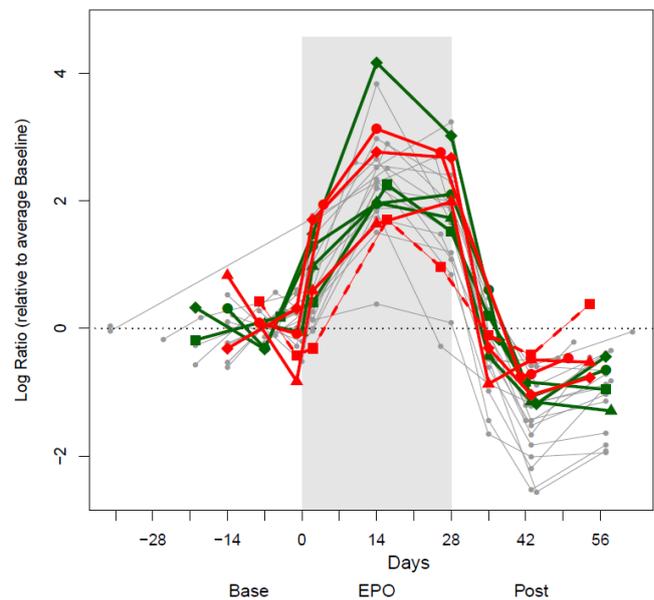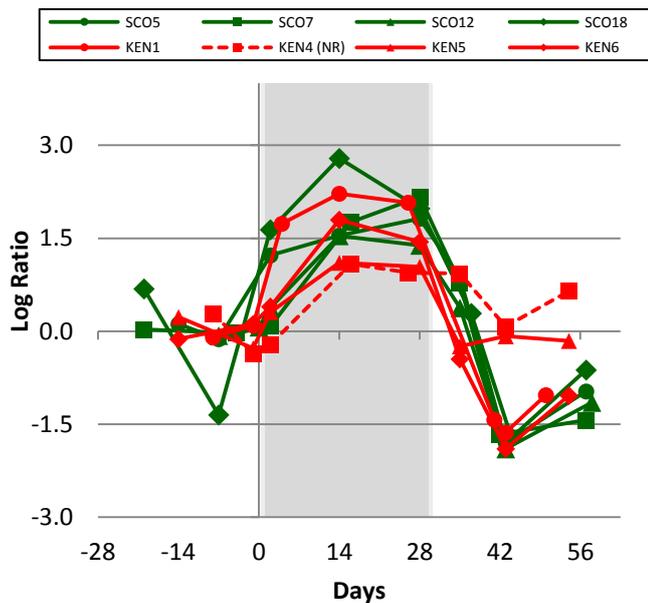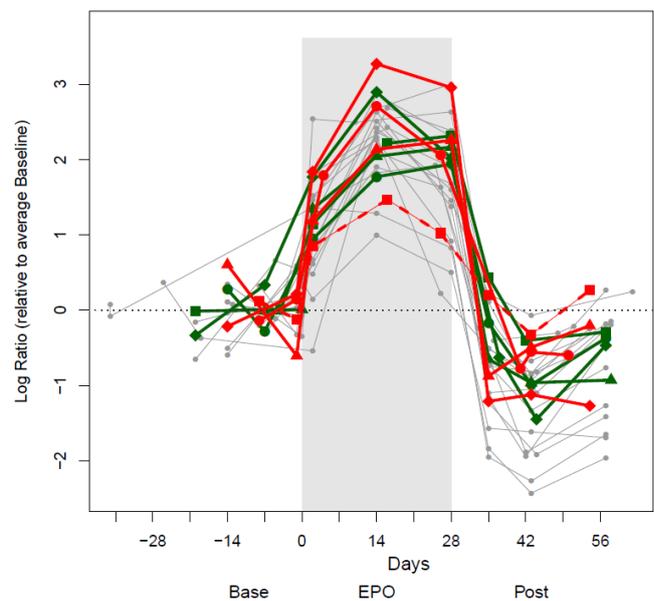
Figure 5: Individual changes in gene expression in two genes selected as a typical example: Delta-aminolevulinate synthase 2 (ALAS2) (top panels) and Family with sequence similarity 46, member C (FAM46C) (bottom panels). Results of the QuantiGene Plex assay and the Illumina microarray are presented in the left and right panels, respectively. Changes are reported in log ratio compared to the average baseline values. Time point 0 represents the first injection. The grey zone represents the administration. Each line corresponds to one subject. SCO (n = 4) and KEN (n = 4) are represented in green and red, respectively.
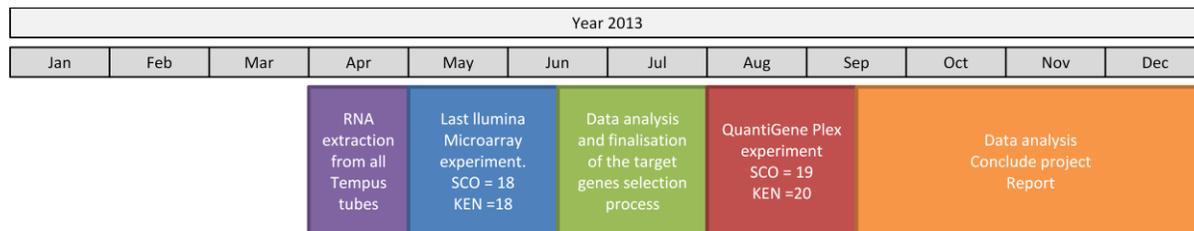
Discussion: In this project, we successfully used gene expression profiling in whole blood to identify genes that are differentially regulated following rHuEpo administration in Caucasian trained males and Kenyan runners living at sea-level and altitude, respectively, leading to the discovery of a "molecular signature" of rHuEpo doping. These results provide the strongest evidence to date that omics technologies such as gene expression have the potential to add a new dimension to the ABP in terms of specificity and sensitivity for rHuEpo detection.

Following the success of the QuantiGene Plex pilot experiment and at the prospect of setting up a potential future test which can reveal the molecular signature of rHuEpo doping, we would like 1) to finalise the microarray experiment, *i.e.* 12 more KEN subjects to be analysed to make a balanced design and 2) to increase the number of target genes to 50 which will allow a more defined and precise molecular signature of rHuEpo doping and we would like to analyse these 50 target genes in all subjects (*i.e.* 39 in total) using the specific, sensitive, time and cost-effective QuanitiGene Plex technology. This study will enable the validation of the molecular signature of rHuEpo doping as well as the establishment of the testing procedure.

BUDGET – Illumina microarray and QuantiGene Plex experiments:

Exchange rate = 0.65

Illumina microarray		
Extraction and amplification kits	£ 6,350.00	$ 9,769.23
Illumina BeadChip	£ 12,170.00	$ 18,723.08
QuantiGene Plex		
Assay kit	£ 3,098.00	$ 4,766.15
Blood Sample Processing Kit	£ 678.00	$ 1,043.08
Quantigene Plex Probeset	£ 42,248.00	$ 64,996.92
Discount 20% QuantiGene Plex	-£ 9,204.80	-$ 14,161.23
TOTAL	**£55,339.20**	**$ 85,137.23**

TIME LINE:

Year 2013											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec

	RNA extraction from all Tempus tubes	Last Ilumina Microarray experiment. SCO = 18 KEN =18	Data analysis and finalisation of the target genes selection process	QuantiGene Plex experiment SCO = 19 KEN =20	Data analysis Conclude project Report

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Appendix G: One page summary to include in WADA's website:

The use of recombinant human erythropoietin (rHuEpo) is prohibited by the World Anti-Doping Agency. An omics-based longitudinal screening approach has the potential to improve further the performance of current detection methods such as the Athlete Biological Passport. For this project, we successfully used gene expression profiling in whole blood to identify genes that are differentially regulated following rHuEpo administration in Caucasian trained males and Kenyan endurance runners living at sea-level and moderate altitude (~2150 m), respectively. Relative to baseline, the expression of hundreds of genes were found to be altered by rHuEpo. In particular, 15 transcripts were profoundly up-regulated during the 4 weeks of rHuEpo administration and subsequently down-regulated up to 4 weeks post administration in both groups. Importantly, the same pattern was observed in all subjects. Furthermore, 30 transcripts were already differentially expressed two days after the first injection and are therefore promising candidate genes to detect microdose rHuEpo doping. The functions of the discovered genes were mainly related to either the functional or structural properties of the erythrocyte or to the cell cycle and its regulation. In summary, this research project successfully identified the blood "molecular signature" of rHuEpo administration and provided a set of candidate genes with potential to be robust biomarkers of rHuEpo doping. These preliminary results provide the strongest evidence to date that omics technologies such as gene expression have the potential to substantially improve and add a new dimension to the current anti-doping methods such the Athlete Biological Passport for rHuEpo detection.