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Glutamine and Glutamate Reference Intervals as a Clinical Tool to Detect Training Intolerance During Training and Overtraining

Rodrigo Hohl[1], Lázaro Alessandro Soares Nunes[1],
Rafael Alkmin Reis[1], René Brenzikofer[1], Rodrigo Perroni Ferraresso[1],
Foued Salmen Spindola[2] and Denise Vaz Macedo[1]
[1]Laboratory of Exercise Biochemistry (LABEX), Biology Institute,
University State of Campinas (UNICAMP),
[2]Laboratory of Biochemistry and Molecular Biology (LABIBI),
Federal University of Uberlândia (UFU)
Brazil

1. Introduction

1.1 Training and overtraining

A training process consists of a sum of repeated exercise sessions with gradual overloads that are performed in a systematised and programmed way. The workload can be manipulated through variables such as weight load resistance, speed, duration, pauses between stimuli, muscular action, movement speed, amplitude, weekly frequency, number of sessions per day, number of exercises per session and the combination of different exercises in the same session.

Exercise triggers the synthesis of several enzymes and structural proteins that adapt tissues, organs and systems to changes in cellular homeostasis, in a task-oriented way and depending on the exercise stimulus. This set of chronic physiological and metabolic changes, currently termed supercompensation, allows for a more efficient and sustainable physiological environment during voluntary physical activity. Supercompensation supplies energy economy for habitual physical activities or enhances the energy supply during exercises of high metabolic demands. Recently, our group demonstrated, using proteomic analyses of rat muscle, that only one stimulus of exhaustive, incremental exercise (approximately 30 min) is enough to produce an acute, generalised, metabolic response in the muscular fibre (Gandra et al., 2010). This probably occurs to minimise the stress that will occur in a subsequent exercise session and, in the long term, the cumulative effects of exercise on gene expression lead to specific muscle phenotypic alterations, which is a major aspect of performance enhancement.

However, supercompensation is only achieved when the ratio between overload and recovery time is individually balanced. Damaged tissue structures resulting from the exercise stimulus are repaired during recovery, when rest and food intake are crucial for the energy supply that is required for the synthesis of new proteins and cellular components.

On the other hand, an excess of rest and a lack of exercise load may cause a loss of phenotypic adaptation, or performance stagnation. Therefore, athletes routinely use a continuous process of intense training to achieve maximal competitive performance (Bompa & Haff, 2009; Meeusen et al. 2006). The training load can be manipulated through substantial increases in duration, frequency, intensity or multiple variables simultaneously, along with a reduction of the regenerative period. However, a persistent imbalance between exercise load and recovery time can also lead to a state of chronic fatigue associated with previously acquired performance decrement, generally called overtraining (OT).

Throughout the years, different nomenclatures have been used to describe this loss of performance in previously well-adapted individuals, such as overtraining, overtraining syndrome (OTS), overreaching, non-functional overreaching (NFOR), staleness and chronic fatigue. Independent of the terminology, decreased performance seems to be the only critical feature of OT in human beings (Halson & Jeukendrup, 2004; Meeusen et al., 2006). Furthermore, OT may cause financial loss and emotional distress to trainers and athletes.

The European College of Sport Science proposed a change in terminology for OT (Meeusen et al., 2006). They defined OT as a continuous process of intense training that can generate different outcomes, depending on performance states. Upkeep, or a possible increase in performance after a brief recovery period (days to weeks), was named functional overreaching (FOR); meanwhile, a prolonged decay in performance, reversed only by a long regenerative period (weeks to months) was named non-functional overreaching (NFOR). Finally, the extreme state of the OT process was named OTS, where recovery may take years or may never happen.

The NFOR and OTS states can be associated with one or more symptoms, including accentuated catabolic state; physiological, immunological and biochemical alterations; increased incidence of injury; and mood alterations (Halson & Jeukendrup, 2004). Still, there is no set of conclusive characteristics that define the NFOR and OTS states. Diagnosis is only possible when a decrease in performance cannot be explained by other factors, such as high levels of muscle microtrauma (which is characterised by increased blood concentrations of muscle injury markers such as creatine kinase and lactate dehydrogenase), contusions, diseases, infections, allergies and abnormal cardiac symptoms (Meeusen et al., 2006). Elite athletes are susceptible to OT outcomes because they are constantly submitted to OT to maintain high physical performance during the training season. However, amateur sportsmen who do not respect the time for recovery between stimuli are also susceptible to undesirable OT outcomes.

There are many theories regarding the biological basis of the training–OT *continuum*, but the underlying mechanisms remain to be validated experimentally. Experimental difficulties that have impeded progress in this field include variability of research studies, the contradiction of applying a training program that aims to reduce functional physiological capacity and the lack of volunteer athletes willing to risk losing a season of training and competitions (Halson & Jeukendrup, 2004). These obstacles limit data collection to anecdotes from athletes who have been diagnosed as overtrained (Halson & Jeukendrup, 2004) due to the intensification of the training process (i.e., OT), which is routinely utilised by athletes who hope to improve their performance. Thus, physiological and psychological limits dictate a need for research that addresses the avoidance of the undesirable outcomes of OT, maximises recovery and successfully negotiates the fine line between high and excessive training loads (Kellmann, 2010).

1.2 Overtraining animal model

Experiments in humans must meet ethical requirements to protect the physical and emotional well-being of the volunteer subjects. Those subjects must also be aware of all possible benefits and disadvantages of the experimental protocol. Therefore, one must consider the risks of possible damage to the athlete's professional and social life when he or she is subjected to an OT induction protocol. Therefore, the study of OT in animal models is endorsed by The American Physiological Society (APS, 2006), which states that '...experimental protocols that use animal subjects are therefore developed when it would not be appropriate to use human subjects for studies of exercise's impact.'

Currently, animal models are used in all biological research areas. Claude Bernard (1865) advanced the principle of studying animal models and showed how findings in animal models could be translated to human physiology. A model is an imitation object that must have similar characteristics to the imitated object and the capacity to be manipulated without the limitations of the imitated object. Therefore, an OT animal model should display a set of similar alterations that would be expected in humans. In this vein, our group standardised an 11-week treadmill endurance training model using Wistar rats, where a gradual reduction in the recovery time between exercise sessions was introduced during the last three weeks (Hohl et al., 2009). Six incremental performance tests to exhaustion were performed during the training protocol, which is described in Table 1.

Experimental Weeks	Performance tests	Training Speed (m/min)	Training Time (min)	Number of Daily sessions	Recovery between training sessions (h)
	1	(--)	(--)	(--)	(--)
1st	no tests	15	20	1	24
2nd	no tests	20	30	1	24
3th	no tests	22.5	45	1	24
4th	2	25	60	1	24
5th to 7th	no tests	25	60	1	24
8th	3	25	60	1	24
9th	4	25	60	2	4
10th	5	25	60	3	3
11th	6	25	60	4	2

Table 1. Overtraining animal model protocol

This OT animal model was characterised by an adaptive training period (1st to 8th week) followed by a period of increased daily training sessions (9th, 10th and 11th weeks). This OT model is also unique because it allows for the comparison of two distinct groups of animals separated by performance *a posteriori*. One group show continuous performance improvement after 60 hours of complete rest, characterising the FOR state; and the other group show improvement followed by a sharp drop in performance that persists for two weeks, characterising the NFOR state (Fig. 1).

Analysis of performance during training was the parameter used both for selection criteria and to define the experimental FOR and NFOR groups. Thus, similar changes observed in the training groups (i.e., FOR and NFOR) as compared to a control group likely reflect the common response to OT, whereas differences that are unique to the NFOR group reflect the intolerance of some rats to OT. This generates a performance drop that is related to the effects of OT on the intrinsic characteristics of each animal. As observed in Fig. 1, although
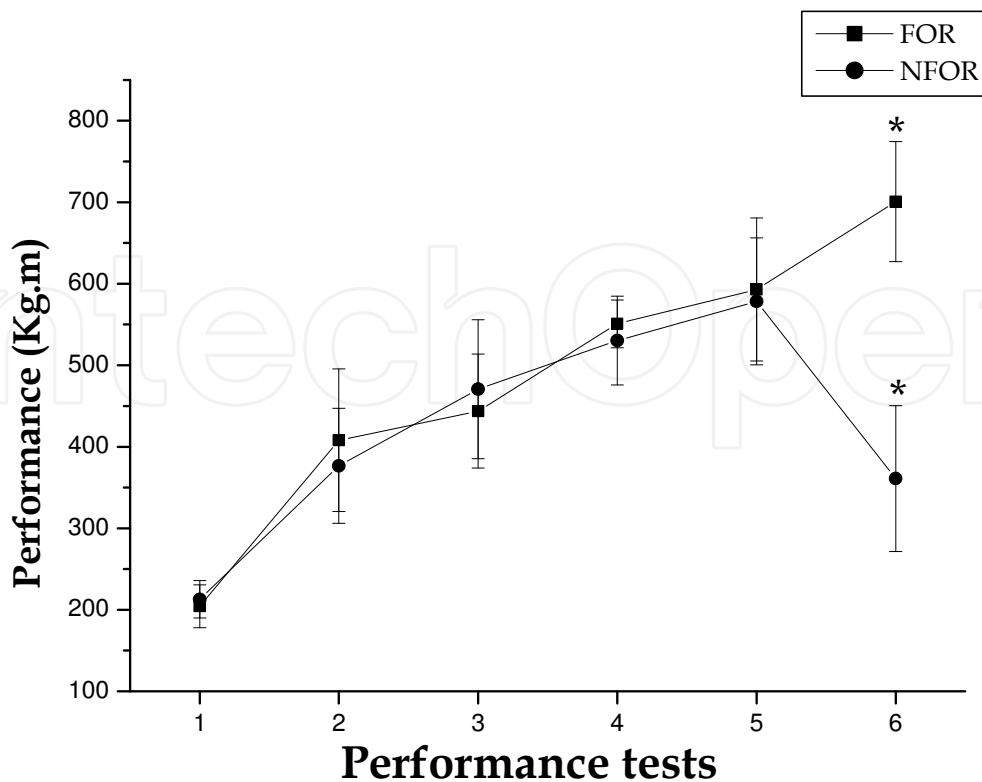
Fig. 1. Performance (mean ± sd) of the FOR (n = 11) and NFOR (n = 8) groups in the six performance tests performed during the 11-week training described in Table 1. * Significant difference between test 6 and test 5 in the paired *t* test analysis of FOR and NFOR groups (p < 0.01 for FOR and p < 0.001 for NFOR).

OT is necessary to maximise the increase in performance, it is also detrimental to the adaptive process for some animals. Lehmann et al. (1993) reported that inter-individual variability in recovery potential, exercise capacity and stress training tolerance explains the different vulnerabilities of athletes to OT under identical training stimuli. Therefore, OT is a process that deserves careful and individualised control of appropriate training loads and recovery times.

The animal model proposed by Hohl et al. (2009) has been a useful tool in seeking tissue and blood biomarkers for use in studying undesirable OT outcomes (i.e., NFOR/OTS) that are associated with an animal's tolerance or intolerance to the same OT protocol. This method can be used to compare the effects of OT in FOR and NFOR group of rats.

1.3 Glutamine and glutamate as potential biomarkers for training intolerance

Some blood biomarkers have been proposed to be associated with OT in humans (Petibois et al., 2002), but there is currently no consensus for all OT cases. One possible biomarker that could be used is the ratio of the concentration of glutamine to glutamate (Gm/Ga) in the blood. A decreased Gm/Ga ratio, due to a decrease in the glutamine blood concentration (Parry-Billings et al., 1992) and/or an increase in glutamate concentration (Coutts et al., 2007; Smith & Norris, 2000) after exercise, has been observed in overtrained humans (Coutts et al., 2007; Halson et al., 2003; Smith & Norris, 2000).

Keast et al. (1995) reported mean plasma glutamine decay from 630 µM to 328 µM in five highly trained male subjects who underwent intensive interval training sessions twice daily

for ten days. The authors concluded that 'reduced plasma glutamine concentrations may provide a good indication of severe exercise stress'. Smith and Norris (2000) reported a mean (± standard deviation (sd)) glutamine concentration of 522 ± 53 µM, glutamate concentration of 128 ± 19 µM and Gm/Ga ratio of 4.15 ± 0.57 'as the extreme values for athletes who have not met conditions of overtraining and are thus managing the training load imposed', and they also proposed that 'overreaching which can also lead to overtraining may occur when the Gm/Ga ratio is 3.58'. Additionally, Halson et al. (2003) reported a mean (± sd) glutamine concentration decrease from 631± 21 µM to 475 ± 40 µM, glutamate increase from 158 ± 18 µM to 235 ± 18 µM and Gm/Ga ratio decrease from 4.38 ± 0.49 µM to 2.13 ± 0.26 µM in 'overreached athletes' after two weeks of intense training loads. The terminologies and statements of 'severe exercise stress', 'who have not met conditions of overtraining' and 'overreaching' suggest that blood glutamine and/or glutamate levels change before the dangerous and undesirable outcomes of OT, here termed NFOR or OTS. We have also found the same pattern of plasma glutamine and glutamate responses in rats subjected to OT (Hohl et al., 2009). Hohl et al. (2009) showed a significantly higher glutamate blood concentration in the NFOR group compared to the FOR group. Moreover, blood glutamine level in the NFOR group suggested a trend to a lower plasma concentration than the FOR group. Therefore, the Gm/Ga ratio in the NFOR group was significantly lower than in the FOR group (3.1 ± 0.2 and 4.5 ± 0.9, respectively), confirming previous studies with humans. Together, these data suggest that changes in glutamine and glutamate levels may be early indicators of some critical aspects of metabolism related to the individual training intolerance.

1.4 Glutamine and glutamate changes due to metabolic maladaptation

Changes in glutamine and glutamate concentrations may not be the direct cause of OT outcomes (Keast et al., 1995), but those changes may be linked to many different aspects of metabolism, which may contribute, in different magnitudes, to the undesirable OT outcomes during the training program. Significant plasma glutamine and glutamate changes could be an indication of undesirable maladaptation in progress, in other words, that the training program is becoming more harmful than helpful to the subject.

In response to deleterious challenges, such as burn damage or surgery, plasma glutamine decreases, despite increased mobilisation from muscle (Lobley et al., 2001). This is probably due to increased metabolic usage by the immune system and the liver, due to immunological challenge. Of note, the skeletal muscle is the main glutamine exporter to the blood stream (Krebs, 1980), and glutamine is mainly metabolised by immune cells such as lymphocytes, macrophages and neutrophils, which all depend on glutaminolysis for cell proliferation (Krebs, 1980).

Smith (2000) proposed that OTS is a response to excessive muscle stress, which may induce a local acute inflammatory response that may evolve into chronic inflammation and can lead to systemic inflammation. Circulating monocytes are activated in response to muscle trauma, which may increase the demand for glutamine. In addition, the increases in pro-inflammatory cytokines IL-6 and Tumor necrosis factor (TNF-α) stimulate glutamine and alanine uptake in human hepatocytes (Fischer and Hasselgren, 1991). Increased glutamine uptake by the liver from blood also favours the synthesis of large quantities of inflammatory-related, acute-phase proteins, such as C-reactive protein and haptoglobin (Marks et al., 1996). Serum proteomic analyses have shown increases in other acute-phase

proteins in NFOR when compared to FOR rats (Lazarim et al., 2010), which may be linked to the decreased blood glutamine observed by Hohl et al. (2009).

Another complementary hypothesis to be considered for blood glutamine reduction is that a decrease in oxidative capacity is caused by muscle mitochondrial damage. The muscle glutamine synthetase (GS) requires α-oxoglutarate as co-substrate for glutamate synthesis that is actually used as GS substrate for glutamine synthesis with ATP and NH_3^+. It was speculated that mitochondrial injuries could limit the availability of α-oxoglutarate formed by the Krebs cycle, thereby diminishing glutamine production inside the muscle (Rowbottom et al., 1995).

This hypothesis is supported by the uncommon reduction in citrate synthase (CS) activity that we found in the NFOR group from the OT animal model protocol (Hohl et al., 2009). The unexpected chronic performance drop associated with lower oxidative capacity in the NFOR group could be related to the increased generation of reactive oxygen species (ROS, e.g., superoxide anion [$O_2^{\bullet-}$], hydrogen peroxide [H_2O_2] and hydroxyl radical [$OH^{\cdot}$]). Ji et al. (1988) have shown that increased ROS production in muscles of rats during prolonged and exhaustive exercise causes an alteration in the intra-mitochondrial redox state. This occurs by the oxidation of thiol groups (-SH) of mitochondrial enzymes (e.g., CS, malate dehydrogenase and aminotransferase alanine), linking this alteration to the reductions in the activities of these enzymes for 48 hours after exercise. We observed oxidative stress in the NFOR rat group red gastrocnemius, along with decreased CS and mitochondrial Complex IV activities, 60 hours after the last training session (at 11th week in Table 1) (Hohl et al., 2010).

The increase in blood glutamate is less understood than the decrease in glutamine during OT. A possible explanation for the increase in blood glutamate in the NFOR/OTS states is that excessive skeletal muscle microtrauma causes a reduction in the electrochemical gradient in the muscle by increasing intracellular Na^+ (Hack et al., 1996).Glutamate is carried in the cell by Na^+-dependent transporters; therefore, increased intracellular Na^+ will result in decreased glutamate carried into the cell (McGivan & Pastor-Anglada, 1994). This problem was verified in hypercatabolism (cachexia) (Hack et al., 1996), which entails a great loss of body cell mass. In addition, excess tissue trauma may be associated with reduced food intake, causing gluconeogenesis to be up-regulated in order to maintain blood glucose level (Smith, 2000). Because alanine and glutamine are the main precursors for gluconeogenesis, glutamine will decrease in this case (Wagenmakers, 1998). Muscle microtrauma and up-regulation of liver gluconeogenesis could link the blood glutamate increase and glutamine decrease in a feedback loop.

Although measuring blood glutamine and glutamate may be useful to monitor the effects of exercise programs, they can only be used to individualise medical/nutritional programs or exercise training interventions if the blood values are increased or decreased in relation to a well-defined reference population. So far, there have been no reports describing the reference intervals for blood glutamine and glutamate that can be applied in the exercise/sport sciences.

1.5 Reference interval as a clinical tool

The results of laboratory tests are often used in the clinic to diagnose, monitor or prevent many different pathological states. The most commonly used interpretation task is to compare individual blood parameter values with reference intervals that have been obtained from a defined population. Reference intervals refer to the range of values for a laboratory test that are observed in a specific population, typically described by upper and lower reference limits.

The International Federation of Clinical Chemistry (IFCC) Expert Panel on Theory of Reference Values in 1986 established the terminology, analytical procedures and statistical analyses of reference intervals (Solberg, 1987a). A *reference individual* is an individual selected for comparison using defined criteria (Solberg, 1987a). For sport science studies, it is important to consider that physical training promotes significant alterations in blood cells, enzyme activities, and protein and metabolite concentrations (Lazarim et al., 2009; Nunes et al., 2010; Sawka 2000). The training characteristics, or sport modalities, can promote different adaptive responses that can be reflected in each analyte. For example, endurance athletes have lower haematocrit, haemoglobin and red blood cell count compared to individuals who perform strength training (Schumacher et al., 2002). In addition, biochemical and haematological biomarkers may be influenced by age, body mass, genotype, ethnicity, sex, diet, circadian rhythm (Ritchie & Palomaki, 2004) and biological variation (Nunes et al., 2010). Thus, the selection of a reference individual should include the training characteristics or sport modality.

The *reference population* consists of all possible *reference individuals* of a *reference sample group*. The IFCC recommend a minimum of 120 subjects to obtain reliable estimates and confidence intervals (Solberg, 1987a). This may be a problem when one decides to estimate reference intervals for team sports, such as soccer, volleyball or individual sports. One alternative is to obtain reference intervals in co-operation with laboratories that use the same methods for screening tests in athletes. Another important issue is the selection criteria for a reference sample group. It is important to consider the training level and adaptive state of each individual. In such a way, a performance test is important to characterise the subjects that will make up an exercised reference sample group. In addition, when the reference sample group is composed of professional athletes, it is important to consider possible variations of blood parameters during the training season and competition periods (Banfi et al., 2011).

The *reference values* are the values obtained in reference individuals for an individual analyte (the constituent that will be analysed) (Solberg, 1987a). The reference values are sensitive to pre-analytical and analytical variation; therefore, sample collection and handling techniques that are adopted should be standardised to minimise the sources of error (Fraser, 2001). Some techniques must be observed to obtain the reference samples: i) taking samples at the same time of day, considering possible circadian variations of the analyte; ii) ensuring that the reference individuals have been subjected to the same conditions (i.e., fasting for at least 10 hours and no consumption of alcohol or medication for at least 2 days before testing, particularly anti-inflammatory drugs); iii) the individual's training load or fitness level should be standardised; iv) taking blood samples with a standard phlebotomy technique (e.g., placing the samples into the same type of collection tubes and preferably having the subjects in a sitting position); and v) no training or exercise for at least 48 hours before the collection to avoid the acute haemodilution effects that can occur on the blood samples (Sawka, 2000). Also, the sample transport and handling should be carefully monitored to avoid haemolysis and to keep the analytes stable.

Analysis of reference samples sometimes requires many methodological steps. Therefore, applying the same equipment, reagents and calibrators is critical to ensure that the results are accurate. The analytical variation can be estimated by calculating the analytical coefficient of variation, obtained from the mean and standard deviation of the quality control analysis. The internal quality control should analyse a sample that simulates the reference values samples (e.g., serum, plasma, whole blood, urine or saliva).

After the reference sample assay, a histogram should be generated to inspect the distribution of the data (Fig. 2). We found that glutamine and glutamate show a Gaussian (Fig. 2A) and non-Gaussian distribution (Fig. 2B), respectively. In the visual histogram analysis, it is also possible to detect aberrant values (*outliers*) and to identify possible data errors. A number of statistical tests should also be performed to detect *outliers*. The IFCC do not recommend any particular method, but the Dixon test is commonly used, and it is relatively 'insensitive to moderate deviation from the Gaussian distribution' (Solberg, 1987b). However, this method often fails when several outliers are present. The *Horn's* algorithm (Horn et al., 2001) attempts to solve this problem by employing two stages. In the first stage, the data are transformed in a Gaussian distribution, and in the second stage, the extreme values are detected based on 50% of the transformed sample (Horn et al., 2001). The aberrant values, which are identified as *outliers*, should be removed following rational criteria: the pre-analytical process should be re-evaluated, the analytical process should be checked or samples re-assayed to discharge possible mistakes (Solberg, 1987b).
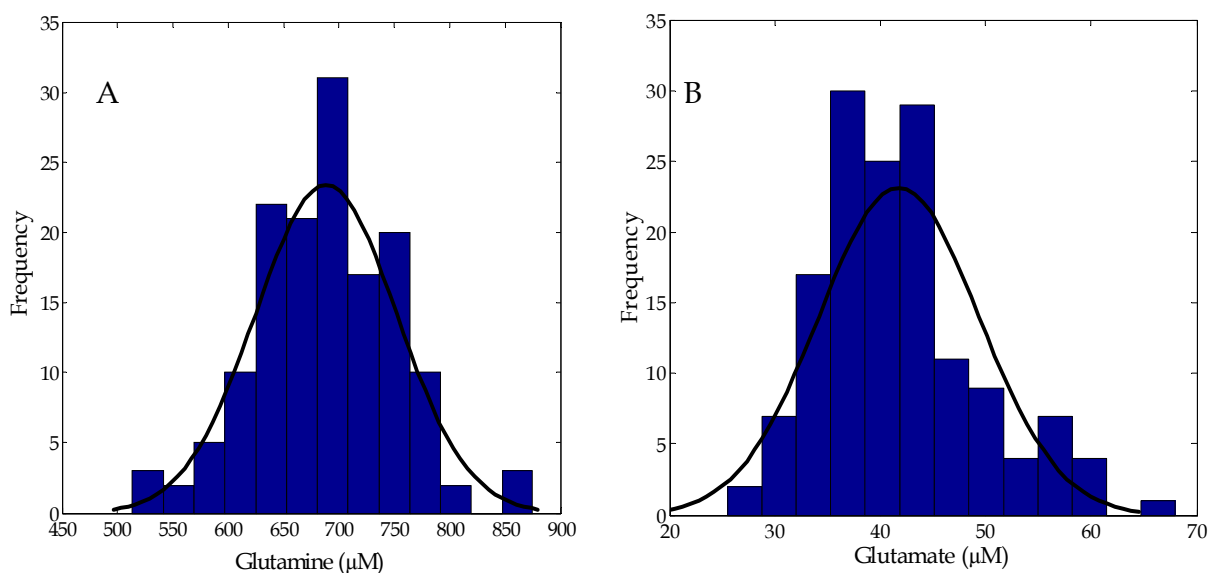

Fig. 2. Histograms representing the reference distributions of glutamine (A) and glutamate (B) in a physically active population (n = 146). The black line represents a normal fit.

Several types of reference intervals have been proposed in the literature: inter-percentile interval, tolerance interval and prediction interval. Most frequently, the reference intervals are estimated to be the lower and upper percentiles in the central 95% of the results (Solberg 1987b). This procedure is recommended because the reference intervals are easily estimated by parametric and non-parametric procedures. Other percentiles (e.g., 90%) can be adopted to narrow the intervals.

The percentiles may be estimated by parametric (Gaussian distribution) or by non-parametric methods (non-Gaussian distribution). There are several non-parametric methods for estimating reference intervals. The rank-based method is simple to apply manually or by using a computer, and it is also recommended by the IFCC and well described by Reed et al. (1971). As parametric estimates are more precise, we can transform the non-Gaussian distribution into a Gaussian distribution by applying statistical techniques (e.g., logarithms or square roots of the values) (Solberg, 1987b). To calculate reference intervals by parametric methods, we first need to test a Gaussian

distribution by applying a goodness-of-fit test, such as an Anderson-Darling or Kolmogorov-Smirnov test (Solberg, 1986). The calculation will use the mean - 1.96 x sd to estimate the 2.5th percentile and mean + 1.96 x sd for the 97.5th percentile of the reference intervals (Horn & Pesce, 2003). The RefVal program, developed by Solberg (2004), is a computer program that performs many statistical routines described above, including procedures and algorithms in accordance with the IFCC recommendations. It has a simple data input routine and intuitive interface.

One difficulty in assessing the effects of training on blood parameters is the lack of appropriate reference intervals obtained from a reference population that practices regular and systematised physical activity and following the IFCC rules. The aim of this study was to obtain glutamine, glutamate and Gm/Ga reference intervals, according to IFCC rules, using automated equipment that does not require extensive laboratory skills. The reference population consisted of a cohort of young men who had increased performance in a 3000-m time trial test after four months of periodic training, when compared with their performances at the beginning of training. Secondly, we present a suggestion for a practical method to follow training effects that combines routine performance analyses with glutamine level, glutamate level and Gm/Ga ratio.

2. Material and methods

2.1 Subjects

Male volunteers (n=526), with an average age of 18 ± 1 years, participated in this study. All volunteers were students in the first stage of physical and educational preparation for a career in the army. The participants responded to a questionnaire about their use of medication and their complaints of pain and injuries caused by training. Those who were using medications or were injured were not included in the study. Volunteer subjects were duly informed about the research and signed a free informed consent form. They participated for nine months (February to October) in a regular and strictly controlled exercise program, which consisted predominantly of aerobic activities (high volume and low intensity) for three hours daily. They trained five days per week, with two days of rest. This work was approved by the Human Research Ethics Committee of the Campinas State University (CAAE: 0200.0.146.000-08).

2.2 Performance test and subject selection for a reference population

All subjects performed four freely paced 3000-m time trial tests during the training period. The tests were performed in February, April, May and October, respectively, with all subjects performing them within the same one-week period. The time trial test is a feasible way to test the endurance capacity of the subjects, considering the large sample size. Each subject ran 3000 m with a numbered wristband. At the end of the 3000-m trial, the subjects placed their wristbands on a pole. A total of five poles were placed at the end line, and one evaluator stopped a memory stopwatch every time a wristband was placed on the pole. Later, the sequences on the wristbands were matched with the stopwatch times.

The students were highly motivated for the time trial test. The 3000-m time trial was graded by members of the army school, and students failed the year if they did not perform the 3000 m in at least 14' 59'' in the last test (October). A maximum grade (10) was obtained when the cadet performed below 11' 30'', and the grade dropped 0.5 per every additional 10''.

Software was developed to facilitate the visualisation, identification and selection of subjects from this large sample. It is based on the use of scatter plots and allows the user, in an interactive way, to observe trends and patterns of the group's results, providing the position of the individual within the group and showing the current results of the subjects compared to previous results (Reis et al., 2011).

The example shown in Fig. 3 illustrates the comparison between the time achieved for a 3000-m time trial performed in February and May. This type of comparison allowed for visualisation of the progress of the students during the training period between tests. By clicking on 'Identity Line', a straight line is drawn with a slope equal to 1 and an intercept equal to 0, which divides the graph diagonally. This line represents the set of identical results in both tests. The points below the identity line represent the students who responded well to the physical training program, as they performed the 3000-m time trial with a lower time in May than in February. There were also a few students positioned above the identity line who, for some reason, performed the test more slowly in May than in February.
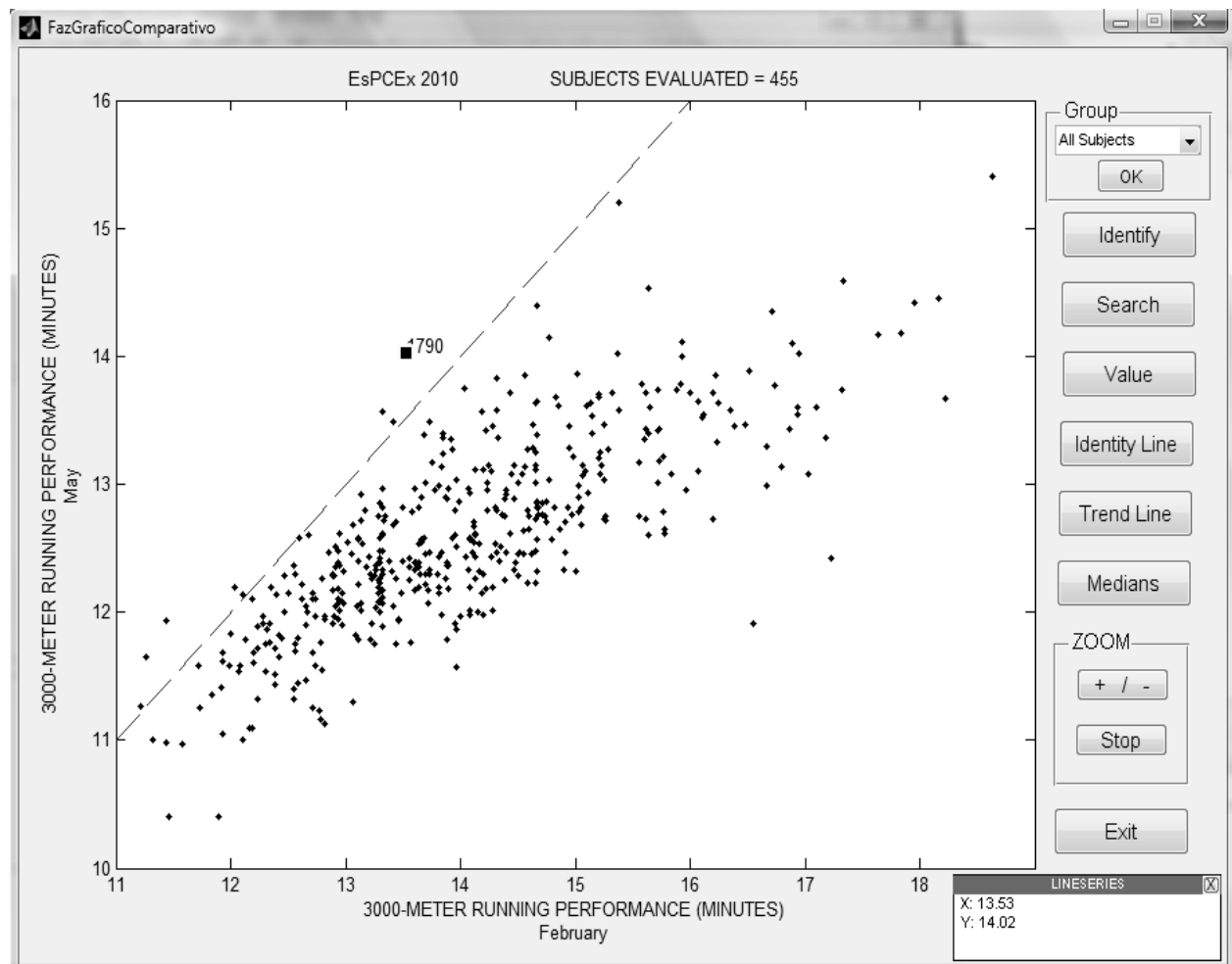

Fig. 3. Comparison between 3000-m time trials performed in February (*x*-axis) and May (*y*-axis). The dashed line represents the identity line. The text box indicates the exact value obtained by subject 1790 on both dates.

Figure 4 shows a zoom of a specific region of the graph and the selected students chosen to compose the reference population (points circled in gray). Thus, the glutamine and glutamate reference intervals presented here represent a population of young, physically active, healthy men who responded to 4 months of training.
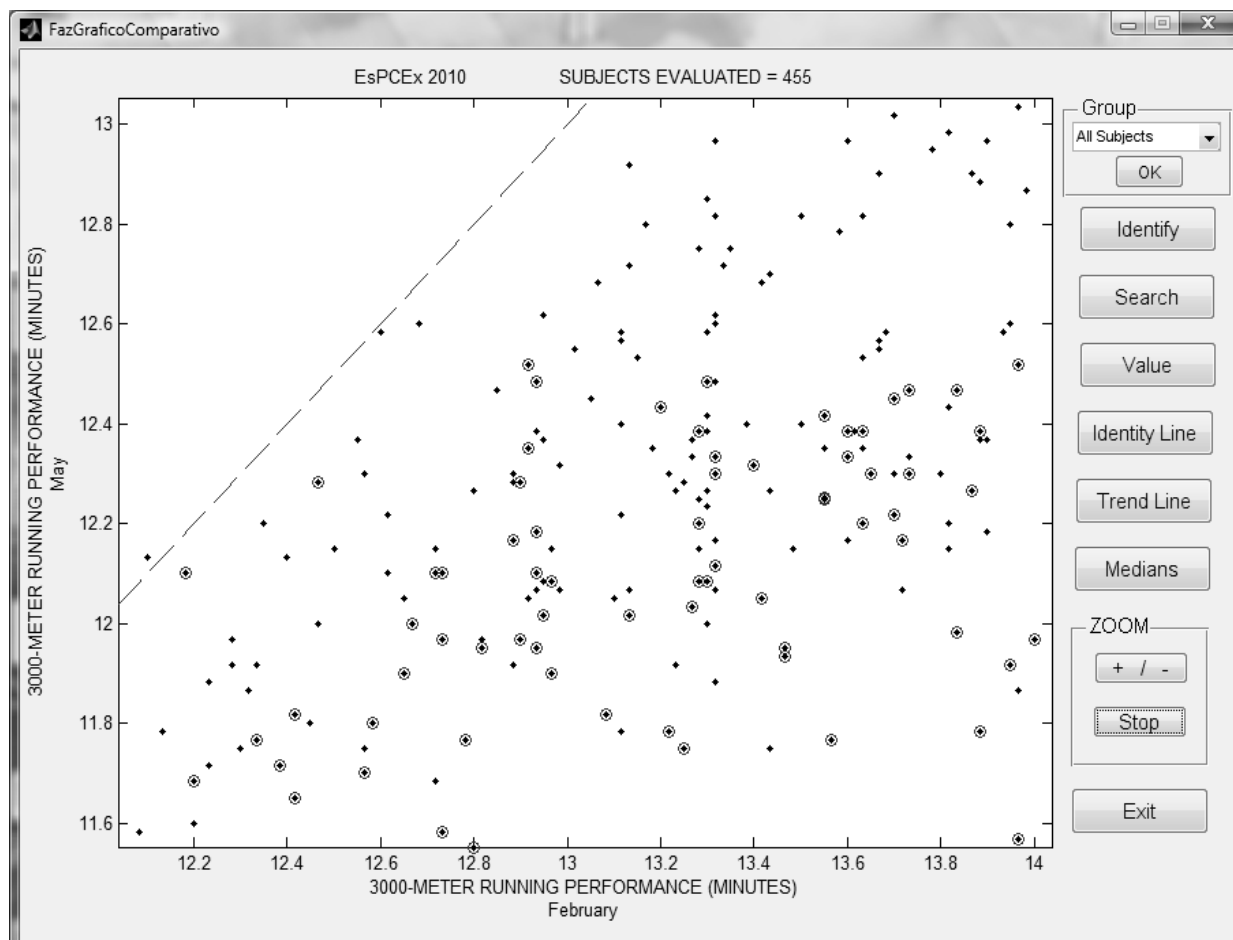

Fig. 4. Zoom applied on Fig.3. Comparison between 3000-m time trials performed in February (*x*-axis) and May (*y*-axis). The points circled in grey represent the individuals selected to establish the reference values for glutamine and glutamate. The dashed line represents the identity line.

The performances of the students in the 3000-m time trial tests between May and October were used to choose candidates to test the use of plasma glutamine level, glutamate level and Gm/Ga ratio as biomarkers of tolerance or intolerance to the same training protocol. Twenty-five subjects who were above the identity line were randomly selected as the non-responders to training (NRT), and an additional 25 subjects who fell below the identity line were randomly selected as responders to training (RT).

2.3 Collection of blood samples

All blood samples were collected after two days of rest to avoid the effects of hemodynamic variations and acute haemodilution that are induced by exercise. Blood samples were collected under standardised conditions. Eight millilitres of venous blood was collected in

heparin tubes with a Vacuette® (Greiner Bio-one) gel separator to obtain plasma for glutamine and glutamate assays. Blood samples were collected in the morning after 12 hours of fasting with subjects in a seated position, transported at 4°C to the laboratory within 30 min, centrifuged under refrigeration (4°C) at 1,800 x g for 10 minutes, and then immediately separated and protected from light. Plasma samples were finally stored at -80°C and analysed within 2 months.

2.4 Assays for glutamine and glutamate measurements

Glutamine and glutamate analyses were conducted with a dual-channel YSI 2700® Select Biochemistry automated analyser (Yellow Springs Instrument Co., Ohio, USA), according to the manufacturer's recommendations. This analyser uses a platinum electrode to measure the current generated by two enzyme-impregnated membranes. When a sample is injected into the sample chamber, the glutamine diffuses to the glutamine membrane, which contains glutaminase and glutamate oxidase. The glutamine is deaminated to glutamate and ammonia by glutaminase. In the presence of glutamate oxidase, glutamate is oxidised to hydrogen peroxide, α-ketoglutarate, and ammonia. The hydrogen peroxide is detected amperometrically at the platinum electrode surface. The current flow at the electrode is directly proportional to the hydrogen peroxide concentration and thus to the glutamate concentration. The glutamate in the sample is also oxidised at the glutamate and glutamine membranes by glutamate oxidase, producing hydrogen peroxide, α-ketoglutarate, and ammonia. Glutamine concentration is calculated as the concentration measured by the glutamine electrode minus that detected by the glutamate electrode.

We used 1 mM glutamine and 0.500 mM glutamate standards to calibrate the machine after every four measurements (sample size 65 µl). The standard solutions (5 mM) were provided by the manufacturer (Yellow Springs Instrument Co., Ohio, USA) and were diluted in Milli-Q water (Millipore Corporation, MA, USA).

The 146 samples used for the reference interval were analysed within two days, and the linearity of the enzyme membranes was evaluated every day before sample analysis. The 50 samples (RT, n = 25; NRT, n = 25) were analysed within the same day with other enzyme membranes and standard kits, after evaluation of membrane linearity. All plasma samples were measured in duplicate, and the mean of the duplicate runs was used in subsequent calculations. The linearity for glutamine and glutamate was calculated with diluted standards in three concentrations: 0.300 mM, 0.500 mM and 0.900 mM for glutamine and 0.05 mM, 0.100 mM and 0.200 mM for glutamate. In all linearity analyses, the correlation coefficient and the slope (r) were approximately 1.

We used one plasma pool to evaluate the within-day coefficient of variation (CVw) and four different plasma pools to evaluate the between-day coefficient of variation (CVb). The CVw of glutamine and glutamate were 0.60% and 1.20%, respectively (n = 20 using one plasma pool). The mean CVb, within three consecutive days, of glutamine and glutamate were 2.0% and 3.8%, respectively.

The accuracy of the YSI 2700D, when analysing the amino acids in non-deproteinated plasma, was evaluated by adding four different standard concentrations into four vials of the same plasma sample. Glutamine and glutamate standard concentrations that were diluted in plasma were thus calculated by subtracting the real glutamine and glutamate concentrations previously measured in plasma. We added 0.600 mM, 0.700 mM, 0.800 mM and 0.900 mM of glutamine standard into four different vials of one plasma sample, and the

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standard concentrations detected were 0.620 mM, 0.692 mM, 0.812 mM and 0.892 mM, respectively. The same procedure was done for glutamate: the added standard concentrations were 0.020 mM, 0.030 mM, 0.040 mM and 0.050 mM, and the standard concentrations detected were 0.021 mM, 0.030 mM, 0.039 mM and 0.053 mM, respectively.

2.5 Statistical analysis

Initially, all data from the 3000-m time trial tests were organised using Microsoft Excel and were checked for consistency between the recorded values and their identities with the evaluated subjects. After this initial organisation, all data were imported into Matlab® 7.0, which is the platform on which the software that was designed for this study was developed. Despite having been developed in this environment, the software was compiled and can run on any computer using Microsoft Windows and does not require the installation of Matlab® 7.0. The software, Origin 6.0, was used to perform statistical analyses and to generate graphs. An unpaired t test was used to compare RT and NRT glutamine and glutamate measurements; $p < 0.05$ was considered significant. The *Horn's* algorithm was applied to detect and remove outliers (Horn et al., 2001). The RefVal program (Solberg 2004), including practical approaches and formulas recommended by the IFCC, was used to calculate the 97.5th and 2.5th percentiles of the subjects and their respective 0.90 confidence intervals. This was achieved by using parametric estimates of the glutamine levels, glutamate levels and Gm/Ga ratios that were obtained from plasma samples of the 146 volunteers after four months of daily, periodic physical activity.

3. Results

Table 2 shows the reference intervals (upper and lower limits) and the confidence intervals for healthy, physically active young men.

Analysis	Reference Interval	0.90 Confidence Interval		Subjects (n)	Outliers	Subjects Ref. Interval (n)
	2.5th - 97.5th	2.5th	97.5th			
Glutamine (µM)	566 - 798	546 - 586	782 - 813	146	514, 867, 875	143
Glutamate (µM)	31 - 59	30 - 32	55 - 62	146	26, 28, 68	143
Gm/Ga	12 - 23	12 - 12	22 - 24	146	10, 10, 26	143

Table 2. Reference intervals for glutamine level, glutamate level and Gm/Ga ratio for healthy, physically active young men.

Figure 5 presents the performance analyses of the test subjects for the 3000-m time trial, over the span of one training year. At the end of the training year (May x October, Fig. 4C) almost all subjects performed below 14' 59'' (cut off) and the 3000-m times were more homogenous than at the beginning of the year (February x April, Fig. 4A). However, the regression line in Fig. 4C approaches the line of identity due to the increase in the amount of time it took some the test subjects (n = 222) to complete the 3000-m trial in October compared to May.
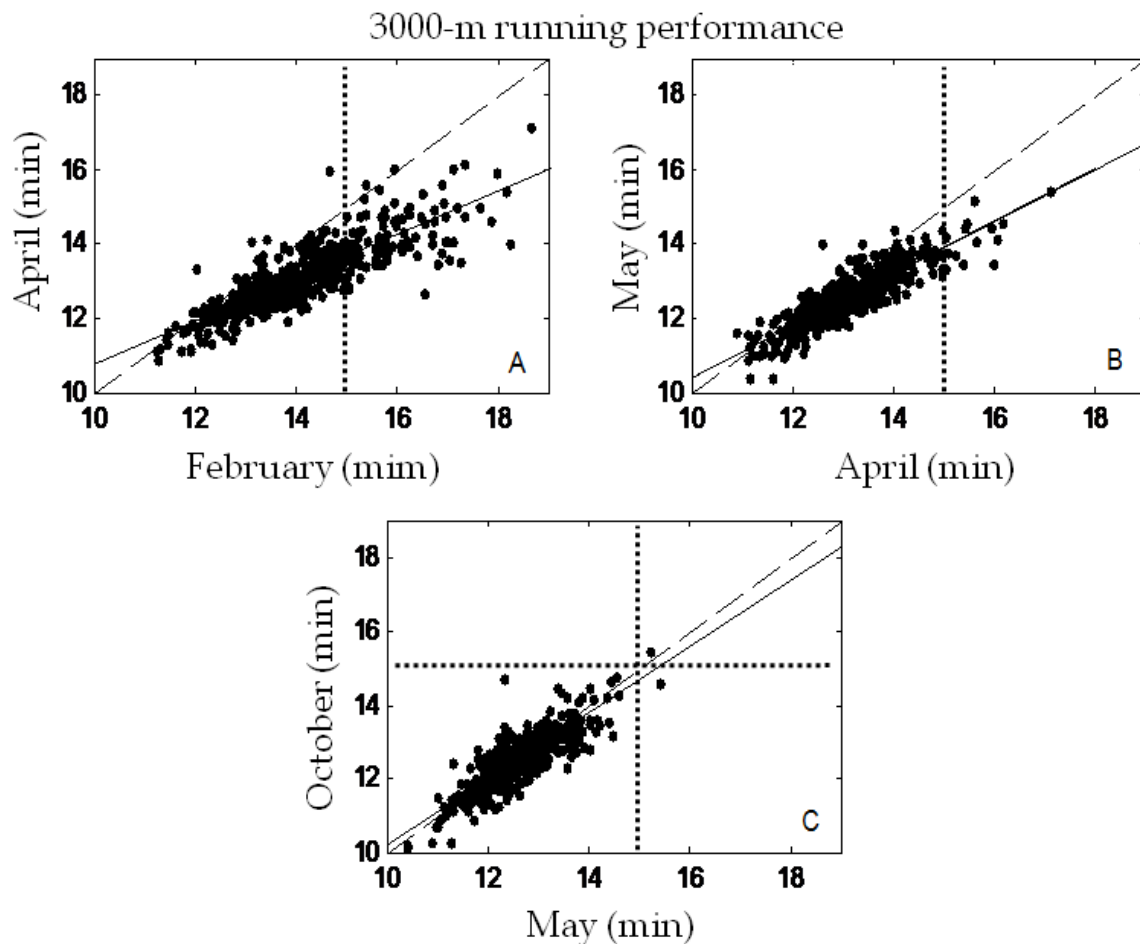
Fig. 5. Comparisons of 3000-m time trial tests over the training year. In these charts, the identity lines are dashed and the regression lines are solid. The identity line is the reference for time changes between the two tests. Subjects who fell below the identity line showed lower 3000-m times from one test to the next, so the performance was improved. The regression line shows the trend between the two 3000-m time trial tests over the year. Dotted lines show the Army School's cutting time in 14'59''.

Table 3 shows the comparisons of glutamine, glutamate and Gm/Ga between the RT and NRT groups. We observed a tendency of lower glutamine, significantly higher glutamate and significantly lower Gm/Ga ratio in the NRT group compared to the RT group.

	RT	NRT
Glutamine (μM)	669 ± 49	645 ± 63
Glutamate (μM)	41 ± 5	46 ± 6 *
Gm/Ga	16 ± 2	14 ± 2 *

Table 3. Glutamine, glutamate and Gm/Ga ratio in the RT group vs. the NRT group. RT: Responders to training (n = 25). NRT: Non-responders to training (n = 25). * Unpaired t test: significant difference between groups (glutamate, p = 0.002 and Gm/Ga, p = 0.0003).

Figure 6 presents the blood glutamine and glutamate levels of responders and non-responders to training in relation to the 97.5th and 2.5th percentile reference interval. Only four NRT subjects had Gm/Ga ratios below the reference interval, and one of them also presented a glutamine concentration below the reference interval (subject "X").
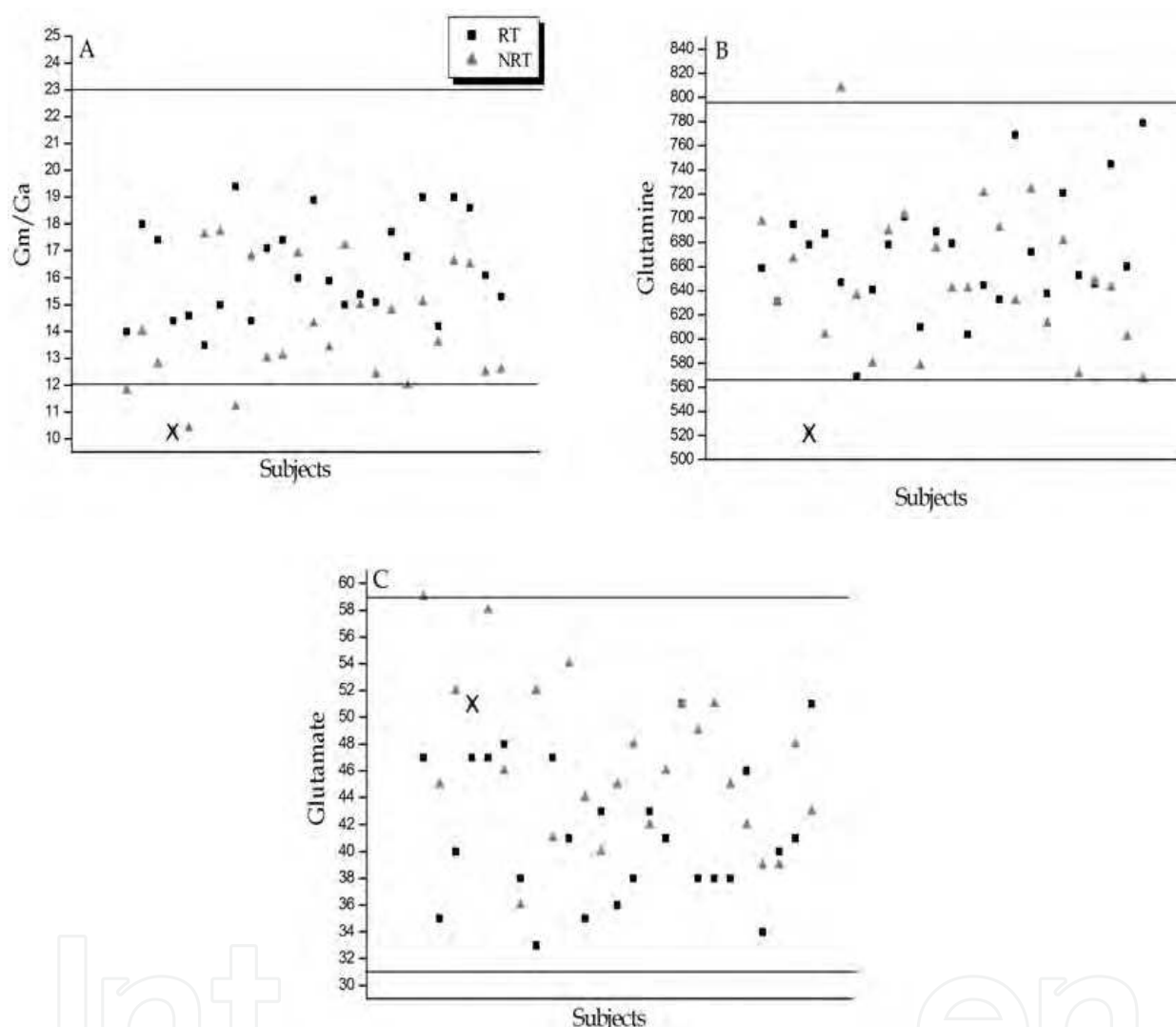

Fig. 6. Gm/Ga (A), glutamine (B) and glutamate (C) plasma levels (μM) of responders and non-responders to training (NRT; n = 25). Solid lines are the reference intervals. **X** identifies one subject with low Gm/Ga ratio and glutamine.

4. Discussion

This is the first study to establish reference intervals for glutamine, glutamate and Gm/Ga ratio in the plasma of test subjects, according to the IFCC recommendations. The reference intervals presented here are not comparable to other studies regarding the subject selection criteria or number (n= 143), sample preparation, sample storage or the equipment used for glutamine and glutamate measurements (i.e., YSI 2700).

4.1 Methodological aspects and confounding factors in measuring plasma glutamine and glutamate

To date, three different methods have been used to measure glutamine and glutamate concentrations: high-performance liquid chromatography (HPLC), the enzymatic method and a glutamine-dependent *Escherichia coli* bioassay (Hiscock & Pedersen, 2002). According to Hiscock and Pedersen (2002), the major difference amongst the three is in the concentrations of glutamine that is detected. When measured by the bioassay, the glutamine concentration is 40% higher than by HPLC. In addition, plasma glutamate measured by the enzymatic assay seems to be more than 200% higher than HPLC. The reason for disagreement amongst these methods is speculative. However, the discrepancies may be verified by noting the plasma glutamate values between 100 and 200 µM measured by the enzymatic assay (Halson et al., 2003; Keast et al., 1995; Smith & Norris, 2000) with values between 28 and 55 µM measured by HPLC (Abdulrazzaq & Ibrahim, 2001; de Jonge & Breuer, 1995; Van Eijk et al., 1994).

HPLC has been widely used in methodological studies that evaluate the stability of amino acids in non-deproteinated, deproteinated and neutralised samples at several storage temperatures (Abdulrazzaq & Ibrahim, 2001; de Jonge & Breuer, 1995; Van Eijk et al., 1994). Although HPLC is considered a reliable method for amino acid measurements, it also requires highly skilled laboratory personnel to guarantee reliability. According to the manufacturer (Yellow Springs Instrument Co., Ohio, USA), the YSI 2700 electrode method is highly correlated with HPLC glutamine measurements (slope 0.94 and $r^2=0.99$), with the advantage of being much more user-friendly than the HPLC method. The reliability tests performed in this study also showed outstanding accuracy in measuring glutamine and glutamate standards in plasma, good linearity and small within-day and between-day variability.

Blood plasma storage and deproteination add systematic errors that can influence inter-laboratorial comparisons of data (de Jonge & Breuer, 1996). Blood acid deproteination is frequently used to stop enzymatic activity; however, glutamine is particularly influenced by sample acid deproteination because a low storage pH accelerates the spontaneous degradation of glutamine in pyroglutamate (Khan et al., 1991). Nevertheless, the systematic error caused by deproteination is negligible when samples are stored below -70°C (Van Eijk et al., 1994). Glutamine and glutamate are stable for at least 6 months in non-deproteinated samples that are stored below -70°C (Van Eijk et al., 1994) but are not stable when stored at -20°C for more than 4 weeks (Abdulrazzaq & Ibrahim, 2001; de Jonge & Breuer, 1996). Therefore, based on those previous studies, non-deproteinated plasma stored at -80°C was used in this study.

Plasma glutamine and glutamate seem to not be affected by sex or age. Van Eijk et al. (1994) used deproteinated plasma and HPLC as their measurement technique. They showed mean glutamine levels between 663 and 693 µM and mean glutamate levels between 49 and 55 µM in healthy males 20–69 years old, with no significant differences in those amino acids between males and females. Planche et al. (2002) showed no differences in plasma glutamine and or glutamate between healthy children (12–71 months) and healthy adults (age not specified) using the YSI 2700. They observed a mean value (range min–max) of 532 (485–577) µM for glutamine and 32 (28–43) µM for glutamate. Both Planche et al. (2002) and Van Eijk et al. (1994) used only 8–12 subjects, which are insufficient numbers to consider those glutamine and glutamate values as references, according to the IFCC, who recommend a

minimum of 120 subjects (Solberg, 2004). However, those glutamine and glutamate values are slightly lower than the upper limits of the reference intervals found in this study (Table 2), probably due to the regular physical training effect. Particularly, glutamine may increase due to endurance training. Rowbottom et al. (1997) identified a positive correlation between the level of plasma glutamine and improved endurance performance. In addition, Kargotich et al. (2006) showed increased plasma Gm, VO$_2$max and time to exhaustion after 6 weeks of endurance training (3 to 6 × 90-minute sessions per week at 70% VO$_2$ max) in active, not well-trained subjects.

Plasma glutamine level is also influenced by acute exercise. The effect of acute exercise on plasma glutamine seems to depend on the intensity and duration of the exercise (Hiscock & Pedersen, 2002). For instance, intermittent, high-intensity bouts of activity decrease plasma glutamine (Walsh et al., 1998), but a single, short, high-intensity bout increases (Babij et al., 1983) or maintains glutamine (Sewell et al., 1994). Other studies have shown that blood glutamine decreases for 2–4 hours after acute exercise lasting more than 2 hours (Castell et al., 1997; Rohde et al., 1996). Therefore, to use the glutamine and glutamate reference intervals as a training monitoring tool, it is advised not to withdraw blood samples right after acute exercise of any type. Otherwise, the effect of acute exercise may overcome the chronic training effect on glutamine metabolism.

As well as being influenced by exercise, blood glutamine level may also change due carbohydrate (CHO) intake. Wagenmakers et al. (1991) showed that both plasma glutamine and glutamate decrease after glycogen depletion, compared to CHO loading during exercise. This change is probably due to increased deamination of amino acids. Blanchand et al. (2001) showed that resting mean plasma glutamine of test subjects was significantly higher after 14 days of rich CHO intake (70% of total energy; mean plasma glutamine of 857 μM) than in subjects with poor CHO intake (45% of total energy; mean plasma glutamine of 610 μM). In practice, athletes and coaches should carefully plan the CHO intake of the athlete because it can also negatively affect performance (Maughan et al., 1997). If performance decreases and glutamine falls below the reference interval, but CHO intake is sufficient for maintenance of liver and muscular glycogen levels during the training program, then intolerance to training may have occurred.

For the reasons expressed so far, the reference intervals for glutamine and glutamate presented here should be used whether the measurement conditions (i.e., storage, preparation and assay technique) are reproduced faithfully. Sufficient CHO energy intake for the maintenance of glycogen stores is recommended in order to avoid glutamine/glutamate changes associated with performance decay related to poor nutrition. Overnight fasting and 8 to 12 hours of recovery from a previous training session should be considered before blood sample withdrawal. Finally, it is advised, for endurance training monitoring, to use glutamine, glutamate and Gm/Ga reference intervals that have been established from endurance-trained subjects, as presented here.

4.2 Training monitoring using self-paced time trials and glutamine and glutamate reference intervals

To test the glutamine and glutamate reference intervals, 25 subjects were randomly selected as responders to training, due to their decreased times in the self-paced 3000-m time trial in October compared to May (subjects below identity line, Fig. 5C). The self-paced time trial is

influenced by an anticipatory component, which, in turn, is influenced by physiological inputs prior to exercise that are related to the fitness level, expected exercise distance/duration and previous experience of the test subject (Tucker, 2009). The army students were experienced in the 3000-m time trial because they had previously performed many trials throughout the year in addition to the four officially valid ones for the army school records. In addition, they were task-motivated, due to the internal competition amongst themselves and also to the army school grading. However, many students showed poorer performance in October compared to May (subjects above identity line, Fig. 5C), and therefore, an additional 25 subjects were randomly selected from this group to test the reference intervals. No additional symptoms, such as mood alterations or increased incidence of injuries, which would also be characteristic of NFOR/OTS, were observed (Meeusen et al., 2006); therefore, those subjects were considered non-responders to training.

The same exercise-training stimulus may be either efficient or insufficient in improving performance and physiological adaptation when applied to many different subjects. An insufficient training stimulus may cause unexpected stagnation or a mild decrease in performance in self-paced time trials of subjects who are not responding (i.e., adapting) to training. The pacing strategy also depends on external factors such as the environment, race situation and the influence of other competitors (Tucker, 2009). Therefore, decreased performance may not be caused only by physiological maladaptation, but may also be a result of stagnation and test variability. In this sense, the army training program does not aim for high performance levels, as in professional athletes; the goal, in this case, is to homogenise the fitness level of young cadets on their first step in an army career. To reduce the differences amongst the 3000-m time trials, all students were subjected to a similar training program and load.

Figures 5A to 5C show that the army training program was effective in improving the 3000-m time trial for those subjects with higher initial 3000-m times (above ~15 min). However, the training program was less effective in decreasing the 3000-m time trial for subjects who performed below 14 minutes. The scattered points behaviour suggest that the army endurance training program was an insufficient stimulus for students already performing the 3000-m time trial below ~14 min early in the season (Figs. 5A and B).

However, the self-pacing time trial is also the result of a complex, physiological, integrative model of exercise. It has been stated that 'pacing is controlled by the brain, which regulates exercise intensity and alters the adopted pacing strategy to ensure that potentially catastrophic derangements to homeostasis do not occur' (Noakes et al., 2005). This definition holds that during self-paced exercise, when one is able to select an exercise work rate, performance is regulated by a central governor (i.e., brain) to prevent changes in physiological systems that may be harmful during exercise (Noakes et al., 2005; Tucker, 2009). We hypothesize that glutamine and glutamate are related to many of the chronic and acute metabolic aspects related to exercise (topic 1.4 in this chapter) which could interfere with the central governor regulation. In this sense, glutamine and glutamate reference intervals may be used as additional tools for deciding between heavier training loads to avoid detraining/stagnation, or extended recovery to avoid maladaptation. In practice, when the performance decreases and the glutamine, glutamate or Gm/Ga ratio falls within the reference interval limits, then an adjustment in the training load should be considered.

On the other hand, when the performance decreases and the biomarker levels fall outside the reference interval limits, then extended recovery should be considered.

4.3 Practical use of glutamine, glutamate and Gm/Ga ratio reference intervals

Any blood biomarker that is measured during training monitoring would be useful when the detectable changes in blood happen before NFOR/OTS outcomes. In this sense, previous studies that have measured glutamine and glutamate before and after a period of intense training loads seem to agree that changes in these amino acids occur before the extreme OT outcomes, independent of the terminologies used to define them (Halson et al., 2003; Keast et al., 1995; Smith & Norris, 2000; Souza et al., 2005). Nevertheless, intense exercise is related to the context of biological individuality and fitness level.

The occurrence of mean lower glutamine, a significantly lower Gm/Ga ratio and higher glutamate in the NRT group compared to the RT group may indicate slight differences in glutamine and glutamate metabolism (Table 2). However, mean glutamine, glutamate and Gm/Ga ratio of the NRT group were all within the population reference interval. Therefore, glutamine and glutamate mean values may be indicative of higher training stress or lower endurance capacity within a group but are meaningless regarding individual intervention.

When the reference intervals were used, only four subjects from the NRT group showed Gm/Ga ratios below the reference interval, and one of them also showed low glutamine (Fig. 6). Of note, all 25 subjects in the RT group showed glutamine and glutamate levels, and Gm/Ga ratios within the reference intervals (Fig. 6).

These results suggest that only those four subjects, and particularly the subject with low glutamine concentration (X-mark in Fig. 6), should continue the training program with close daily or weekly monitoring of their mood profiles. The remaining 21 NRT subjects probably were not responsive to the training program in the last four months; therefore, the 3000-m time trial test probably reflects stagnation and the inherent variation of the test.

5. Conclusion

For most trainers and sportsmen, the main sign that an athlete has developed NFOR/OTS is sustained poor performance and fatigue. Poor performance and fatigue, however, can also be due to many other factors, such as inadequate training sessions and poor nutrition, respectively, as well as to extraneous factors such as loss of confidence, pressure outside of the sport, and sleep disturbances. The reference intervals of glutamine, glutamate and Gm/Ga ratio presented here may therefore be useful tools to monitor adaptation to training and to thereby identify those athletes who show early signs of OT before prolonged fatigue. In addition, glutamine and glutamate blood concentrations may identify those athletes who are intolerant to a training program versus those who are not responsive to the training program because of insufficient workload.

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7. References

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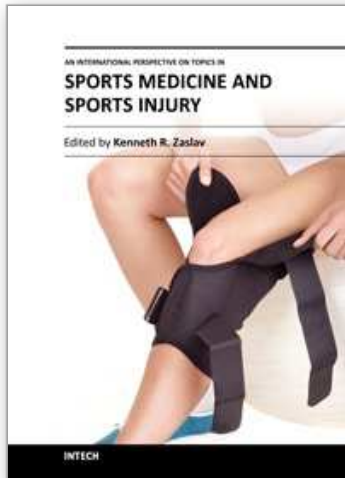
An International Perspective on Topics in Sports Medicine and Sports Injury
Edited by Dr. Kenneth R. Zaslav

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