PASSCLAIM[1] – Physical performance and fitness

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■ **Summary** *Objective* The aim of the EC Concerted Action PASS-CLAIM was to develop a set of methods and procedures for assessing the scientific support for function-enhancing and health-related claims for foods and food components. This paper presents a critical review of the existing methods to evaluate the different aspects of physical performance and fitness needed to support claims on foods and food ingredients intended to enhance specific physiological functions. *Design and Results* Based on an inventory of labelling claims on available sport nutrition products, seven physiological functions in the field of physical performance and fitness were identified: 1) strength and power, 2) endurance, energy supply and recovery, 3) hydration/re-hydration, 4) flexibility, 5) tissue growth, 6) free radical scavenger capacity and 7) immune function. For each function the existing methodology was reviewed critically and judged on suitability to generate scientific support for physiological function claims on foods. *Conclusions* A database of methods including advantages and disadvantages of use has been generated for considering the scientific support of claims on foods and food ingredients relating to physical performance and fitness. It will contribute to the formulations of guidelines for assessing the scientific support of enhanced function or reduced disease risk claims on foods.

■ **Key words** physical performance – fitness – sport nutrition – physiological functions – function and health claims – exercise

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1 Process for the Assessment of Scientific Support for Claims on Foods

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Introduction

Most sport nutritionists recommend that athletes consume a balanced diet of macronutrients and micronutrients to provide adequate energy, regulate metabolic processes properly and maintain an optimal body mass specific to their sport. In general, dietary guidelines for healthy eating developed for the average population are also applicable to athletes. However, considerable research effort has been expended to determine whether or not athletes can benefit from particular foods or food ingredients beyond the general recommended dietary guidelines. With the introduction of the carbohydrate-electrolyte sports drinks about two decades ago, a complete new functional food market as well as the science behind it to substantiate claims has been developed [1]. With the development of new methods and techniques in the field of exercise physiology and nutrition over the years, the field of sports nutrition can be considered as one of the first developed markets of functional foods with type A "Enhanced Function" claims [2]. On the other hand it also shows the potential reverse side of the development of a functional food market if not well regulated. A wide variety of sports foods and drinks have been launched labelled with an abundance of performance and health benefit claims. Frequently these claims are not substantiated with valid scientific data.

In this paper relevant data concerning the science to substantiate claims in the field of physical performance and fitness have been reviewed. Based on an inventory of existing claims in the sport nutrition market, a number of physical functions have been identified and described more in detail. Despite the fact that one can argue about the relation between a specific physiological function and a claim, the following general physiological functions have been identified:

- Strength and power;
- Endurance, energy supply and recovery;
- Hydration, re-hydration;
- Flexibility;
- Tissue growth;
- Immune function.

In relation to physical performance the number of physiological functions is much larger. However, related to the existing list of claims these are of less importance except for the area of free radical scavenger capacity or prevention of oxidative stress. Since this topic will be discussed in detail in one of the forthcoming individual theme groups (ITG) of PASSCLAIM on diet-related cancer it was decided that only a short description of the field should be included in this paper.

Each section is introduced by a limited list of existing labelling claims found on food products in the market followed by a paragraph about the definition used, the physiological background as well as the relevance in relation to the existing claims. Most efforts have been given to review the available methods of measurement including specific aspects of study design such as subject selection. Of particular importance is the level of fitness of the target group. Generally, elite athletes do not volunteer for this type of validation studies to substantiate claims. In most situations well-trained individuals will be a good alternative target group to study. However, if a food product with a claim is focused on an untrained population group, one should be more specific in the subject selection, since results found in the well-trained population cannot be generalised to an untrained population nor vice versa.

For each physiological function field the available methods have been tabulated in a standardised form including information about reproducibility, validity and specific remarks about advantages and disadvantages to substantiate the claim of interest.

This framework of information about techniques and methods will contribute to the formulation of guidelines for assessing the scientific support for claims of foods and food ingredients.

Strength and power

■ CLAIMS

- Increases muscle strength
- Increases muscle force or power
- Enhances sprint and/or jump performance
- Increases muscle mass/volume
- Reduces muscle fatigue
- Enhances recovery

■ Definition, physiological background and relevance

Definition of muscle strength and power

Muscle strength and power are important determinants of performance in short maximal exercise requiring high muscle force production, such as weight lifting, throwing and jumping, or sprinting. A majority of the athletes involved in strength and power sports ingest nutritional supplements with the express purpose of enhancing muscle strength.

Muscle strength can be defined as 'the maximal amount of torque (S. I. unit: Newtonmeter, Nm) or force (S. I. unit: Newton, N) that a muscle or muscle group can voluntarily exert in one short maximal effort, when type of muscle contraction, movement velocity and joint angle are specified'. By analogy, muscle power can be defined as 'the maximal amount of work per unit of time that can be performed in one short dynamic effort' [3].

Muscle force production is the endpoint of a series of intermediary physiological processes. However, inde-

pendent of the mode of contraction (isometric, concentric, eccentric), muscle strength is very closely correlated with physiological cross-sectional area (CSA), which can be defined as the area perpendicular to the muscle fibre direction [4]. Muscle CSA is regulated by the balance of protein synthesis versus protein breakdown, termed net muscle protein synthesis. Furthermore, maximal force output at a given CSA depends on the capacity of the central and peripheral neuromotor system to activate available musculature [5]. The above intermediary physiological processes are covered in the sections on Tissue growth, and will not be further discussed in detail in this section.

Energy metabolism during short maximal exercise

Phosphocreatine breakdown through the creatine kinase reaction and glycogen breakdown to lactate (glycogenolysis/glycolysis) are the primary pathways of ATP provision during short exercise events requiring maximal force and power output. For example, during a 6-second sprint, during which power output corresponds to ~250% of VO2max, PCr hydrolysis and glycogenolysis each contribute ~50% of the total ATP requirement with very little contribution from oxidative phosphorylation [6, 7]. The relative contribution of PCr to energy turnover increases as the exercise duration becomes shorter. Conversely, during maximal exercise lasting 5 to 6 s to about 90 s to 2 min, glycogen degradation to lactate rather than the creatine kinase reaction is primarily involved in the regeneration of ATP [8].

ATP turnover rate under normal conditions is not a limiting factor in short 'explosive' exercise performance (throwing, jumping, weight lifting etc.). Muscle structure (physiological CSA, muscle length, muscle architecture), neural factors (neuromotor drive, co-ordination) as well as skill ability are the predominant factors limiting performance. However, during sprint exercise causing PCr depletion and involving substantial activation of glycogenolysis, the rate of ATP breakdown often exceeds the rate of ATP synthesis, which results in net ATP breakdown and increasing intracellular content of the end products of adenine nucleotide degradation, notably inorganic phosphate (Pi), ADP, AMP, IMP and ammonia. A small proportion of the IMP formed is converted to inosine and further to hypoxanthine, which appear in the venous effluent of the muscle [9, 10]. There is substantial evidence to indicate that H$^+$ production (~pH drop) from glycolysis/glycogenolysis in excess of available buffer capacity, in conjunction with intracellular accumulation of ADP and Pi resulting from adenine nucleotide degradation, are implicated in fatigue during short maximal exercise [11].

■ Methods of measurement

Analytical versus global assessment of force and power

Muscle strength can be evaluated either in an 'analytical' way or by a 'global' approach [3, 12]. In the analytical setting, ideally the force or power production by a single muscle is measured. Conversely, the global approach involves the measurement of force and power output by the concerted action of several muscle groups in a whole body exercise task (e.g. weight lifting, sprinting, jumping).

Strictly taken, analytical strength measurements in humans would require the invasive procedure of attaching a force transducer to either the muscle or the tendon [13]. For obvious reasons such a method is not useful as a routine clinical measure. A valid alternative for this 'single muscle' approach is the measurement of torque production in a mono-articular movement during which force generation results from activation of a single group of prime mover muscles (arm flexion, knee extension). For example, force output measured during maximal unilateral dynamic knee-extension or arm flexion on an isokinetic dynamometer largely reflect the strength of the knee-extensor muscles and arm-flexors muscles involved, respectively. Force output measured is not limited by factors other than muscle strength, such as the level of skill acquisition. As a result, learning effects in these kind of simple force/power tasks are very small and in general are eliminated by a single habituation session.

Conversely, during more complex exercise tasks or 'skills', as a rule technique and co-ordination play a pivotal role in determining the force and power output measured. Learning effects are often considerable from one test to the other and can mask changes in muscle strength even after multiple habituation sessions. Thus, vertical jump performance not only depends on the contractile performance of the prime movers (leg extensor muscles) but also to an important degree on co-ordination of agonistic, synergistic and antagonistic muscle groups, or other 'disturbing' factors such as for instance operation of the stretch-shortening cycle. The larger the 'skill' factor in the test, the less performance depends on muscle strength per se.

Based on the above rationale the analytical approach is the method of choice to evaluate the effects of nutritional supplements on muscle strength.

Analytical force measurements

Analytical assessment of muscle strength is performed on an isokinetic dynamometer. This allows for a strict standardisation of the type of muscle contraction (isometric, concentric, eccentric), the movement velocity, the movement range (joint angle) as well as the contrac-

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tion history [3, 12]. This standardisation is essential because the force and power generating capacity of muscle depends on these factors. Furthermore, measurement of torque is critically dependent on the alignment of the joint axis with the axis of the measurement device. Therefore, adequate measures must be taken to guarantee an identical positioning of the subject's leg or arm relative to the dynamometer axis from one measurement session to another [14]. Finally, reliability of torque measurements on an isokinetic dynamometer as a rule decreases as contraction velocity increases. Therefore, reliability of the specific dynamometer used must be investigated at the contraction velocities relevant to the experiment.

Peak isometric force (Fmax) and peak contraction velocity (Vmax) stand for two distinct basic factors of muscle performance during a maximal muscle contraction. From a functional point of view, the force-velocity relationship of a muscle probably provides the most optimal syntax of muscle performance capacity because it characterises the gradual transition from Fmax to Vmax via a well-known hyperbolic relation. Thus, from a theoretical point of view an ideal assessment of muscle strength should involve determination of the force-velocity curve in the full range from Fmax to Vmax. However, available isokinetic dynamometers allow for reliable measurements of torque at angular velocities up to not more than ~300°/s, whilst Vmax is higher by at least a factor 3. Due to these and other practical constraints, assessment of muscle strength for the purpose of evaluating the efficacy of a nutritional supplement can be limited to a series of measurements which includes maximal isometric torque (Fmax) at a specified joint angle, as well as a series of maximal dynamic torque measurements with a specified movement amplitude and well within the reliability range (contraction velocity) of the dynamometer used. Torque values are expressed both as absolute values and relative to Fmax (%).

The minus of analytical strength tests is that validity to predict performance in strength and power skills relevant to athletic performance is often moderate to poor. Athletic strength and power skills are never limited to contraction of a single muscle group, but involve the concerted action of multiple muscle groups. Skill ability and neuromuscular co-ordination thus become major determinants of performance.

Strength and power 'skills'

As outlined above, strength and power skills do not specifically assess muscle strength, yet they can provide valuable information as to the effect of nutritional supplements on athletic performance. In order for such to be true, test reliability must be proven in a test/retest experimental design and in the target population under consideration. A skill which yields highly reliable data in trained athletes does not necessarily do so in untrained subjects. A test which is appropriate to use in young subjects may be inadequate in older ones. Thus, the skill used as a test must match the target population. Whether the test is a laboratory test or a field test does not matter, provided test reliability meets the above criterion. As a rule laboratory tests are more reliable than field tests because external conditions can be more easily controlled in a lab situation. A number of laboratory tests for strength and power have been proven to yield reliable assessment of force and power output [12, 14]. This includes various jump performances, weight lifting tasks, and sprint tests on a bicycle or rowing ergometer or treadmill (Table 1). In fact, sports skills often can be simulated in well-standardised laboratory conditions to increase test reliability without impairing test validity [14–17]. The reliability of force and power skills has been addressed in detail in a recent review [14]. Furthermore, a very popular skill to test maximal muscle strength is the 'one repetition maximum' (1RM), which means the maximum weight that can be lifted once in weight lifting exercises such as the bench-press, knee-extension, leg press, etc. The practical advantage of the 1RM tests is that it does not require the expensive equipment needed for adequate analytical strength tests. A very well experienced investigator must be able to find the 1RM load of the subject within 5 attempts within 5 % accuracy [18]. However, 1RM tests are much more technique-intensive than analytical strength tests and they are less accurate.

Anaerobic power and capacity

Anaerobic power refers to the work output per unit of time (Joule/s or Watt), whilst capacity refers to the total work output (Joule) effected during an all-out 'anaerobic' exercise bout. As a rule anaerobic power and capacity are tested in a maximal exercise bout lasting a couple of seconds to about 2 min, during which power is measured and work is calculated based on the power/time curve. The reliability of anaerobic tests has been addressed in detail in a recent review [14]. As outlined above, the longer the duration of the test used, the smaller the input of ATP provision from phosphocreatine breakdown ('alactic anaerobic metabolism') and the greater the energy production from glycogen breakdown to lactate ('lactic anaerobic metabolism'). Different types of anaerobic tests can be used to evaluate the effects of nutritional supplements on anaerobic metabolism (Table 2). In constant-work tests ('time trials') the subject completes a set amount of work or a set distance as quickly as possible. In constant-duration tests an individual performs as much work or covers as much distance as possible in a set time. In the shorter tests (30 s or less) power rises to a peak within the initial seconds, whereafter it decreases. Thus, peak power, fatigue (loss

Table 1 Measuring strength and power

Method	Reproducibility (CV in %)	Precision	Advantages	Disadvantages	Reference
Arm-flexion or knee-extension on an isokinetic dynamometer	1–3 % on the condition that 1) positioning of arm/leg is strictly standardised, 2) a reliable dynamometer is used and 3) best of 2–3 attempts is used	Very good on the condition that 1) arm/leg positioning is strictly standardised, and 2) a reliable dynamometer is used	Learning effects negligible No skill required Suitable for any individual Independent of training status or age	Expensive equipment Test reliability depends on the quality of the ergometer used Poor reproducibility if arm/leg positioning is not well standardised	[35] [20]
Other exercises on an isokinetic dynamometer	Moderate	Good	None	Idem arm-flexion and knee-extension + Technique intensive	[14]
Jump tests (Vertical jump, …)	2–9 % markedly improved if best of 2–3 attempts is used	Good	Inexpensive	Technique intensive Learning effects Reliability depends on training status	[14]
One repetition maximum (leg press, bench press, squat, …)	Good for these weight lifting exercises requiring little technique	Moderate	Inexpensive	Technique intensive Learning effects Requires experienced investigator Reliability depends on training status	[14]
Sprint test (constant duration 1–5 s)	2–7 %	Good	Inexpensive	Reliability depends on training status	[14]

Table 2 Measuring anaerobic power and capacity

Method	Reproducibility (CV in %)	Precision	Advantages	Disadvantages	References
Arm-flexion or knee-extension on an isokinetic dynamometer	1–3 % on the condition that 1) positioning of arm/leg is strictly standardised, 2) a reliable dynamometer is used and 3) best of 2–3 attempts is used	Very good on the condition that 1) arm/leg positioning is strictly standardised, and 2) a reliable dynamometer is used	Learning effects negligible No skill required Suitable for any individual Independent of training status or age Easy to perform metabolic measurements (knee-extension)	Expensive equipment Test reliability depends on the quality of the ergometer used Poor reproducibility if arm/leg positioning is not well standardised	[20] [35]
Other exercises on an isokinetic dynamometer	Moderate	Good	None	Idem arm-flexion and knee-extension + Technique-intensive	[14]
Constant-power sprint test to exhaustion within 5–30 s	1 %–7 %	Good	Exaggerates differences	Does not reflect 'real' sports performance	[14]
Constant-duration sprint test (5–30 s)	1 %–6 %	Good	Reflects 'real' sports performance Possible to perform postexercise metabolic measurements	Learning effects Reliability depends on experience and training status	[14]
Constant-work sprint test (5–30 s) ~ Time trial	1 %–4 %	Good	Reflects 'real' sports performance Possible to perform postexercise metabolic measurements	Learning effects Reliability depends on experience and training status	[14]

of peak power) and mean power during the test can be measured. Finally, in constant-load tests the subject maintains a constant power output to the point of exhaustion, defined by the inability to maintain the preset power, speed or cadence. Provided adequate measures are taken to optimise test reliability (see Specific study design aspects), either of the above test types, lasting between 10 s and 2 min, can be used to evaluate the effects

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of nutritional supplements on anaerobic power/capacity. Test types are equally valid and reliability measures are comparable. The above tests can be administered as a whole body exercise test (e. g. running, rowing, cycling) or as an analytical muscle test on an isokinetic dynamometer (see section on Analytical force measurements) [19–21].

Evaluation of muscle metabolism during short maximal exercise

The method of choice to assess potential effects of nutritional supplements on energy metabolism during exercise requiring high force and power output is to assay a selection of the above mentioned energy substrates (PCr and glycogen) and their metabolic end products (ADP, AMP, IMP, Pi, ammonia, H^+, lactate) in muscle tissue sampled by appropriate needle biopsy procedures [22, 23] before and after exercise. The appearance of metabolites (inosine, hypoxanthine, ammonia, lactate) in the venous effluent of the muscle also can provide valid information as to the rate of adenine nucleotide degradation and glycolysis during high intensity muscle contractions [10, 24]. Metabolites can be assayed by enzymatic biochemical assays [25, 26] or by HPLC [9, 10]. 31P-NMR spectroscopy can serve as a non-invasive alternative to measure muscle adenine nucleotides as well as PCr content and pH. However, the use of NMR spectroscopy is limited to isometric exercise, or very low velocity concentric exercise, and there are a number of reservations with regard to the validity of some of the measurements/calculations made [27, 28].

Fatigue

Fatigue can be defined as 'the loss of peak force or power output'. Thus, claims with regard to 'reduced muscle fatigue' must be substantiated by scientific evidence to show that either the loss of peak force or peak power output is reduced. This requires the use of an exercise protocol which causes substantial fatigue at least in the placebo control condition. Any of the above recommended tests, provided it fulfils the above criterion, either administered as a single test or as an intermittent protocol [19–21, 29, 30], can be used to evaluate fatigue during strength and power exercise. It is recommended that force, torque or power data be expressed both as absolute values and relative to the peak values measured. Indeed, increased muscle strength/power in general is associated with greater absolute endurance yet reduced relative endurance [12, 31].

Recovery

In the context of the current discussion, recovery can be defined as the rate at which maximal force/power output is restored to normal after prior fatigue-inducing exercise (see section on Fatigue). This requires the use of an intermittent exercise protocol, wherein maximal force/power output is substantially depressed, and exercise bouts are interspersed by adequate recovery intervals. For this purpose, any of the above recommended exercise types can be used, depending on the specific question addressed with regard to a given supplement.

As described above, PCr breakdown through the creatine kinase reaction, as well as glycogenolysis/glycolysis are the primary energy substrates fuelling ATP resynthesis during short exercise bouts involving maximal force/power output. However, during intermittent exercise PCr contributes an even larger fraction of the ATP supply, because it becomes increasingly difficult to restimulate the glycogenolysis/glycolysis pathway for ATP provision as the number of exercise bouts increases [6]. Thus, during intermittent exercise the rate of PCr resynthesis conceivably becomes an important factor to limit PCr availability at the time of initiating the subsequent maximal exercise bout. The half-life of muscle PCr resynthesis in mixed-fibred human muscle is in the range of 30–40 s with near full resynthesis being effected within 2 min [6, 32]. Thus, because depletion of PCr stores probably is one of the primary limitations of force/power production during short maximal exercise, an exercise protocol involving short maximal exercise bouts (5–15 s) interspersed by 30 s to 2 min rest intervals is recommended to evaluate the effect of a nutritional supplement on muscle PCr resynthesis [32, 33].

During exercise involving glycogenolysis as the primary source of energy provision [6], the rate of post-exercise lactate elimination is important to the restoration of normal performance level. Post-exercise lactate elimination can be studied by investigating the time course of muscle lactate elimination as well as the fall of arterial blood lactate (see section on Lactate) during a ~1 h time window immediately following a short all-out exercise bout(s) causing the muscle and blood lactate concentration to substantially increase. The physical activity level during this recovery period must be strictly standardised (rest or well-controlled low intensity exercise).

Following single bouts of exercise with maximal muscle force and power output, the muscle nucleotide pool is rapidly replenished via the purine metabolic salvage pathway in conjunction with the purine nucleotide cycle. However, during training involving many repeated bouts of such exercise, purine salvage and nucleotide cycling may fail to compensate for the massive rate of nucleotide degradation [9, 34, 35]. Thus, a fraction of the nucleosides and bases are lost for the muscle cells and need to be replenished by de novo nucleotide synthesis. The latter is a slow process, which can cause the muscle ATP content to be reduced for several days after exercise. In this context, any claim with regard to replenishment

of the muscle adenine nucleotide pool must be substantiated by studies showing that the recovery of muscle ATP content, following exercise training inducing a net depletion of the adenine nucleotide pool, is enhanced by the administration of a supplement.

■ Specific study design aspects

Subject selection

Due to practical limitations intrinsic to exercise performance studies, studies as a rule are performed on a small number of subjects [8–12]. The majority of studies thus have low statistical power and high incidence of statistical type I and type II errors. Therefore, subjects must be very carefully selected so as to obtain experimental groups with very homogeneous distributions for age, gender, race and training background (athletic status). Conclusions of the study must be limited to this well-defined target population. Furthermore, athletes perform more reliably in short all-out exercise than non-athletes [14]. This implies that more subjects are needed to obtain a similar degree of statistical power in studies using untrained individuals than in studies using well-trained subjects. Finally, initial performance level is always a primary determinant of the potential of performance measures to be enhanced, either by a nutritional supplement or by any other intervention. Therefore, it is important to assign subjects to placebo and experimental groups to obtain groups with similar distributions for the primary biological marker of interest. Randomisation based on the matched-pair principle is recommended.

Test reliability

For obvious reasons, only tests with reliability proven in the population defined can be used to evaluate the effects of food supplements on exercise performance. Issues related to evaluation of reliability in exercise performance testing have been extensively addressed in some recent reviews [14, 36]. It is also important to mention that in case ergometers or treadmills are used to measure the high power (speed) outputs typical to short all-out exercise, particular emphasis must be put on the calibration of the device used. Reliability of tests to an important degree depends on the quality of the ergometer used as well as on the use of adequate calibration procedures. Therefore, researchers must perform a reliability study, and/or investigate the stability of the load of a particular ergometer with a dynamic calibration rig, prior to using the ergometer to assess the effects of a nutritional supplement. Another important point is that consistent use of a standardised and adequate warming-up protocol can significantly increase the reliability of anaerobic test results [14].

■ Summary of strength and power measurements

The method of choice for assessing the effect of a supplement on muscle strength and power involves torque measurements during maximal arm-flexions or knee-extension by means of a reliable isokinetic dynamometer. Type of muscle contraction (isometric, concentric, eccentric), angular velocity and joint angle must be strictly standardised and specified. Such simple mono-articular movements require little technique, if any, which implies learning effects from one test to the other to be negligible on the one hand, and makes the test procedure suitable for any population independent of age or training status. Using the best of 2–3 attempts substantially increases test reliability. Any other strength/power skill is more technique intensive than the mono-articular arm-flexion or knee-extension movement. The more technique intensive the skill used, the higher the risk that the force/power output measured is limited by technique-dependent factors (skill technique, co-ordination) rather than by muscle strength per se. As a result, the reliability and validity of complex strength skills (e. g. jumping, weight lifting, sprinting) largely depends on training status relative to the skill used. Accordingly, in untrained subjects learning effects often impair test reproducibility. Supplements claimed to enhance muscle strength often at the same time also are claimed to improve 'anaerobic' performance, either by increasing peak power output, and/or by reducing fatigue. Constant-duration tests are the method of choice to assess anaerobic performance. This involves measuring peak power, loss of peak power ('fatigue') and mean power output during a maximal exercise test with a fixed duration of 10 s to 2 min. The constant-duration tests can be administered either as a whole body exercise test (e. g. sprinting, rowing, cycling) or as an arm-flexion/knee-extension test on an isokinetic dynamometer.

Endurance, energy supply and recovery

■ Claims

- Improves endurance
- Improves endurance capacity
- Improves fat oxidation
- Spares muscle glycogen
- Improves energy balance
- Improves substrate delivery
- Reduces lactic acid accumulation
- Improves transport of oxygen to working muscles
- Increases blood flow
- Reduces gastro-intestinal distress

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■ Definition, physiological background and significance

Many nutritional supplements claim to improve endurance performance or they claim to affect the factors that are known to be related to endurance performance. Endurance is typically defined as resistance to fatigue. This could be resistance to fatigue during brief intense exercise but also during sub-maximal prolonged exercise of several hours. For the purpose of this review we will define endurance as resistance to fatigue during a mode of exercise where the primary cause of fatigue is induced by substrate depletion or central factors. Typically endurance exercise is therefore 30 min or longer. When exercise is longer than 4–5 hours we will refer to this exercise as ultra-endurance exercise.

In order to understand how nutrition supplements can improve endurance performance, it is important to understand the underlying fatigue mechanisms. In other words, what are the factors that cause fatigue during endurance exercise. Studies in the 1960s demonstrated that one of the main factors was substrate depletion [37, 38]. Muscle glycogen depletion seemed to coincide with fatigue and athletes with high muscle glycogen stores exercised for longer at a given exercise intensity compared to those with low muscle glycogen stores. In order to decrease muscle glycogen breakdown and to delay or prevent muscle glycogen depletion various methods have been employed to increase fat oxidation at the cost of carbohydrate oxidation [39–41]. In addition it was shown that with carbohydrate ingestion blood glucose concentrations and high rates of carbohydrate oxidation could be maintained during exercise resulting in increased time to exhaustion [42, 43]. It is generally believed that a rapid delivery of carbohydrate may enhance performance because endogenous carbohydrate stores will be spared [44].

Especially in ultra-endurance events performance may be hindered by gastro-intestinal problems [45] and it is likely that reducing gastro-intestinal problems in those events would result in improved performance. In multiday events recovery becomes an extremely important factor and maintaining energy balance may be an important issue. Claims are generally built around these factors and the biomarkers to support such claims will be discussed below.

■ Methods of measurement

Measuring endurance performance or endurance capacity

In the literature the terms endurance performance and endurance capacity are often used as synonyms. However, endurance capacity refers to the exercise time to volitional fatigue, whereas performance relates to completing a certain task (running a certain distance, cycling a certain amount of work) as fast as possible (Table 3). The latter is of course a more realistic approach since there are very few events where athletes are asked to exercise as long as they can. However, in the scientific literature we find more studies that have measured endurance capacity as the time to exhaustion when exercising at a constant workload or speed. The advantage of this technique is that it is relatively easy to control and the constant workload allows comparison of metabolic and other measurements in an experimental and a control condition. Some studies have used a time trial following a bout of constant workload exercise. Such designs make it possible to perform metabolic and other relevant measurements and also a valid performance measurement. Performance trials have been de-

Table 3 Endurance performance

Method	Reproducibility (CV in %)	Precision	Advantages	Disadvantages	Reference
Time to exhaustion	~8–26%	Fairly good	Metabolic measurements possible Exaggerates differences	Does not reflect sports	[43] [48]
Time trial	1–3%	Good	Reflects "real-life" situation	Varying workload and therefore impossible to perform metabolic measurements Experience required	[48]
Constant load followed by a time trial	1–3%	Good	Reflects "real-life" situation Constant workload at the start allows metabolic measurements	Experience required	[48]
Constant duration test	3–5%	Good	Simulates "real-life" situation	Experience required Variable workload and therefore not possible to perform metabolic measurements	[48]
Simulated intermittent tests	2–5%	Good	Simulates "real-life" situation	More difficult to control	[46]

veloped for the treadmill (self-paced runs for a fixed distance), intermittent running (Loughborough Intermittent Shuttle Run test; [46], cycle (self-paced time trial [47, 48]), rowing ergometer and various other intermittent sports such as squash [49] and tennis [50].

A performance test must be reliable (reproducible) and valid. The validity of a test refers to the extent to which an individual's test performance reflects true performance. The reliability refers to the consistency of performance when an individual performs the test repeatedly. A test with poor reliability is unsuitable for tracking changes in performance between trials and only tests with high reliability can have high validity [14].

Endurance performance or endurance capacity tests in the literature can be divided roughly into 3 categories:

- Constant work tests, usually referred to as time trials.
- Constant duration tests, these are similar to time trials but instead of completing a pre-set amount of work, as much work as possible is performed in a given time period.
- Constant power or constant load tests, these tests measure endurance capacity.

Studies have investigated the reliability of various test protocols and have found coefficients of variation between 1 and 3 % for performance trials (constant work test [47, 48, 51–53]) and up to 27 % for time to exhaustion measurements [48]. Protocols with constant duration seem to have coefficients of variation between 2 and 3 % [48, 54, 55]. It can be concluded that time trials (constant work tests) and constant duration tests are the most reliable measurements and are also likely to be more valid indicators of true performance. However, it must also be noted that it may be easier to detect differences between two experimental conditions using a time to exhaustion test. For a detailed analysis of the reliability of endurance performance tests the reader is referred to a recent meta-analysis by Hopkins et al. [14].

Size of the effect and physiological importance

Of course even the smallest improvement is relevant to an athlete. However, such small differences are sometimes difficult to measure because the reliability or sensitivity of the test is inadequate. Recently Hopkins et al. [56] argued that tests of endurance power suitable for assessing the smallest worthwhile changes in running performance for top runners need a coefficient of variation smaller than or equal to 2.5 % and smaller than or equal to 1.5 % for tests simulating half or full marathons and shorter running races, respectively [56]. To pick up small but relevant differences in performance a test protocol needs to be very reliable.

Study criteria

It is crucial that performance tests are conducted under strictly controlled conditions. For example the feedback to subjects needs to be minimal and encouragements should be standardised. Heart rate, work rate, speed and time should not be shown to subjects in studies with multiple trials. Experiments should not be conducted in pairs and they should be performed in quiet labs with no distractions. Trained subjects will generally give more reliable results than untrained subjects, especially when using time trial protocols. A familiarisation trial is necessary as a learning effect is generally observed. Hopkins et al. [14] reported that performance increased between the first and second trial on average by 1.2 % (likely range 0.5 to 1.9 %), whereas the increase in subsequent trials was only 0.2 % (likely range –0.3 to 0.7 %).

Equipment can also cause reduced reliability and a clear relationship was found between the quality of treadmills and ergometers and the observed variability in the measurements. For an overview of some of the available methods to measure performance or endurance capacity and their advantages and disadvantages see Table 1.

Summary of performance measurements

Distinction must be made between exercise capacity and exercise performance. The first refers to time to exhaustion and the second refers to a more real-life true performance measurement. Tests have been developed to measure performance very reliably. However, control of all external variables is crucial in these tests.

■ Measuring fat oxidation

Respiratory exchange ratio

Fat oxidation can be measured using indirect calorimetry. Gas exchange measurements not only allow an estimation of the energy expenditure but also of the substrate mixture used. Krogh and Lindhard [57] in the beginning of the 20th Century used the inherent differences in chemical properties of carbohydrate, fat and protein to obtain information about fuel utilisation. The respiratory quotient reflects the relative contribution of carbohydrate and fat to total energy expenditure. Absolute rates of fat oxidation can then be calculated using stoichiometric equations [58].

The application of respiratory exchange ratios (RER) is based on the premise that exchange of oxygen and carbon dioxide at the mouth represents the processes that occur in the tissues that oxidise the fuels. This assumption is valid at rest and during exercise up to about 80–85 % VO_2max above which RQ measured at the

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mouth does not always reflect the oxidation processes in cells (due to hyperventilation and excess CO_2 output). At these high exercise intensities, fat oxidation is thought to be negligible. Normally fat oxidation increases from low to moderate intensities, peaks around 64 % VO_2max in trained individuals and becomes negligible around 80 % VO_2max [59]. In order to demonstrate an effect of an intervention on whole body fat oxidation it is advisable to measure fat oxidation over a wide range of exercise intensities as discussed in a recent paper by Achten et al. [59].

Gas exchange measurements will only allow conclusions to be drawn about total whole body fat oxidation but the source of fatty acids cannot be identified. Whether the fatty acids oxidised are derived from adipose tissue (FFA), or triglycerides (VLDL or IMTG) can only be determined with the use of isotopic tracers or magnetic resonance spectroscopy or imaging techniques.

Isotopic tracers

Although deuterium tracers ($^2H_2$-palmitate) have been used to estimate plasma fatty acid oxidation, it must be noted that oxidation can only be measured using carbon isotopic tracers (1-^{13}C-palmitate or U-^{13}C-palmitate). By primed continuous constant rate infusion of trace amounts of the isotope, not only rate of appearance and disappearance of plasma FFA can be determined but also the oxidation of plasma derived FFA [60]. More recently it was discovered that plasma FFA oxidation may be underestimated by up to 50 % with this technique because ^{13}C is trapped in exchange reactions with the TCA cycle [61,62]. For example, some ^{13}C-carbons may be incorporated into the glutamate/glutamine pool via α-ketoglutarate (α-KG), or into phosphoenolpyruvate (PEP) via oxaloacetate (OAA) [62]. This label fixation results in a decreased recovery of label in the expired gas and in order to correct for this loss, the acetate correction factor has been proposed [62]. This correction is based on the assumption that acetate has immediate access to the TCA cycle and is instantly oxidised. The percentage of label (^{13}C or ^{14}C) not recovered in expired CO_2 represents the amount of CO_2 trapped in exchange reactions with TCA-cycle intermediates (TCAI) and the bicarbonate pool. The label loss is dependent on the metabolic rate. At high oxygen uptakes (> 35 ml/kg·min) less label is trapped and recovery of the 1-^{14}C-acetate label was found to be 85–90 % [62]. Similar results were obtained by Schrauwen et al. [61], when ^{13}C-palmitate [63] was used. This implies that previous studies in which the participants performed at low absolute exercise intensities may have underestimated plasma FFA oxidation rates.

In aerobically trained individuals, intramuscular triglyceride stores (IMTG) are in contact with the mitochondria and are thought to serve as an important fuel source during exercise, particularly prolonged moderate-intensity exercise [40, 64–66]. For instance, it has been estimated from isotope tracer studies in men and women that IMTG can contribute as much as 20–30 % of energy expenditure during prolonged submaximal exercise [67, 68]. It must be noted however that IMTG oxidation is calculated by subtracting plasma FFA oxidation from total fat oxidation and ignoring a possible role for plasma triglycerides as a substrate. To obtain more direct measurements of IMTG content and breakdown NMR spectroscopy could be helpful.

Nuclear magnetic resonance spectroscopy

Recently, several research groups have developed the technology to non-invasively measure intracellular lipids in human muscle by ^{1}H-NMR spectroscopy. In skeletal muscle two compartments of triglycerides/fatty acids can be distinguished: one that is associated with lipid within fat cells, or extramyocellular lipid (EMCL), and the other that is confined to the skeletal muscle cytoplasm, intramyocellular lipid (IMCL) [69, 70]. Measurement of IMCL by NMR has recently been validated in *in vivo* animal and human models [69]. The reliability of IMCL measurements is generally good (6–12 %) whereas the determination of EMCL has a slightly higher variation (18–23 %) [69–71]. Using this technique it was possible to demonstrate decreases after exercise (2 h treadmill running at 67 % VO_2max) and it was possible to detect the difference of nutritional interventions [71].

Muscle biopsies

Traditionally, researchers have measured IMTG concentrations from muscle biopsies [65, 72]. However, there is considerable variation in the measured IMTG concentration in repeated muscle biopsies. The coefficient of variation for this measurement is 20–26 % and IMTG during a continuous exercise bout at 65 % VO_2max is usually smaller than the error of the measurement [72]. This variation is most likely due to local differences in IMTG concentration and potential contamination with intracellular adipocytes. The error of the measurement is small as within biopsy variability is relatively small (coefficient of variation 6 %). Wendling et al. [72] concluded that only changes greater than about 24 % may be detectable with this method.

Plasma FFA and glycerol

It is common practice to measure plasma FFA and glycerol as indicators of fat oxidation and lipolysis. It must be noted that increased plasma FFA concentrations or glycerol concentrations do not necessarily reflect higher fat oxidation rates. Plasma FFA concentrations only re-

flect the net balance between FFA release into the plasma and FFA uptake by different tissues. Plasma glycerol is a fairly good indicator of whole body lipolysis but cannot be used for quantification.

Size of the effect and physiological importance

Reductions in fat oxidation have been observed after carbohydrate feeding, with increasing carbohydrate content in the diet. Other factors like the exercise intensity and exercise duration also affect fat oxidation rates. Training not only lowers fat oxidation but also changes the source of the fatty acids oxidised. Although marked reductions in fat oxidation can be observed with nutritional interventions, increases in fat oxidation as a result of an intervention are usually smaller and more difficult to detect [39, 64, 73]. The importance of increased fat oxidation rates is subject to current debate. When discussed in relation to weight loss it must be regarded in the context of the 24-hour energy expenditure and 24-hour fat oxidation, as well as the energy and fat intake. When a claim is made that increased fat oxidation during exercise to reduce the breakdown of carbohydrate, the relevance is unclear unless it can be demonstrated that glycogen depletion can be prevented.

Study criteria

Fat oxidation can change dramatically as a result of relatively small changes in the dietary fat intake. The coefficient of variation for rates of fat oxidation, measured by RER at a given exercise intensity are between 10 and 30 %, whereas the CV of the intensity at which maximal fat oxidation occurs has a coefficient of variation between 6–10 % [Achten et al., personal communication]. In order to minimise variation in the measurements and decrease the coefficient of variation of all fat metabolism-related parameters.

Fat oxidation during exercise is more difficult to measure in non-active individuals compared to athletes because the rates of fat oxidation are lower relative to the measurement error.

For an overview of methods to measure fat metabolism see Table 4.

Summary of fat oxidation measurements

Fat oxidation can be measured in several ways. If total fat oxidation is of interest indirect calorimetry is the logical method of choice. However if the source of fatty acids needs to be determined, more sophisticated methods involving stable isotopes or NMR are needed.

■ Muscle glycogen sparing

Glycogen

Muscle glycogen is an important substrate for muscular work. When the exercise intensity is 65–90 % it is usually

Table 4 Fat oxidation

Method	Reproducibility (CV in %)	Precision	Advantages	Disadvantages	References
Respiratory exchange ratio	~3–5 %	Good	Metabolic measurements possible Exaggerates differences	Difficult to measure in the field Does not give information about the source of fatty acids oxidised	[236]
Stable isotopes (^{13}C-palmitate infusion)	?	Good	Gives an indication of the source of the fatty acids oxidised Useful in combination with indirect calorimetry Relatively non-invasive	Can only be used in steady-state conditions Can only be measured in lab conditions Is a whole body measurement and not specifically muscle Specialised equipment and personnel needed to perform analysis	[93]
Muscle biopsy	26 %	Good	Direct measurement of intramuscular triglyceride concentration	Invasive Large variability depending on the site of the biopsy Samples easily contaminated with adipose tissue	[72]
^{13}C-NMR	6 %	Good	Direct measurement of intramuscular triglyceride concentration Can distinguish between intra- and extramyocellular triglycerides Non-invasive	Potential problems with localisation Expensive Can only be performed in specialised hospital units	[70]

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the most important substrate. However, our body only stores relatively small amounts of muscle glycogen, approximately 400–1000 g (depending on diet and muscle mass) and it has been demonstrated that these stores can be reduced significantly in only 10 min [74]. Studies in the 1960s demonstrated that muscle glycogen depletion is one of the main causes of fatigue [37, 38]. Muscle glycogen depletion coincided with fatigue and when a high carbohydrate diet was consumed and muscle glycogen stores were elevated subjects' exercise time to exhaustion was increased compared to a mixed diet and normal glycogen stores. Since then, there has been enormous interest by exercise physiologists to study the role of muscle glycogen and a variety of techniques have been used to measure muscle glycogen concentration, muscle glycogen breakdown or both.

Muscle glycogen is also thought to be important in recovery and many studies have investigated the effects of nutritional interventions on the rate of muscle glycogen synthesis after depletion.

Measuring glycogen

There are several ways to measure glycogen breakdown during exercise (Table 5). The most commonly used method is rather invasive and involves muscle biopsies. This technique was redeveloped in the mid 1960s [37, 75] and requires a small surgical procedure. The muscle glycogen concentration may depend on the location of the biopsy within a muscle and on the muscle fibre type. There is considerable variation in muscle glycogen content even in biopsies taken from the same site. The coefficient of variation for this method in human skeletal muscle is typically about 10 % [72]. Despite this rather large variation the technique has been proven useful in

detecting muscle glycogen breakdown during exercise and glycogen synthesis in the recovery period.

When ^{13}C- or ^{2}H-labelled glucose is infused at a constant rate the Rd glucose can be determined. In most conditions the Rd glucose will be equal or be very similar to the rate of plasma glucose oxidation [76]. The difference between total carbohydrate oxidation and plasma glucose oxidation is believed to be muscle glycogen oxidation. The advantage of this technique compared to muscle biopsies is that repeated measurements of muscle glycogen breakdown can be obtained, whereas with biopsies there are always limitations to the number of biopsies that can be taken. The disadvantage of the indirect tracer methods is the fact that no measure of muscle glycogen concentration is obtained but only breakdown rates.

^{13}C-NMR spectroscopy is another method to measure muscle glycogen. The development of these techniques has made it possible to measure muscle glycogen concentration non-invasively using naturally labelled glucose, and more recently using labelled substrates (especially glucose) it has been possible to measure fluxes through the glycolytic and glycogen synthesis pathway.

Size of the effect and physiological importance

Although with the available techniques relatively small changes in muscle glycogen can be picked up it is not clear what changes are relevant to performance. There is a wealth of data that suggests that endurance capacity is impaired when muscle glycogen concentrations are below a critical level (< 30 μmol/g ww), but at concentrations above that no changes in performance have been observed. Recent studies have shown no effect of elevating muscle glycogen concentrations from high

Table 5 Muscle glycogen

Method	Reproducibility (CV in %)	Precision	Advantages	Disadvantages	References
Muscle biopsy	~10%	Good	Direct measurement	Invasive Medical staff needed to perform procedure Can only be used in lab environment Limited number of samples can be taken Local differences in muscle glycogen concentration	[72]
^{13}C-NMR	?	Good	Direct measurement Non-invasive	Potential problems with localisation Expensive Can only be performed in specialised hospital units	[237]
Stable isotopes (^{13}C-glucose infusion)	?	Good	Gives glycogen breakdown rates Frequent sampling possible Less invasive	Expensive Does not give glycogen concentration Is a whole body measurement and not specifically muscle Specialised equipment and personnel needed to perform analysis	[76]

(500–600 µmol/g ww) to very high concentrations (600–900 µmol/g ww) [77].

Summary of muscle glycogen measurement

Muscle glycogen is traditionally measured using the muscle biopsy technique. Although this technique is generally accepted there is considerable variation in the measurement and the number of time points that can be investigated is limited. If muscle glycogen breakdown is the main variable, stable isotopes form a good alternative. The development of NMR techniques has made it possible to measure muscle glycogen concentration non-invasively using naturally labelled glucose, and more recently using labelled substrates (especially glucose) it has been possible to measure fluxes through the glycolytic and glycogen synthesis pathway.

■ Energy balance

Energy balance

The energy balance is defined as the energy intake minus the energy expenditure and can therefore only be assessed by two different and independent methods: one for energy intake and one for energy expenditure. There are several ways to measure or estimate energy intake and expenditure. Unfortunately most ways to measure energy intake are rather inaccurate and prone to error [78] as will be discussed below. The methods to measure energy expenditure range from direct but complex measurements of heat production (direct calorimetry; not discussed here), to relatively simple indirect metabolic measurements (indirect calorimetry) and from very expensive tracer methods (doubly labelled water) to relatively inexpensive and convenient estimations of energy expenditure (heart rate monitoring and accelerometry).

Energy intake

There are several methods available to estimate or measure daily energy intake (Table 6a). These techniques include a 24-h recall, a 3 to 7 day dietary survey, and the duplicate food portion method. The advantages and dis-

advantages of the most important methods will be discussed below.

■ **24 h recall.** This is the most common technique of assessing food intake. A trained interviewer asks people on one or more occasions to describe the food, drinks and dietary supplements that have been consumed during the previous 24 h. The advantages of this technique are that this is easy to administer, it is time efficient and inexpensive. On the other hand, this technique has several disadvantages: Underreporting is common, even when the person is interviewed by a skilled dietician. This technique generally underestimates energy and nutrient intake by about 20 %! Overweight persons tend to underestimate their portions whereas underweight people tend to over-report portions [79]. Also, the technique relies heavily on memory, which makes it unsuitable for certain groups, like the elderly.

■ **Dietary records.** A dietary record is a relatively simple and reasonably accurate method to determine the total daily energy intake. The daily log of food intake for 3 to 7 days should represent a normal eating pattern. When only 3 days are used, it is often recommended to include two weekdays and one day during a weekend. The 7 day dietary record may be more representative of the normal diet but the disadvantage of the longer recording period is that recording errors are more likely to occur. Also compliance is decreased when the duration of the recording period is 4 days or more [80, 81]. Experiments have shown that calculations of energy intake made from records of daily food consumption are usually within 20 % of the actual energy intake although there are reports that this method may underestimate energy intake by 20–50 %. Without instruction, food records will lack sufficient detail to be useful for most research purposes. Errors occur mainly due to memory failures. In general, the stereotypical intakes are recorded accurately but the uncommon foods are sometimes poorly registered. Most errors are made in reporting the frequency of consumption. Errors in estimating the portion size are very common as well. Food may be weighed or the portion sizes may be estimated. When portion sizes are estimated using household measures the errors are usually larger but the respondent burden is consid-

Table 6a Energy intake

Method	Reproducibility (CV in %)	Precision	Advantages	Disadvantages	Reference
Diet recall	Poor	Fairly good	Time efficient Can be used in large populations	Varying workload and therefore impossible to perform metabolic measurements Experience required	[238]
Dietary record	Fairly good	Fairly good		Experience required	[238]
Duplicate food	Fairly good	Fairly good	Simulates "real-life" situation	More difficult to control	[238]

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erable. When food is not weighed, the mass of various foods may be under- or overestimated considerably. Errors as large as 50 % for foods and 20 % for nutrients have been reported [78].

Training and instructing the person who is registering his food intake can reduce some of these errors. Certain categories of people tend to underestimate or overestimate their food intake. It has been shown, for example, that overweight persons tend to underestimate their portions whereas underweight people tend to overreport portions [79]. Errors may also occur when individuals do not report their food intake accurately enough, which makes coding of the specific food type very difficult.

■ **Duplicate food collection.** The most accurate method is the duplicate food collection method. This method involves preparing two portions of food and saving a duplicate of everything that is consumed. The second portion will then be collected and put in a blender for chemical analysis of the nutrients of interest. This method can become very expensive when a wide range of nutrients is investigated because of the costs associated with the chemical analyses. However, when energy intake is the only variable of interests the costs are moderate. The method also places a burden on the individual, is likely to affect the food choices made and underreporting is common [82]. However, of all the methods this is likely the most accurate.

Energy expenditure

■ **Respiration chamber.** For a measurement of a complete energy balance a respiration chamber was developed. The advantage of this technique is that besides ac-

curate information about gas exchange (and thus energy expenditure) it is also possible to have accurate control over the energy intake (Table 6b). In addition, potential energy losses in stool can be analysed. The respiration chambers are the only way to accurately perform energy balance studies [83]. However, the respiration chamber has a big disadvantage in that it interferes with everyday life since not all activities can be performed inside the chamber.

■ **Doubly labelled water.** For measurements of energy expenditure in free-living conditions doubly labelled water is considered the gold standard. The principle of the doubly labelled water technique is based on the administration of a bolus dose of two stable isotopes of water: ^2H_2O and $H_2^{18}O$. These two stable isotopes are used as tracers and the slightly higher mass atoms ^2H and ^{18}O can be measured in various body fluids (urine). ^2H_2O is lost from the body in water alone and $H_2^{18}O$ is lost in water but also as $C^{18}O_2$ in breath. With increasing energy expenditure and oxygen consumption the $H_2^{18}O$ excretion increases relative to the ^2H_2O excretion. The difference of the two tracer excretion rates therefore represents the carbon dioxide production [84]. The intra-subject variation of this method is relatively small (3–6 %) [85–88]. Both the tracer and the analysis by isotope ratio mass spectrometry make this a very expensive technique. It is not suitable to study large populations.

■ **Heart rate monitoring.** In an attempt to avoid some of the problems associated with the measurement of energy expenditure in free-living physical activity, various less expensive and less complicated methods have been developed. One of these methods is based on heart rate,

Table 6b Energy expenditure

Method	Reproducibility (CV in %)	Precision	Advantages	Disadvantages	References
Indirect calorimetry Respiration chambers	~3–5 %	Good	Accurate When using chambers energy intake can also be monitored	Performed in respiration chambers when recorder over 24 h May interfere with normal activity patterns Inaccurate during very intense exercise (> 85 % VO$_2$max)	[83, 236]
Doubly labelled water	4–6 %	Good	Can be used in free-living conditions	Very expensive	[88]
Heart rate monitoring	10–15 %	Moderate	Can be used in free-living conditions Relatively inexpensive	Inaccurate during low and high intensity exercise Heart rate can be influenced by external factors such as temperature, hydration status etc Less suitable for use in individuals and small groups	[83] [83]
Accelerometry	10–20 %	Moderate	Suitable for large populations Can be used in free-living conditions	Indirect measurement Less suitable for use in individuals and small groups	[83]

based on a linear relationship with oxygen uptake at submaximal exercise intensities. At very low and very high exercise intensities (supramaximal) this estimation may be less accurate. To use heart rate for the estimation energy expenditure, the individual relation between HR and VO_2 (and EE) needs to be determined. Measurements of oxygen uptake can then be used to calculate energy expenditure at several different heart rates. The main limitation of the use of heart rate for measuring energy expenditure is the almost flat slope of the relationship at low expenditure levels [89]. At rest, slight movements can increase the heart rate, while energy expenditure (i. e. oxygen consumption) remains almost the same. There appears to be general consensus that while the HR method provides satisfactory estimates of average EE for a group, it is not necessarily accurate for individual subjects [90–92]. For instance Spurr et al. [92] compared 24h EE by calorimetry and with the HR method in 22 subjects. The maximum deviations of the values of EE between the two methods varied between +20% and –15%. However, when the data were compared using a paired t-test, no significant differences were found. Also, the heart rate-VO_2 relationship is influenced by several factors including environmental conditions (temperature, humidity), altitude, body position, anxiety (at low work rates) and so on). Nevertheless, in some conditions heart rate can provide a very convenient and relatively inexpensive estimate of energy expenditure. This method therefore gives a reasonable estimation of the energy expenditure.

■ **Accelerometer.** Another way of estimating someone's activity level is through accelerometry. The number and the degree of the accelerations give an indication of someone's activity level. Accelerometers can record accelerations on one, two or three axes. A single axis or single plane accelerometer measures only acceleration in the vertical direction. Triaxial accelerometers measure accelerations along three axes and are likely to be more accurate. Generally accelerometer readings (usually expressed as activity counts or in kJ or kcal) correlate well with energy expenditure. However, for some movements, especially those in free-living conditions, the accelerometer tends to underestimate the true energy expenditure. Given the low weight and compactness of the modern accelerometers this instrument is a very convenient and easy method to estimate an individual's activity level in a free-living situation. The disadvantage is of course that it does not provide direct measurements. For an overview of the methods to measure energy expenditure see Table 6b.

Summary of energy balance measurement

The only way to accurately measure energy balance is with a respiration chamber. Food intake can be accurately controlled and energy expenditure accurately measured. However this method cannot be used in free-living conditions and a good but expensive alternative for the measurement of energy expenditure is doubly labelled water. Other methods are available but they are less accurate. There are also several methods to estimate energy intake. The duplicate food collection methods may be the most accurate but may interfere with food choices and eating patterns. The 3–7 day food diaries are a good alternative although underreporting is a common problem.

■ Substrate delivery

Substrate delivery

Since endogenous carbohydrate stores are limited depletion of these stores may occur within several hours of exercise. Depletion of carbohydrate stores (liver glycogen and/or muscle glycogen) have been recognised as causes of fatigue. Prevention of glycogen depletion can enhance performance and it is generally believed that an increased delivery of substrate from exogenous sources is beneficial.

Measuring substrate delivery

Carbohydrate delivery from exogenous sources can be measured accurately and reliably using (radioactive or stable) carbon isotopes [93]. The oldest method to trace ingested CHO is adding a ^{14}C-glucose tracer to a CHO beverage and measuring ^{14}C in expired gas samples using a scintillation counter. The advantage of this technique is that it is relatively inexpensive compared to the use of stable isotopes. In addition, shifts in background enrichments which may occur when using stable isotopes (see below) are not a problem, because the background level of ^{14}C is negligible.

An obvious disadvantage of this technique is the fact that it exposes the subject to radioactivity. The radiation dose given, however, is usually low (< 40–80 µCi is consumed), and is calculated to correspond to 0.02–0.03 rem, 200–250 times lower than the permissible dose.

Studies in which stable isotope methodology was used to measure exogenous carbohydrate oxidation have used ^{13}C-enriched substrates. Some of these studies have used naturally enriched CHO (derived from C4 plants such as corn and cane sugar). These plants have a naturally high abundance of ^{13}C. When ingesting these CHO during exercise, breath $^{13}CO_2$ will become enriched and together with a measure of the total CO_2 production rate, exogenous CHO oxidation rates can be quantified. In addition to the problems described above, there is another complication with this technique, namely shifts in substrate utilisation may result in a change in back-

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ground enrichment. Because CHO is usually more ^{13}C-enriched than fat, glycogen stores may display higher ^{13}C-enrichments than endogenous fat stores. Any change in shift in endogenous substrate utilisation can therefore cause a change in the background ^{13}C-enrichment independent of ingested CHO. These changes occur for instance in the transition from rest to exercise and typically an increase in [13]CO_2 in the expired gases is observed. The magnitude of the error depends on the ^{13}C-enrichment of the ingested CHO relative to the ^{13}C-enrichment of endogenous glycogen stores. It has been shown that subjects with a diet in which most CHO are derived from C4-plants (typical Northern American or Canadian diet) have higher ^{13}C-enrichments in their muscle glycogen stores compared to Europeans whose diet is typically derived from C3 plants such as potato and beet sugar. By adding a tracer to the CHO, the shift in background remains the same but the relative error is reduced. Another way around the problem is to perform control trials with an identical protocol but with ingestion of CHO with a low natural abundance. The background ^{13}C-enrichments can then be used to correct the calculated exogenous CHO oxidation.

The reliability of these measurements of exogenous substrate oxidation is good (coefficient of variation 3–7 %) and most variation is due to day-to-day variation in the VCO_2 measurement. An overview of the methods is provided in Table 7.

Study criteria

Most of the variation in measurements involving ^{13}C labelling is caused by the variation in RER measurements and the same limitations to this technique as apply to indirect calorimetry. This implies that these techniques can only be used during exercise at intensities below 85 % VO_2max where lactate accumulation occurs. Studies have shown, however, that exogenous substrate oxidation is similar in very well-trained compared to untrained subjects and therefore the selection of subjects is less important [94, 95].

Summary of substrate delivery measurements

[13]C labelling of exogenous substrates in combination of indirect calorimetry is the method of choice for measurements of substrate delivery. The method has been validated and produces reliable results if external variables are adequately controlled and if the tracer used traces the tracee (an overview is provided in Table 5).

■ Modification of lactate accumulation

Definition, physiological background and relevance

Lactic acid which appears in the muscle and blood during exercise is a product of pyruvate/lactate conversion in the process of glycolysis which regulates cytosolic NADH + H/NAD equilibrium thus making possible anaerobic generation of ATP. The blood lactate levels are a result of the balance between the lactic acid production in muscle and elimination from the blood stream, the main pathways of blood lactate elimination being 1) the uptake in liver for oxidation and gluconeogenesis and 2) uptake in active as well as inactive muscles for oxidation. Lactate levels in arterial blood are considered to be a good reflection of the whole body status of lactate production and elimination. While low levels of exercise are not associated with arterial lactate increase the high exercise intensities induce a rise in blood lactate and metabolic acidosis. Thus, during low-intensity exercise, muscle lactate production is in balance with lactate elimination while this balance is disturbed during higher exercise intensities during which the lactate production is enhanced more than lactate elimination [96, 97]. The increase of lactate production during the high intensity exercise is associated with a need of additional energy formation over what can be provided by aerobic pathways. In these conditions conversion of pyruvate to lactate becomes increasingly important for an adequate regeneration of ATP. The metabolic acidosis resulting from the increase of lactic acid is supposed to be one of the mechanisms of muscle fatigue limiting the duration of high intensity exercise. Between the two states of blood lactate balance there is a transition area of intensities of exercise in which the shift to lactate accumulation occurs. This area can be identified with those named "lactate threshold", "onset of blood lactate accumulation", "maximal steady state of blood lactate level" or, the most frequent but perhaps the most imprecise, "anaerobic threshold".

The muscle and blood lactate accumulation in response to high intensity exercise can be modified by training status (the more trained subject having the lower lactate levels at the given intensity of exercise), by

Table 7 Substrate delivery

Method	Reproducibility (CV in %)	Precision	Advantages	Disadvantages	References
[13]C substrate oxidation	Good	Good	Direct measurement of substrate oxidation	Can only be measured in a specialised laboratory Relatively expensive	[93]

modifications of oxygen supply to the exercising subject (hypo- or hyper-oxia) and/or by administration of exogenous nutritional or other products. The claims of such products to modify the lactate accumulation should be substantiated by methods enabling the detection of the lactate accumulation and, eventually, the associated metabolic acidosis.

Methods of measurement of blood lactate accumulation

The most simple and most widely used method of blood lactate monitoring is the measurement of lactate concentration in the blood samples. Four sites are commonly used for blood sampling: forearm arteries, forearm veins, ear lobes and finger tips and the role of sampling size is mentioned below. The blood sample is analysed mostly by enzymatic methods for blood lactate concentrations [98]. The lactate levels in arterial blood are considered to be a "golden standard" for evaluation of lactate balance as they do reflect the whole body status of lactate production and elimination.

■ **Lactate threshold.** A possible approach for evaluation of the status of blood lactate accumulation is to determine the area of exercise intensities which represent the transition between the intensities corresponding to the steady state of blood lactate and those which induce the blood lactate increase. This can be examined during the continuously rising steps of exercise intensities of 3–4 min duration with lactate concentrations determined at the end of each work load and at the peak of exercise (the blood should be sampled between the 3rd to 6th minute after the end of exercise). Several criteria have been suggested in order to express the exercise intensity which is associated with the blood lactate accumulation and several examples are listed in Table 8 (examples 1, 2, 3). Alternatively, the blood lactate response may be examined during several longer (10 to 20 min) discontinuous incremental work loads and transition zone determined (Table 8, example 4) (see also [99]).

Reproducibility of the lactate threshold determination – expressed as VO_2 – was shown to be of the order of $r = 0.93$ [100] if the method of individual calculation was used. However, the reproducibility is not good for the blood lactate concentration at the threshold area which suggests that the fixed blood lactate levels of 2 and/or 4 mmol/l are not good indicators of the area of onset of blood lactate accumulation [100].

A similar approach using exercises of increasing intensities can be applied to field conditions. A simplified approach – which avoids the repetitive blood sampling – is used in the field conditions, e.g. to monitor the training-induced improvements: it includes the determination of the lactate concentrations at the end of a single standard fixed-pace exercise bout.

■ **Bicarbonate measurement.** The concentration of lactic acid is closely, and reciprocally, related to HCO_3-concentration. HCO_3-concentration decreases as a result of immediate buffering of hydrogen ions of lactic acid. Measurement of the decrease of blood HCO_3-concentration might, therefore, provide indirect information about blood lactate accumulation during exercise [101].

■ **Ventilatory threshold.** An indirect – and completely non-invasive – method to determine the transition (threshold) area is represented by analysis of gas exchange using indirect calorimetry. The exercise-induced lactic acidosis results in the consumption of HCO_3-ions for buffering the hydrogen ions (see here above) and, consequently, in the rise of CO_2 production. When measuring VCO_2 as a function of VO_2, then the exercise intensity at which the VCO_2 increase is accelerated corresponds to the area of onset of metabolic acidosis and, thus, of blood lactate accumulation (designated as "ventilatory threshold") [101]. Reproducibility of the ventilatory threshold determination – expressed as VO_2 – was shown to be of the order of $r = 0.95$ [100].

Table 8 Criteria for estimation of blood lactate (BL) accumulation

Example No	Blood lactate level (mmol/l)	Criteria and designation and protocol	Reference
1	4	Anaerobic threshold: VO2 or the work load associated with BL of 4 mmol/l Graded test with 3 min loads	[239] [240]
2	4	Onset of blood lactate accumulation Expressed in VO2, work load or corresponding heart rate. Graded test 4 min loads	[241]
3	3.5–5	Individual lactate threshold: The starting point of the increase in lactate accumulation expressed in VO2 or work load Graded test with 3 min loads	[100]
4	2–7	Maximal steady state of blood lactate level: maximal intensity during which the blood lactate remains stable Test with two 20 min submaximal intensities	[242]

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Methods for estimation of lactate concentrations in the muscle

Blood lactate is only the reflection of lactate production in the muscle and elimination in muscle and liver. The muscle lactate can be determined either by invasive muscle biopsy method or non-invasively using nuclear magnetic resonance (NMR) in vivo spectroscopy. The biopsies can be performed only after the exercise is stopped. NMR spectroscopy necessitates the fixed position of the exercising limb and, thus, can be used with exercises such as knee extension or specific forearm exercises. Indirectly, the information about the muscle lactate production can be obtained by measuring the muscle pH using selective electrodes inserted directly into the muscle or, again, using the NMR spectroscopy in vivo.

Specific study design aspects

■ **Role of sampling site and of exercise mode.** Blood lactate levels differ depending on the sampling site and results of several comparative studies are listed in Table 9. Arterial blood lactate is considered to be the golden standard. Sampling from hyperemised ear lobe or finger tip represents lactate levels in arterialised blood. The levels of lactate in venous blood are strongly variable according to the sampling site in relation to the working muscle. Thus, variations between the sites may vary according to the mode of exercise: they are different for bicycle ergometer, treadmill and arm-crank ergometer [102]. It is concluded that lower lactate levels are found in venous blood than in ear-capillary, finger-capillary or arterial blood when exercising on a bicycle ergometer, but e. g. for arm-crank ergometer the venous blood lactate is higher than the ear-capillary level [102].

■ **Subjects selection.** Subjects of any study of blood lactate accumulation should be well defined and standardised with respect to various conditions which were reported to affect the blood lactate response to exercise. The training status affects greatly the exercise intensity associated with blood lactate accumulation (review [96, 97]). Numerous studies have shown that the response of blood lactate is influenced by previous diet, mainly by the status of body glycogen stores [96, 103]. Age was reported not to influence the relationship between the on-set of blood lactate accumulation and performance [104] and does not affect the reproducibility of the threshold determination [100]. The results on the influence of sex on the latter relationship are not unequivocal [97].

Summary of lactate accumulation measurements

The blood lactate accumulation during exercise may be assessed by monitoring the evolution of blood lactate concentrations during exercise with increasing steps of intensities. The alternative is the measurement of blood lactate concentrations at the end of a single standard fixed-pace exercise bout which should be used for later comparisons within the same individual. The easiest approach is to measure the lactate concentration in arterialised blood sampled at the ear lobe or fingertip.

■ Oxygen transport

Definition, physiological background and relevance

Oxygen transport is usually understood as a process by which oxygen, after having been transferred from the alveoli into mixed venous blood in the pulmonary capillaries in exchange for carbon dioxide, is carried in systemic arterial blood to the tissues. Adequate oxygen transport is critical for the delivery of oxygen to the working muscle and, consequently, for aerobic metabolism of energy substrates needed for muscle work.

The amount of oxygen transported to the tissue is a product of the cardiac output (i. e. amount of blood pumped into circulation/per min) and the content of oxygen in arterial blood (Ca_{O_2} = systemic arterial oxygen content). The majority of blood oxygen is bound to the protein carrier haemoglobin, forming thus oxyhaemoglobin, and only a small part is dissolved in plasma. Consequently, oxygen blood content is strongly dependent on haemoglobin content in blood and, furthermore, on the degree of oxygen binding to haemoglobin (Sa_{O_2} = oxygen saturation of arterial Hb, equal to a ratio of the amount of haemoglobin bound to oxygen to the total amount of haemoglobin, expressed in %). Saturation of Hb increases, in a non-linear manner, with increasing partial pressure of oxygen in arterial blood (Pa_{O_2}). Furthermore, during the given levels of blood

Table 9 Comparison of lactate values from various sampling sites

Example No	Exercise type	Result	Reference
1	Discontinuous incremental cycling	E >, = V	[243, 244]
2	Continuous incremental cycling	A > V	[245, 246]
3	Continuous incremental cycling	F > V	[247]
4	Continuous incremental treadmill, cycling	Va >, < A	[248]

A arterial; *V* venous; *F* finger tips; *E* ear lobe; *Va* venous arterialised

Hb, oxygen affinity of Hb and, thus, oxygen saturation of Hb is modified by changes of blood temperature, blood acidity (pH), blood carbon dioxide content (pCO_2) and, also, by the content of the compound 2,3-diphospho-glycerate (2,3-DPG) in blood red cells. In addition, haemoglobin content in blood and, in general, erythropoesis depend on the adequate status of body iron stores and might be reduced upon long-term depletion, ultimately leading to anemias.

During exercise, there are increasing demands on oxygen transport to the working muscle. The increase in oxygen transport is realised through

- Increase in cardiac output
- Redistribution of blood flow towards working muscles
- Facilitation of the release of oxygen from oxyhaemoglobin in the muscle capillary bed due to exercise-induced increase in local temperature, partial pressure of CO_2 and, eventually, acidity.

The claims of improving oxygen transport and, consequently, improving maximum aerobic capacity, might refer to any of the above mentioned mechanisms: 1) to improvements in cardiac performance, i. e. cardiac output, 2) modifications of muscle blood flow, 3) modifications of content and features of oxygen carriers (haemoglobin/oxyhaemoglobin) in blood. As the first two points are treated in other sections of this paper the following remarks concern point 3. Numerous studies have shown the positive relationship between haemoglobin levels and individual aerobic capacity [105, 106].

Methods of evaluation of the variables related to blood oxygen transport

Blood haemoglobin concentration is measured with commercial devices using a spectrophotometric method and expressed in g of Hb per l litre of whole blood.

■ **Blood gases.** *In vitro.* The variables measured *in vitro* directly in samples of arterial blood are partial pressure of oxygen (Pa_{O_2} and blood oxygen saturation (Sa_{O_2}). Polarography methods using oxygen sensitive electrodes are used for Pa_{O_2} measurement, while Sa_{O_2} is either measured directly using spectrophotometric technique or calculated using Pa_{O_2} and acid-base variables. Alternatively, these variables can be measured in arterialised blood drawn from a hyperemised ear-lobe or finger tip.

■ **In vivo.** Sa_{O_2} can be measured in a non-invasive way using a commercially available transcutaneous pulse oximetry applied to the ear-lobe or to the finger tip. Pa_{O_2} and Sa_{O_2} may also be measured using intravasal catheterisation directly in the relevant vessels using intravasal fibroptic catheters with polarographic oxygen microsensors.

■ **Calculated parameters.** Arterial blood content (Ca_{O_2}) and blood oxygen capacity are useful parameters for evaluation of the amount of oxygen in blood and are calculated using the measured Pa_{O_2}, Sa_{O_2} and Hb values.

■ **Parameters evaluating status of body iron stores.** Determination of serum ferritin concentration (μg/l) and of saturation of serum transferrin (%) provide bases for this evaluation.

Subject selection and experimental conditions

At rest the variables mentioned above should be in the normal range for normal subjects. There is no convincing evidence suggesting that their values are regularly different in trained and sedentary populations [107], in spite of some studies finding lower indices of body iron status in trained subjects, especially females [108]. Hb concentrations are lower in females. The Hb concentration is affected by the hydration status of the subject and is markedly increased during exercise due to hemoconcentration.

■ **Size of effect.** A significant relationship between total Hb mass and maximum aerobic capacity and/or ventilatory threshold was shown in numerous studies (e. g. [105, 106]) in untrained and trained persons, numerous experiences with a positive effect of increased Hb with respect to performance are known from sports practice or have been reported [106]. When evaluating the inverse effect, i. e. reduction of Hb concentration, reduction of Hb by 15 % produced an 18 % reduction in leg oxygen uptake [108].

Summary of oxygen transport measurements

Blood haemoglobin concentration and haemoglobin oxygen saturation in arterialised blood are appropriate approaches for assessment of blood oxygen transport capacity. A non-invasive way of measurement using transcutaneous pulse oximetry applied to the ear-lobe or to the finger tip may be of great value in field conditions.

■ **Effect on skeletal muscle blood flow**

Definition, physiological background and relevance

Muscle blood is defined as a volume of blood passing through a given volume of muscle per unit of time and is expressed in units ml/g tissue/min or l/min depending on the mode of measurement.

Skeletal muscle blood flow (BF) is one of the essential mechanisms controlling the supply of oxygen and energy substrates to the muscle. Knowledge of the BF mag-

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nitude is, thus, of essential importance during the estimation of substrate fluxes and energy turnovers in the muscle. Skeletal muscle BF remains fairly constant at rest. Physical exercise promotes a linear increase in the skeletal muscle blood flow with exercise intensity [109–111] through several mechanisms: the main ones being the exercise-induced increase in cardiac output and exercise-induced peripheric vasodilation. Impairment of the capacity to increase blood flow during exercise severely limits exercise capacity. An extreme example of this situation is claudication, i. e. feeling of pain in the lower extremities during exercise, in patients with peripheral atherosclerotic disease of the lower extremities. On the other hand, an increase in the rest and/or exercise-induced BF might improve metabolic rates in the muscle and, thus, contribute to improvement of exercise performance.

Methods of blood flow measurement

When interpreting the results of BF measurements it is important to take into account a large heterogeneity of skeletal muscle blood flow (it can be demonstrated e. g. by differences in BF between the muscles containing predominantly red twitch fibres and those with the white ones). Furthermore it is important to realise that muscle blood flow oscillates at rest and even more during the exercise depending on the changes of blood pressure (and muscular contractions) and the values obtained during a given period of measurement are averaged values of BF.

The majority of blood flow to a limb perfuses skeletal muscle and skin. Some methods provide information of the regional blood flow in the whole limb (e. g. estimating arterial inflow and venous outflow) while others measure the local blood flow at the specific site of the muscle. An overview of the methods is provided in Table 10.

■ **Regional blood flow measurement.** Traditional methods are represented by those using *dye indicators* and measuring the dilution of the dye assuming that, during steady state, the dilution is related to BF. The practical realisation of the method in lower extremities represents a continuous infusion of the known concentration of the dye (e. g. indo-cyanine green = ICG) in the femoral artery or distal part of femoral vein, sampling the blood in the proximal part of the femoral vein and subsequent

Table 10 Methods for muscle blood flow measurement**

Method	Reproducibility* (CV%)	Precision	Advantages	Disadvantages	References
Regional blood flow					
ICG dye dilution		Good, invasive	Good temporal resolution No limitation by movement	Arterial/venous catheterisation Dye infusion	[249] [250]
Thermodilution	6%	Good, less invasive	No blood sampling Good temporal resolution No limitation by movement	Venous catheterisation Saline infusion	[110] [251] [109]
Ultrasound Doppler	6%	Good during rest, need of experienced investigator during exercise	No blood sampling Continuous measure High temporal resolution	Need of fixed vessel position	[252] [109]
Magnetic resonance velocity		Good for small muscle groups	No blood sampling Continuous measure High temporal resolution	Need of fixed vessel position High cost	[113] [253]
Local blood flow					
Laser Doppler		Difficult to interpret	No blood sampling	Intramuscular insertion Semiquantitative	[254]
Radionuclide clearance		Reasonable	No blood sampling Continuous measure Good spatial resolution	Radionuclide administration motion artefacts (reducible with portable detector) Imprecisions in clearance	[255] [111]
Positron emission tomography		Good, Repetitive measurements not feasible	Continuous measure Good temporal and spatial resolution	Need of fixed vessel position Radionuclide administration High cost	[256]
Near infra-red spectroscopy		Reasonable, invasive	Good temporal and spatial resolution No limitation by movement	Arterial/venous catheterisation Dye infusion	[257] [253]

* Assessment of reproducibility of the methods is very questionable due to the high biological variability of the muscle blood flow at rest and during the exercise
** We are indebted to Dr Jens Bulow for his valuable advice in the evaluation of this table

determination of the dye concentration in the blood sample. The method provides accurate values of BF for the intermittent periods of time at rest and during exercise regardless of the intensity of exercise, as the results are not influenced by the motion itself. However, the requirement of continuous infusion and repetitive blood sampling represent some limitations for its use outside the well-defined laboratory setting.

The same principle as that described above is used in the *thermodilution method*. Decrease of the blood temperature induced by the infusion of saline of the known temperature is measured using a thermistor inserted into a peripheral vessel. The method does not necessitate the sampling at the venous site and again, can be used – with the limitations mentioned above – even during intensive exercise.

Venous occlusive plethysmography is broadly used for measurements of resting blood flow. This method is not found to be appropriate for measurements during exercise.

A unique method for BF measurement is *Doppler ultrasound*. It measures the ultrasound frequency shift which is related to blood velocity and enables continuous blood flow measurements. Due to high temporal resolution it is able to detect rapid changes in flow during exercise. However, the site of interest must be in a fixed position during measurement which limits the use of the technique in the case of typical intensive dynamic exercise.

Magnetic resonance imaging (MRI) is a sophisticated method of measuring blood velocity together with the vessel cross section. The temporal and spatial resolution of the method is high, it enables the measurement of BF even in deep vessels, it is non-invasive, the main disadvantages being the motion artefacts and, of course, the high cost of equipment and running.

■ **Local blood flow measurement.** Blood flow at the specific site of the limb/muscle is determined. Traditional methods are represented by radionuclide clearance techniques. They are based on the measurement of the wash-out of a substance labelled with radionuclide and administrated in the region of interest. The theoretical base is given by the assumption that the clearance of a molecule injected into the specific site of the muscle is dependent solely on the local blood flow (if no significant recirculation takes place). The decline of radioactivity is measured with an external detector above the site of radionuclide administration. The method is subject to numerous presumptions concerning the post-injection distribution of the radionuclide in the muscle and other tissues present in the muscle (connective tissue, fat). The method has been limited by motion artefacts. A commercially available portable detector, recently introduced, might resolve this problem.

The semiquantitative relative changes of the local blood flow can be estimated by *microdialysis technique using ethanol wash-out*. The method consists of detection of substance flow across a semi-permeable membrane of a probe inserted into the muscle. It is used primarily for studies of muscle metabolism. If ethanol is perfused through the probe, the difference between its concentration in the inflow perfusate and outflow dialysate is assumed to be proportional to the local blood flow. The method gives information about the changes of BF but cannot determine its absolute magnitudes. While the method has been used for evaluation of the blood flow in adipose tissue during exercise, the use in muscle is difficult due to danger of damage or breaking of the probe during muscle contractions.

The *laser Doppler* method is based on the Doppler principle (see Doppler utrasound above) applied to laser light. The optic fibre inserted into the muscle serves as a source of light and detector of the Doppler frequency shift. The blood velocity is determined as a resulting value while the absolute value of BF cannot be determined. The use of the technique during exercise is problematic due to movement artefacts.

Near infrared spectroscopy is an extension of the dye dilution methods and seems to be a promising technique. Using infrared optotes it measures the decline of the dye concentration in the muscle and requires the dye infusion and arterial blood sampling.

A highly sophisticated method is represented by *positron emission tomography (PET)*. It gives information on the local distribution of radionuclide-labelled substances, the distribution being flow dependent. The absolute values of local muscle BF are calculated from the local radioactivity detected by the positron camera in the tomographic mode and arterial concentrations of the labelled substance. Nevertheless, if used for exercise studies it necessitates a stable position of the measured muscle and, thus, could be used for exercise of the type of knee extension. In addition, the PET device and the running costs of the method are extremely expensive.

Methods of choice

The description of the methods given above clearly demonstrates that numerous methods are available for BF measurements at rest but, during exercise, many of them are limited by motion artefacts (plethysmography, PET, MRI).

During onset of exercise and exercises with fixed position of the vessel, i.e. the limb, Doppler ultrasound seems to be ideal, due to its high temporal resolution and, hence, capacity to detect precisely quick dynamic changes of blood flow [109, 112, 113]. During the peak, i.e. intensive exercise, the ICG dye method and/or thermodilution method seem to be most appropriate – as they are not influenced by motion artefacts – in spite of the severe limitation given by the necessity of intra-ves-

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sel infusion and, eventually, blood sampling (not necessary with thermodilution). MRI and PET methods offer unique possibilities for measuring the deep single vessel BF and, respectively, local distribution of BF, their main limitation being the high cost of equipment and running. Methods using the radionuclide (mostly ^{133}Xe) clearance are still widely used for quantitative assessment: their diagnostic value, limited by motion artefacts, might be improved with portable detectors fixed on the moving limb. A number of necessary theoretical assumptions are other limitations of the latter method.

Subject selection and experimental conditions

There exists a large inter-individual and intra-individual variability of the blood flow. Hence the size of sample population should be sufficiently large. The choice of subjects with respect to their training status might be important as training influences the blood flow response to metabolic interventions. In addition, the response of the blood flow might be substantially affected by external conditions and subject's condition (heat stress, hydration, previous exercise) [114] and, consequently, the standardisation of these parameters is essential for a correct interpretation of data.

Exercise elicits an important rise in BF in skeletal muscle, the range being from two to five-fold increase for exercise of low intensity up to the 25-fold increase for high intensity exercise [109–112]. Metabolic interventions, e. g. hyperinsulinemia, elicit a 1.5- to 2-fold increase in the limb BF and this change might be already clinically relevant as it is associated with an increase in substrate (glucose) extraction [115].

Summary of muscle blood flow methods

During the intensive exercise, the ICG dye method and/or the thermodilution method seem to be the most appropriate in spite of the severe limitation given by the necessity of intra-vessel infusion and, eventually, blood sampling. The Doppler ultrasound method may be valuable during the onset of exercise if performed by an experienced investigator.

■ Gastro-intestinal distress

Measuring gastro-intestinal distress

Gastrointestinal discomfort is a common complaint amongst endurance athletes with 40–90 % of athletes reporting such problems [45, 116]. Gastro-intestinal distress is usually measured using questionnaires. Although there is no commonly accepted standard, the existing questionnaires all seem very similar. Subjects are asked to rate a number of complaints including de-

gree of fullness, stomach pains, regurgitation, intestinal cramps, flatulence, diarrhoea/loose stools. Often these complaints are ranked in different categories: severe and non-severe or upper and lower gastro-intestinal tract problems.

Unfortunately it seems difficult to provoke the same complaints observed in field events in controlled laboratory studies. Therefore it is difficult to perform well-controlled studies of gastro-intestinal distress during exercise. However, from the literature it seems clear that the food intake 3 days prior to the test should be standardised because this may also impact upon the observed complaints.

Hydration status: markers of hydration status and assessment of rehydration efficacy

■ Claims

- Improved hydration status
- Faster rehydration

■ Definition and relevance

When the body is fluid replete, it is said to be in a state of euhydration, but there are no good markers of euhydration. Water loss leads to a state of hypohydration, which can be redressed by fluid intake. Where fluid losses are large and time for replacement is short, as occurs during exercise, rapid and effective rehydration is important for the maintenance of exercise performance.

Although the endpoint is improved exercise performance, better hydration status, faster rehydration during exercise, more complete rehydration after exercise, and better maintenance of physiological homeostasis as evidenced by measures of cardiovascular and thermoregulatory function and subjective perception of effort are all important parts of this process. These physiological measures are covered in the sections on Carbohydrate replacement and Endurance performance and will not be discussed in detail here.

■ Markers of hydration status

There is no good marker of absolute hydration status, but changes in hydration status can be assessed. Loss of fluid leads to a decrease in body mass, an increase in the osmolality of body fluids, and an increase in the osmolality and sodium concentration of plasma. A hypohydrated individual, in an attempt to conserve water, will produce small volumes of urine, and the solute load will be contained within a small volume of urine with a high osmolality. These changes provide opportunities for the

assessment of hydration status. Body mass is a useful and sensitive measure of acute fluid loss, but lacks value in assessing changes in body water content over a longer time scale [117]. For many of the other putative markers, the individual variability precludes the use of absolute values to indicate hydration status, but changes in hydration status may be identified if a baseline value has been established. For many of the intermediate markers that have been proposed, the precision of the measurement itself is good. This means that repeat measures made close together or repeated measurements on the same blood or urine sample will show little variability. If, however, measurements are separated by a period of days or weeks, the large biological variability will mean that reproducibility is poor. For example, the osmolality of a urine sample can be measured with a coefficient of variation of less than 2%, but morning urine samples collected on successive days typically show a variability of about 50–100% [117].

Urine markers of hydration status

It is apparent that urinary measures of colour, specific gravity and osmolality are more sensitive at indicating moderate levels of hypohydration than are blood measurements of haematocrit and serum osmolality and sodium concentration [118]. Collection of urine has the further advantage of being a largely non-invasive, distress-free technique for all individuals whereas blood collection can prove stressful for some individuals. Urine indices of hydration status may be of limited use in identifying changes in hydration status during periods of rapid body water turnover [119]. Urine osmolality rather than specific gravity should be the preferred index of urine concentration, as specific gravity is influenced by solutes such as urea, glucose and protein [120]. The precision of measurement of urine osmolality is good, with a coefficient of variation that is typically 1–2%, but the association between osmolality and hydration status is affected by many factors that are specific to the individual. It has recently been demonstrated that the osmolality of the first urine of the day to be excreted, collected before ingestion of food or drink, varies according to hydration status [117] such that hypohydrated individuals produce urine with a greater osmolality. Specific gravity and conductivity measures have been shown to give similar results [121]. A urine osmolality of more than about 900 mosmol kg^{-1} can reasonably be taken as indication of a hypohydrated state [117], but at present there is no "gold standard" measure of hydration status and indeed debate still arises as to how a state of euhydration should be defined and determined.

Monitoring the volume of urine excreted and observing the frequency of urination have been suggested to athletes as useful tools for self-monitoring of hydration status. This is particularly useful around times of changes in lifestyle such as moving to warm weather venues for training camps etc. when comparisons can be made to the individuals' normal pattern. Athletes have also been advised to take note of their urine colour every day and use it as an indicator of their hydration status. Urine colour can, however, be influenced by a number of factors unrelated to hydration status, including foods, medications and illness. Urine colour, estimated from a colour chart, has been demonstrated to provide a reasonable "in the field estimate" of hydration status in association with exercise [125] when compared to measures of urine osmolality and specific gravity.

Blood indices of hydration status

Haemoglobin concentration and haematocrit, serum osmolality and sodium concentration and the concentrations of blood borne protein and hormones including testosterone, adrenaline, noradrenaline, cortisol, ANP have been investigated in relation to hydration status. Measurements of haematocrit or haemoglobin concentration are possible indices of hydration status, provided that a reliable baseline is established. However, to ensure comparable results, a standardisation of posture for a short time (15–20 min) prior to blood collection is necessary to distinguish between the postural changes in blood volume, and therefore in haematological indices, which occur [122, 123]. Also, acute exercise, such as endurance running, has been shown to cause an immediate fall in the calculated plasma volume followed by an expansion which is maintained for at least 72 hours [124]. This will cause problems in the interpretation of data from athletes training on a daily basis.

Francesconi and his colleagues [118] investigated military personnel during field training over a period of 44 days and reported that even when the subjects had lost more than 3% of their body mass and had a high urine specific gravity, there was no change in haematocrit or serum osmolality measurements. The authors concluded that plasma volume is defended by the body in an attempt to maintain cardiovascular stability, and therefore plasma variables are not affected by hypohydration until a certain degree of body water loss (which must be more than 3% of body mass loss) occurs. Similar findings were again reported by Armstrong et al. [125] who reached the same conclusion. This is far more than the level of dehydration that will have a negative impact on exercise performance, so it may be concluded that this measure is too insensitive to be useful in an exercise context.

Hoffman et al. [122] reported that hypohydration to the extent of a body mass loss of up to 5.1% did not influence plasma testosterone, cortisol or adrenaline concentrations but plasma noradrenaline concentrations responded to the hydration changes. This, the authors

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suggest, makes plasma noradrenaline concentration a possible contender as a sensitive marker of exercise-heat stress, but the sensitivity and reproducibility are not defined.

Bioelectrical impedance analysis

Bioelectrical impedance analysis (BIA) can give an estimate of total body water, and its cellular divisions if a multi-frequency device is utilised. Changes in hydration status in an exercise situation may be detected if the procedure is carefully standardised, but the precision and sensitivity of the method remain to be established [126]. The posture of a subject when BIA measurements are made and the time for which they have been in that posture, and prior exercise may all influence the results obtained, as may food or drink consumption before making measurements [127].

Pulse rate and blood pressure

Alterations in the response of pulse rate and systolic blood pressure to postural change have been demonstrated in clinical settings of dehydration and rehydration [128]. However, at present there seems to be no record of their use in an exercise situation and indeed it is probable that they may not be sensitive enough to be of value in association with exercise-induced dehydration.

Summary of the hydration markers

A wide variety of indices have been investigated to establish their effectiveness as markers of hydration status in an exercise situation. The choice of marker will be influenced by the sensitivity and accuracy with which hydration status needs to be established, together with the technical and time requirements and expense involved. Furthermore, many of the measures outlined above are influenced in the short term by exercise per se, and may only be successfully implemented for the identification of dehydration some time after the cessation of exercise. In Table 11 an overview of the different markers is given.

■ How to assess rates of rehydration

In most exercise situations, the duration is limited and ingested fluids should therefore be available with little delay if they are to be effective. There is unlikely to be any benefit from ingestion of fluid, which remains in the gastro-intestinal tract at the end of exercise. Ingested fluids remain temporarily in the stomach before being emptied into the small intestine, which is the site of absorption of most of the water and nutrients ingested. Only after the processes of gastric emptying and intestinal absorption have occurred are ingested water and nutrients available to the body, so the extent and rate of these processes will determine the availability of in-

Table 11 Markers of hydration status

Method	Reproducibility	Precision	Advantages	Disadvantages	Reference
Body mass	Good	Poor	Simple, inexpensive Useful for acute hypohydration	Lacks precision Not meaningful as an absolute assessment	[117]
Bioimpedance analysis	Moderate	Moderate	Easy to measure	Lacks precision Does not give an absolute measure of hydration	[128]
Urine osmolality	Poor, unless collection conditions are standardised	Precision of measurement is good, but physiological variability is high	Easy to measure if collected under controlled conditions, Gives a good index of hydration status	Not absolute; baseline must be established; depends on diet and other factors	[117]
Urine specific gravity	Poor, unless collection conditions are standardised	Precision of measurement is good, but physiological variability is high	Easy to measure if collected under controlled conditions Gives a good index of hydration status	Not absolute; baseline must be established; depends on diet and other factors	[120]
Hb/Hct	Good	Poor	Inexpensive Gives specific information on acute changes in blood and plasma volume	Requires blood sampling; baseline must be established	[258]
Hormone markers	Moderate	Moderate	Responsive to changes in hydration status	Not specific Highly variable	[122]
Pulse rate/blood pressure	Poor	Poor	Easily measured	Non-specific Too much influenced by other factors to be useful	[128]

gested water and solutes and hence the speed and effectiveness of rehydration. The composition of the ingested fluid will influence the rates at which these physiological processes occur, and thus will determine, at least in part, its efficacy.

Gastric emptying rate

A number of methods are available to assess the rate of gastric emptying, and these may be applied at rest or during exercise. There are many reports in the literature of factors influencing the rate of gastric emptying of liquid and solid meals, and many of these investigations have produced conflicting results. This is due, at least in part, to the different techniques that have been used to measure gastric emptying, so it is important to consider the different techniques involved. Most studies on emptying rates of liquids have relied on gastric aspiration techniques in which the stomach contents are recovered at a fixed time interval after ingestion of the test drink. This method assumes a linear rate of emptying. However, use of scintigraphic techniques, in which the movement of a non-absorbable radioactive tracer added to the drink can be followed by obtaining an image of the test drink, shows that emptying follows an exponential time course [129]: repeated sampling gastric aspiration techniques provide more direct evidence confirming these observations [130]. Comparison of aspiration and scintigraphic techniques by making simultaneous measurements with both, shows that the two methods give similar results [131].

Method of choice

The method of choice for assessment of the gastric emptying rate of liquids is undoubtedly the double sampling aspiration technique. This method is quantitative and reproducible, and can, in spite of the relatively large reported variability (see Table 12) reliably detect small differences in gastric emptying rates using realistic subject numbers [132, 133]. Because serial measurements are obtained, the use of repeated measures statistical analysis, with time as one factor, improves the sensitivity of the method. The single time point aspiration method may give a false picture as it assumes a linear rate of emptying over the measurement period used. Because the emptying pattern is exponential rather than linear, interpretation of the results is strongly influenced by the time at which measurements are made. Where sampling at a single time point has been used, different investigators have chosen different intervals in the range 10–60 min between drink ingestion and sampling, making comparisons between drinks and between studies difficult. Epigastric impedance and scintigraphy both have the advantage of being non-invasive, but the former method is confounded when there are differences in the amount of gastric secretion provoked by different test drinks, and the latter method involves exposure of the subject to ionising radiation.

Experimental design and subject selection

■ **Size of effect.** The rate of gastric emptying of any given solution and the absorptive capacity of the intestine vary greatly between individuals, so comparisons between studies and between laboratories are difficult, but within-subject repeatability allows detection of differences in emptying rates to be identified.

■ **Subject selection.** The measured rates of gastric emptying and intestinal absorption vary greatly between individuals, so the sample population should be sufficiently large to reflect this inherent variability. The biological basis of this variability is not known, but gender, body size, training status, habitual diet, nutritional status and other factors are all likely to be involved. Based on the data of Vist (personal communication 1994) there is a 90 % probability that a 25 % difference in emptying rate can be detected if eight subjects are used. This variance seems high, but it includes the large inter-individual variability as well as the intra-individual variability introduced by differences in prior diet, activity and other factors.

Table 12 Gastric emptying methodology

Method	Reproducibility (CV%)	Precision	Advantages	Disadvantages	Reference
Double sampling aspiration method	29%: [261] 31%: [262]	Good	Quantitative	Limited to subjects who tolerate intubation	[261]
Single timepoint aspiration method	Unknown	Poor	Limited intubation time	Pattern of GE not seen	[262]
Scintigraphy	31%: [265] 19% [266]	Good	Acceptable to subjects Use of small volumes possible	Radioactive tracer used Takes no account of secretion or reflux Not quantitative Underestimates GE	[265]
Epigastric impedance	Unknown	Good	Non-invasive	Not quantitative	[266]

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■ **Experimental design.** Both gastric emptying and intestinal absorption are affected by a variety of stresses, including exercise. Values obtained from resting subjects may therefore differ from those obtained during strenuous exercise [134] or during intermittent exercise that includes short periods of high intensity exercise [135]. It does appear, however, that the rank order of emptying when different solutions are compared is the same at rest and during exercise. Given the large interindividual variability in emptying and absorption rates and the apparent differences between laboratories, it may not matter much whether studies are conducted at rest or during exercise.

Intestinal fluid absorption

Water absorption occurs largely in the proximal segment of the small intestine, and although water movement is itself a passive process driven by local osmotic gradients, it is closely linked to the active transport of solute. The method of choice for measurement of intestinal absorption of water involves placement of a triple lumen tube in the region of interest. The test solution, containing a non-absorbable marker, is perfused at a fixed rate within the physiological range for gastric emptying (usually between 5 and 15 ml/min): a sample of the intestinal contents is aspirated via the second tube from a point 10–20 cm distal to the perfusion port, and the change in composition gives a measure of the effects of mixing of the test solution with the endogenous secretions. Aspiration via the third tube from a site a further 20–60 cm along the intestine allows net exchange of solute and water in the test segment to be calculated: in the test segment, steady-state conditions should be established. The method was described in detail by Leiper and Maughan [129].

This technique allows reliable measures to be made of the net flux of water and solutes in a well-defined region of the gut. Although the technique is reliable and reproducible in itself, there are some important limitations. Because the test solutions are added directly to the jejunum, the role of the stomach in moderating the delivery rate and in modifying the composition of ingested fluids is ignored. A constant perfusion rate is normally used in perfusion studies, and this may represent an unphysiological situation. A more recent modification to the technique involves repeated ingestion of the test drinks orally in an attempt to maintain a constant rate of gastric emptying, with simultaneous aspiration from three or four sampling sites located in the small intestine. The effects of solute secretion may be important, and there is evidence that, when electrolyte free solutions are ingested orally, there is a rapid secretion of sodium such that equilibrium is rapidly reached [136, 137]: if this is indeed the case, it confounds the evidence available from the perfusion method that indicates an increased water uptake when sodium containing fluids are perfused [129]. The technique also looks only at a small part of the whole intestinal surface that is available for absorption in the intact individual: thus, concentrated solutions stimulate water secretion in the upper part of the intestine, but absorption will still occur in the distal regions.

A number of animal models are available, involving either isolated perfused gut segments, inverted gut sacs, or in situ perfusion models. Each of these has advantages for studying the physiological processes involved in water and solute transport, but these methods have limited relevance to the intact human intestine.

Method of choice

The method of choice for estimation of water uptake in the human intestine is likely to be the triple lumen segmental perfusion model, used without an occlusive balloon (Table 13). The major limitation to this method is that the data obtained are limited to the section of gut that is being perfused. Thus net secretion may occur in the perfused segment, but net absorption occurs because of absorption lower down the gut. Some differences are seen between absorption rates as measured in the duodenum and the jejunum [138, Leiper, unpublished data], but the rank order is generally the same when similar solutions are compared.

Table 13 Intestinal absorption methodology

Method	Reproducibility (CV%)	Precision	Advantages	Disadvantages	Reference
Triple lumen perfusion	17%: [142]	Good	Quantitative	Limited to subjects who tolerate intubation Test solutions are contaminated by secretions Data apply only to a limited segment of the gut Radiation exposure	[267]
Occlusive balloon perfusion	Unknown	Good	Quantitative	Limited to subjects who tolerate intubation Balloon may cause discomfort Data apply only to a limited segment of the gut Radiation exposure	[267]
Whole gut perfusion	Unknown	Unknown	Quantitative	Subject acceptability Non-physiological?	[268]

The considerations that apply to the use of the intestinal perfusion method for assessment of the rehydration potential of different drink formulations are similar to those associated with measurement of gastric emptying.

Experimental design and subject selection

■ **Size of effect.** The rate of gastric emptying of any given solution and the absorptive capacity of the intestine vary greatly between individuals, so comparisons between studies and between laboratories are difficult, but within-subject repeatability allows detection of differences in emptying rates to be identified. Given the negative effect on exercise performance of even low levels of dehydration (less than 2 % of body mass [139]) and the small amount of fluid (less than 400 ml) typically ingested during exercise [140], differences in absorption of a few ml per cm of gut per hour may well be meaningful in terms of fluid replacement.

■ **Subject selection.** The measured rates of gastric emptying and intestinal absorption vary greatly between individuals, so the sample population should be sufficiently large to reflect this inherent variability. The precision of measurement of the markers used to assess absorption is good (less than 2 %), but the biological variability is high. Based on the data of Leiper and Maughan [141] there is a 90 % probability that a 50 % difference in the rate of water and solute absorption can be detected if eight subjects are used. This estimate is based on repeat measurements made on 15 subjects at intervals of up to 18 months, so a large part of the variability is inherent physiological variability rather than analytical imprecision.

■ **Experimental design.** Intestinal absorption is affected by a variety of stresses, including exercise. Values obtained from resting subjects may therefore differ from those obtained during strenuous exercise [134]. A limited number of perfusion studies have been carried out during exercise, but it does appear that the rank order of water absorption when different solutions are compared is the same at rest and during exercise. Given the large inter-individual variability in absorption rates and the apparent differences between laboratories, it may not much matter whether studies are conducted at rest or during exercise.

Tracer methodology

Recognising that the combined effects of solute content on both gastric emptying and intestinal absorption are more important than the separate effects on each of these processes, a number of studies have used tracer methodologies to assess the net effect on fluid availability. The tracer of choice is deuterium oxide, and the rate of accumulation of the deuterium tracer in the circulation gives a measure of the availability of ingested fluids. The deuterium concentration may be measured using infrared spectroscopy or mass spectrometry: mass spectrometry gives a sensitivity of less than 1 ppm and a cv of less than 1 % at an enrichment level of about 80 ppm [142], whereas infrared spectroscopy has a sensitivity of 10 ppm and a cv of less than 4 % at an enrichment level of 290 ppm [Leiper, unpublished results]. The somewhat lower analytical sensitivity and precision of infrared spectroscopy can be overcome by the use of a larger tracer dose and by repeated measurements.

There are, of course, limitations to the use of this method, and quantitative data cannot be obtained as the exchange of water between the intestinal lumen and the body water compartment is a bi-directional process. The technique has been shown to be reproducible and to give results that are consistent with the known effects of solute content on the rates of gastric emptying and intestinal absorption (Table 14). Rehrer et al. [143] found a reasonably good relationship ($r = 0.63, p < 0.01$) between the rate of gastric emptying (as measured by a double sampling aspiration method) and the rate of accumulation of an added deuterium tracer in the circulation.

Post-exercise rehydration

Restoration of fluid and electrolyte balance is a key aspect of recovery after any exercise that has resulted in a significant amount of sweat loss. Because there is no agreed absolute measure of hydration status, most investigations have focused on the efficacy of various formulations in returning subjects to their pre-exercise hydration status. For this to be meaningful, it is important that the pre-exercise status should be standardised as far as possible. This involves control of diet and exercise in the pre-exercise period, and a standardisation period of 48 hours is probably necessary: trials should be carried out at intervals of one week, allowing the subject to be studied on the same day of the week on each trial. Stan-

Table 14 Tracer methodology

Method	Reproducibility (CV%)	Precision	Advantages	Disadvantages	Reference
Deuterium accumulation in the circulation	21 % [271]	Good precision of measurement, but large biological variability	Integrates effects of gastric emptying and intestinal absorption	Not quantitative	[270]

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dardisation of the initial hydration status is better achieved if subjects are measured in the morning rather than later in the day, and euhydration is more likely to be achieved if subjects ingest a standardised volume of fluid (perhaps 500 ml) 1–3 hours before reporting to the laboratory.

Any combination of exercise, heat and humidity can be used to induce a standardised level of hypohydration, and the effects of administration of a fixed volume of fluid on whole body water balance (as assessed by urine loss) and body water compartments (as assessed by plasma volume) can be measured over the subsequent few hours. An alternative model, which takes account of the effects of drink palatability on volitional intake, allows subjects free access to the test drinks for a fixed period of time. A recovery period of at least four and preferably six hours after the end of the ingestion period is necessary for a steady state, as indicated by a return of urine output to basal levels, to be achieved.

There is only one published study where the reproducibility of these measures has been reported. Maughan et al. [144] required eight subjects to complete three trials, on two of which the same fluid was administered in the same volume after exercise-induced dehydration equivalent to 2.1 (SEM 0.0)% of initial body mass. Subjects then ingested, over a 60 minute period, a volume of fluid (a commercially available sports drink) in a volume equal to 1.5 times the body mass loss. Median (range) cumulative urine output over the subsequent 6 hours amounted to 934 (550–1403) ml on one trial and to 954 (474–1501) ml on the other trial. Other variables (plasma volume change, plasma and urine electrolyte concentrations and osmolality) were similar on both of these trials. In contrast, where food and fluid were given (such that the total water intake was the same, but the solute content was higher) a lower cumulative urine output (665 (3096–1190) ml, p < 0.01) was observed.

These methods have been used to show that the efficacy of restoration of fluid balance after exercise is achieved only if sweat electrolyte losses are replaced. In a study involving 6 subjects where the sodium concentration of drinks was varied systematically [145], the mean difference, relative to a trial where a sodium-free drink was given, in whole body fluid balance at six hours after the end of the exercise period was 330 ml when the sodium concentration was 26 mmol/l, 687 ml when the sodium concentration was 52 mmol/l, and 787 ml when the sodium concentration was 100 mmol/l. These differences are substantial when compared with the volume of fluid ingested commonly during exercise, and are physiologically relevant as evidenced by a better restoration of exercise capacity [Shirreffs and Maughan, personal communication].

■ Summary of hydration status and rehydration efficacy measurement

Physical activity, especially in warm environments, is associated with an increased fluid loss from the body as sweating is invoked to limit the rise in body temperature. A water loss of more than 1–2 % of body mass impairs exercise performance and has adverse subjective effects. Fluid intake can offset the negative consequences of dehydration and can improve exercise performance. Hydration status is difficult to define because of the absence of any clear marker. Urine measures (colour, specific gravity, osmolality) are generally better than blood or other markers. Fluids aimed at replacing losses usually include carbohydrate as an energy source and to stimulate intestinal water transport, but high carbohydrate concentrations slow the gastric emptying rate. Gastric emptying is relatively easy to measure, and aspiration techniques are most reliable. Intestinal water and solute exchange can be measured in defined segments of the gut by multi-lumen perfusion methods. Each of these techniques measures part of the rehydration process, and addition of isotopic tracers for water may give a better estimate of the availability of ingested fluids. Most sports drinks are ingested after exercise to promote rehydration and recovery, and efficacy of these drinks at restoring fluid balance in the hours after exercise can be calculated by assessing intake and all routes of loss.

Flexibility

■ Claim

- Increased flexibility

■ Definition, physiological background and relevance

Flexibility is defined as bending ability, which is determined by joint mobility and muscle elasticity. Poor flexibility in joints may cause musculo-skeletal problems because of increased bending stress. It is not possible to assess flexibility by a single test [146]; in contrast, all tests presented below are site specific. It should also be noted that extreme flexibility should never be a goal for health-related fitness.

■ Methods of measurement

Side-bending of the trunk measures the extent of lateral flexion of the thoracic and lumbar spine [146, 147]. Trunk-side bending seems to be associated with poor perceived health and back dysfunction [147, 148]. The

inter-rater reliability and test-retest reproducibility of side-bending are good [149, 150]. According to Suni et al. [150], the coefficient of variation (CV) for test-retest reproducibility is 4.7 %.

Active knee extension range of motion (ROM) is a method to assess hamstring muscle extensibility [151]. The results (endpoint ROM angle in degrees) are measured by an inclinometer. The sit-and-reach test evaluates trunk flexibility and hamstring "tightness" [146]. Results are expressed in cm. It is believed that poor results of hamstring muscle extensibility are associated with back problems, but this opinion is controversial [146, 151].

Shoulder-neck mobility measures functional mobility restrictions of the shoulder-neck region [150]. The results are scored using an ordinal scale (0, 1 or 2). The test is used more as a screening test, because the 3-point scale limits the use of the test in the assessment of change [150].

All of the above mentioned tests are graphically described in, e. g. Oja and Tuxworth [146], Suni et al. [150] and [147]. The safety and feasibility of the shoulder-neck mobility, side-bending and active knee extension range of motion is good, since more than 90 % of subjects aged 52–57 years had no contraindications for the tests [151].

■ Summary of the flexibility measurements

All flexibility tests are site specific. Side-bending of the trunk measures the extent of lateral flexion of the thoracic and lumbar spine. The inter-rater reliability and test-retest reproducibility of side-bending are good. Active knee extension range of motion (ROM) is a method to assess hamstring muscle extensibility. The sit-and-reach test evaluates trunk flexibility and hamstring "tightness". Shoulder-neck mobility measures functional mobility restrictions of the shoulder-neck region This test is used more as a screening test, because its 3-point scale limits the use of the test in the assessment of change. The safety and feasibility of the shoulder-neck mobility, side-bending and active knee extension range of motion is good.

Tissue growth

■ Claims

- Increased protein synthesis
- Increased protein mass
- Increased muscle mass
- Increased fat-free mass
- Reduced fat mass or body fat percentage
- Reduced intra-abdominal (or trunk) fat mass

■ Definition, background and relevance

The human body can be organised into components using several different levels of organisation with increasing complexity [152]. In the context of this report, the most important organisation levels are tissue level (the body is divided into adipose tissue, skeletal muscle, organs, skeleton and the remaining tissues) and molecular level (lipids, water, proteins, glycogen, minerals). Most methods to assess body composition are based on the latter approach. It should be noted that lipids in the molecular level organisation include both intracellular and adipocyte lipids, whereas adipose tissue in the tissue level organisation includes only lipids in the adipose tissue.

Protein, muscle and fat-free mass may be important in sports events where absolute strength and power are important for performance. Maximal strength is partly determined by the cross-sectional area of the muscle, partly by nerve-muscle interaction. In contrast, light body weight and low fat mass (or low fat percentage) may give an advantage in disciplines where the body has to be raised against gravity (e. g. running, jumping for height or length) or where a lean body is regarded as an aesthetic necessity (e. g. ballet, gymnastics). However, it should be noted that the relations between body composition and performance are not linear and that elite athletes may differ considerably as regards body composition.

■ Methods of measurement

Protein synthesis

The traditional nitrogen balance technique has nowadays been largely replaced by stabile isotope tracers in assessment of protein turnover, synthesis and catabolism [153]. The clear advantage of the stabile isotopes is that synthesis and catabolism can be separated, whereas the nitrogen balance method only indicates the difference between synthesis and catabolism. On the other hand, stable isotope techniques are influenced by the chosen tracers and the assumptions used to estimate whole body metabolism from the metabolism of a single tracer.

Whole body protein metabolism is studied by using [13C]-leucine, [15N]-glycine, [15N]-lysine or [2H5]-phenylalanine as a tracer [153]. The principle of analysing stable isotopes is based on mass spectrometer which enables separation of neutral molecules according to their mass and charge. The stable isotopes can be incorporated either as a continuous infusion or as a flooding dose. The latter technique is faster and technically easier, but it seems that the continuous infusion gives more valid data [154].

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Two main types of mass spectrometers are used in stable isotope analyses, namely the isotope-ratio mass spectrometer (IRMS) and the organic mass spectrometer in combination with a chromatographic separation device (GC-MS). The precision (CV %) is ±0.0001 and ±0.5 for IRMS and GC-MS, respectively [153].

Body composition

Excluding carcass analysis, body composition can not be measured directly. All methods used are based on a similar functional relationship

$$C = f(Q) \tag{1}$$

where C is an unknown body component and Q is a measurable quantity. Q may be a physical, chemical or a biological property, or a known body component (derived from a property based method). The mathematical functions (f) can be divided in two classes (Table 15): the mechanistic or biological models have a functional relation to a known component. The relationships are based on data from (chemical) analyses. The descriptive mod-

els are all based on statistically derived regression equations (prediction equations) against a mechanistic reference method, using a well-characterised subject group.

Both the measurement of a property and the equation used may lead to systematic bias and/or random errors. A systematic bias shifts the mean value of a population. For instance, a change of the measurer may lead to a systematic over- or underestimation of the measured property. A similar systematic shift is usually the consequence of a change of the equation or method used. A random error does not affect the population mean, but it increases the variance and range of results. Poor measurement technique with low reliability is one source of random error. Since all equations are based on either biological assumptions or regression equations, the standard error of estimate (SEE) in the equation also leads to random errors. Since the descriptive models are based on regressions against mechanistic models, both the random errors in the latter and the SEE in the regression itself affect the magnitude of random error. Consequently, the random error in the descriptive meth-

Table 15 Classification and characteristics of human body composition methods (modified from van Marken Lichtenbelt and Fogelholm [157])

Method	Measured quantity (property)	Reproducibility (CV%)	Precision	Advantages	Disadvantages	References
Underwater weighing (2-compartment model)	Body weight under water, residual lung volume	2–4 %	Good	Accurate, if the basic assumptions are met	Time consuming Composition of FFM and measurement of RV affect the results	[156, 160]
Multicompartment models	See: underwater weighing, DXA and dilution techniques		Good	Perhaps the most accurate technique	Time consuming Expensive	[156]
Dual-energy X-ray absorptiometry (DXA)	Attenuation of low- and high-energy X-ray beams	< 2 %	Good	Easy for the subject, good reproducibility	Choice of device and software version affect the results Expensive device	[168, 163]
Dilution techniques	Concentration of the tracer in body fluids (e. g. urine)	1 %	Good	Easy for the subject, good reproducibility	Affected by hydration status	[165, 166]
Urine excretion of protein metabolites	24-h excretion of creatine or 3-methylhistidine in urine		Poor	Easy for the subject	Several other factors (diet, muscle trauma, etc.) have an effect on urine excretion	[170]
Anthropometry, including skinfolds	Thickness of subcutaneous fatfolds, circumferences, etc.	ca. 3 %	Moderate	Inexpensive Fast to measure	Fat distribution affects the results Variation between measurers (technicians) is potentially large	[161, 176]
Bioimpedance (BIA)	Electrical resistance	2–3 %	Moderate	Fast to measure Quite inexpensive	Hydration status and choice of the regression affect the results	[178, 180]
Near-infrared interactance	Interactance of two wavelengths	2 %	Moderate	Fast to measure	Choice of regression (e. g. inclusion of physical activity) has a large effect on the results	[156, 184]

FFM fat free mass; *RV* residual volume

ods is typically double compared to the mechanistic models.

The biases and errors of the body composition methods lead to the following concerns:

- Results from different methods or equations may be systematically different.
- Results obtained in different laboratories or by different measurers may be systematically different.
- Poor measurement techniques lead to increased random errors and enlarged population variance, which may decrease the statistical power and lead to false negative findings.
- The random errors of the equations make it difficult to reliably distinguish individual differences in body composition, unless the differences are large enough to overcome the SEE of an equation.

■ **Mechanistic models.** Underwater weighing, also referred to as hydrostatic weighing, involves the application of Archimedes principle of water displacement: the differences between the mass of a body in the air (Ma) and in water (Mw) is equal to the volume of the body ($V = Ma - Mw$) [155]. Data on volume allows estimation of density ($D = Ma/V$). To obtain a valid measure of body density, Ma needs to be corrected for the displacement caused by the air in the lungs (residual volume, RV), air in the intestines and water density. An accurate measurement of RV is important, because even small errors have a considerable effect on D [156].

A new technique for measuring body volume, the air replacement plethysmograph, may overcome many of the practical problems of underwater weighing [157]. However, this technique still needs more validation in a variety of subjects.

Body density is typically converted to body composition by a two-compartment (2C) model, for instance, by the Siri [158] equation:

$$\text{Body fat, \%} = (495/D) - 450 \qquad (2)$$

The day-to-day coefficient of variation (CV) for underwater weighing (2C model) may be less than 2%, if the lung volume is measured accurately [159, 160]. However, higher CVs (4%) have also been reported [161].

The Siri [158] equation is based on the assumptions of constant density of fat mass (FM) and the fat-free mass (FFM). Since the density of FFM is affected by the relative proportions of water, proteins and minerals, the assumptions of the 2C model are not always met. Variation in particularly the body hydration status, but also bone mineral mass and density, may lead to erroneous results [162, 163] which may be solved by using 3- or 4-compartment models (body density combined with total body water and/or body mineral mass) [158]. The 4-compartment model may be regarded as the best available method for assessment of the molecular composition of the human body.

The dilution techniques make use of the dilution of a known amount of a tracer that is administered to a subject [88]. For determination of total body water (TBW), stable isotopes (H^2 or O^{18}) are used as tracers. Once TBW is known, FFM can be calculated by assuming that water occupies a relatively constant fraction of FFM (73.2%) [165]. However, it has become clear that the real hydration factor varies between 70 and 76% even in healthy people [156]. Moreover, the hydration may acutely be changed after exercise, dieting, dietary manipulations (e.g. creatine) and in several diseases. It should also be noted that the result may vary according to the time used for equilibration (e.g. 4, 6 or 10 h) [142]. The technical CV is about 1% [165, 166].

The dual-energy X-ray absorptiometry (DXA) is based on measurement of the ration of tissue attenuation of X-rays at two energies [167]. The imprecision (CV) of DXA is less than 2% for FFM and BF% measurements [163, 168]. However, the most evident problem of DXA is that results vary according to different manufacturer's instruments, different software versions and even different instruments by the same manufacturer and the same software version [169].

Muscle mass may be estimated by 24-h urinary creatinine excretion and urinary 3-methylhistidine (3MH) [170]. Because of day-to-day variations, creatine excretion should preferably be measured from three consecutive 24-h urine specimens [170]. Measurement of 3MH is the primary in vivo method to measure skeletal muscle (myofibrillar) protein breakdown [171]. The problem with both of the above mentioned methods is that dietary protein adds to the body pool and causes an increased and variable excretion which must be taken into consideration when data are evaluated [170]. Also skeletal trauma increases excretion.

■ **Descriptive methods.** Anthropometric measurements include body weight, height, skinfold thickness and circumferences. When gender is taken into account, body composition may be predicted from weight, height and age [172]. An unusually high or low relative muscle mass leads to increased random error. Also hydration status, by its effect on body weight, is a potential source of error, although not as evidently as, for example, underwater weighing and dilution techniques.

Skinfold thicknesses are the thickness of a double fold of skin and subcutaneous adipose tissue at specific cites on the body, taken by spring-loaded calipers [173]. The sum of usually 4–7 skinfold thicknesses is the measured property, which is placed in a regression equation to predict body density [174]. The accuracy of skinfold measurements is dependent on two factors: the measurement itself (technical error) and the characteristics of the equation. The reliability of measurements is dependent on the skills of the measurers; within-subject reliability CVs between 2.4 and 3.4% [161, 175, 176] have

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been reported. Nevertheless, even trained technicians may consistently take larger or smaller skinfolds, which leads to systematic biases between laboratories and technicians [169, 176].

The SEE of equations lead to random errors on an individual level [156]. The variation is caused by, e. g., the distribution of subcutaneous fat over the body and the ration between subcutaneous and visceral fat. The magnitude and direction of random errors are difficult to predict on an individual level. However, data suggest that the body fat content may be under- or overestimated on an individual level by even more than 10 % [163].

The bioelectrical impedance (BIA) technique is based on the principle that the electrical conductance of the body is mainly determined by the water compartments and its solutes [177]. In BIA, a known alternating current (500–800 mA) with a single frequency (50 kHz) is led through the body, usually from one foot to the hand at the same side of the body. The voltage is measured and resistance (or impedance) calculated. Since BIA measures water, not fat, hydration status affects the results: an excess amount of water leads to overestimated FFM and vice versa. Therefore, BIA measurements should be carefully standardised (e. g. time after previous meal, exercise, timing within a menstrual cycle). The choice of equation is a source of systematic bias, and individual differences in, e. g. hydration status and skin conductivity lead to random errors. The day-to-day repeatability (CV) for body composition is about 2–3 % for the most often used frequency 50 kHz [176, 178–180], and about 1 % for repeated measurements within a single day [180, 181].

Most commercial BIA apparatuses have a built-in equation, often including age, body mass, height and sex [182]. In these cases, the relation between the resistance measurement and FFM (or FM) becomes obscured by the many variables involved.

The near-infrared interactance (NIR) is based on the assumption that interactance measurements of two wavelengths can characterise body composition [183]. In addition to optical densities, the method uses sex, age, weight, height and sometimes also physical activity level to predict body composition. The accuracy of NIR seems to be rather comparable to skinfolds in normal-weight subjects, but NIR clearly underestimates body fat in obese subjects [169]. NIR equations with physical activity level are particularly prone to errors, because wrong estimation of an individual's physical activity may change the result by several percentage units [158]. The within-day repeatability (CV) has been reported to be about 2 % [184]. An overview and characteristics of the body composition methods are given in Table 15.

Site specific measurements of muscle and fat mass

Muscle and fat mass in specific sites (e. g. upper arm, abdomen, thigh or calf) may be measured by scanning techniques (computerised tomography, magnetic resonance imaging) or by anthropometry. The former may be regarded as direct and accurate, but the equipment is very expensive. These methods also allow quantification of regional and total tissues, e. g., quantification of abdominal and visceral adipose tissue, or quantification of lean tissue and muscle. Anthropometrical measurements, in contrast, are indirect, but fast and cheap.

Computerised tomography (CT) is a radiological technique [156]. An image can be generated from the intensity of attenuated x-ray beams. The image allows separate recognition of bone, adipose tissue and fat-free tissue. The drawbacks of this method are ionising radiation and high costs. Because of the exposure to ionising radiation, this method does not allow a wide or repeated application on healthy subjects [185].

Magnetic resonance imaging (MRI) uses magnetic moments of hydrogen atoms. The radio frequency used does not have any known risks to the subjects. Compared with CT, MRI has a longer acquisition time and it is also more susceptible to artefacts produced by, e. g. respiratory and bowel movements [185]. The repeatability CV for subcutaneous fat tissue has been reported to be 2–4 %, whereas the CV for visceral fat determination is much higher (as high as 10–15 %), meaning rather poor repeatability. However, much lower CVs (about half compared to the numbers given above) have also been reported [186].

An indirect estimation of fat and/or muscle tissue at a specified site can be measured by using anthropometric measurements, including circumferences and skinfolds [187]. Abdominal fat distribution is demonstrated by waist circumference alone, or by the ratio between waist and hip circumferences. In sports science, muscle cross-sectional area is often much more relevant to performance than anatomical distribution of fat. An estimate of limb muscle plus bone area can be calculated from

$$A = (C\text{-}\pi S/10)^2/4\pi \qquad (3)$$

where A is muscle plus bone area (cm^2), C is limb circumference (cm) and S is the appropriate limb skinfold or mean skinfold [187]. Because skinfolds are not symmetrically distributed on both sides of a limb (for instance, the triceps skinfold is much larger than the biceps skinfold), it is recommended that both sides are measured twice and the mean result is used to indicate site-specific skinfold (S).

Specific study design aspects

The assumptions and regression equations used in assessment of body composition are dependent on several background variables, such as age, sex, obesity status and race. It is particularly important to notice that the variation of fat-free mass composition (bone, water, protein) affects most body composition assessment methods. Athletes, for instance, may have high bone density, which might make the assumptions for the 2C model of underwater weighing invalid. Techniques with water as a basic tissue of measurement (e. g. dilution methods, bioimpedance) are obviously very dependent on water balance. Therefore, use of these methods requires careful standardisation as regards physical activity, eating and phase of menstrual cycle.

The random errors of descriptive methods are larger compared to mechanistic models. This, in turn, leads to larger variation within a given study population, which decreases the statistical power. Consequently, a prerequisite for the use of descriptive methods is an adequate number of subjects, which is usually about double compared to mechanistic models.

Since most methods to measure body composition and muscle mass are not fully comparable [169], a use of "historical controls" (results from another study) is not possible. A control group should be assessed in the same laboratory, by the same method and by the same technician. Systematic differences between methods are of less importance in prospective settings, but even then the same technician and strict standardisation of the conditions of measurement are prerequisites of at least adequate repeatability.

■ Summary of the tissue growth measurements

Claims related to tissue growth are increased protein synthesis, increased protein, muscle or fat-free mass or reduced whole body or intra-abdominal fat mass. Protein synthesis is nowadays measured by stabile isotope tracers. This technique separates protein synthesis and catabolism, whereas the older nitrogen balance technique only indicates the difference between catabolism and synthesis. However, the stable isotope techniques are influenced by the chosen tracer and the assumptions used. Underwater weighing, air replacement plethysmograph, dilution techniques and dual-energy X-ray absorptiometry (DXA) are mechanistic body-composition models, i. e. they have a functional relationship with body composition. Skinfold thicknesses and bioimpedance (BIA) are descriptive models, i. e. based on statistically derived prediction equations against a mechanistic reference method. Intra-abdominal fat is quantified by using computerised tomography or magnetic resonance imaging techniques. The between-subject accuracy is

better in mechanistic than in descriptive models. Repeatability is best in DXA and hydration techniques, but other mechanistic models do not differ from descriptive models. Regardless of the method, systematic differences between any two methods or even between two prediction equations within the same technique may be considerable.

Free radical scavenger capacity and prevention of oxidative stress

■ Claims

- Reduces damage by free radicals
- Prevents oxidative stress

■ Physiological background and relevance

It is generally recognised that free radicals are formed during oxidative energy production processes. Oxygen consumption for aerobic energy production increases about 20-fold and so does free radical production since both processes are quantitatively interrelated. It has been estimated that about 2 to 5 % of the total electron flux during normal metabolism "leaks off" to generate free radicals. This stress-related response can be further augmented by increased body temperature and stress hormone levels. Other sources of free radical production and/or processes in which free radicals may be involved are the breakdown of ATP during energetic stress, auto-oxidation of catecholamines and production of nitric oxide. Also muscle damaging events, leading to post exercise inflammation and muscle pain, and endurance exercise in polluted air, such as running a major city marathon on a hot summer day in the smog are suspected to involve free radical actions.

The human body has a number of ways to eliminate free radicals rapidly. There are a number of enzyme systems that are able to quench most of the radicals; in addition there are a number of antioxidant compounds that circulate in body fluids and/or are present in tissues and cells that can help reduce free radical damage. Several components of the enzymatic defence systems require certain minerals and trace elements as an integral part of their structure to function properly. However, the usefulness of vitamin, mineral and trace element status, as measured in blood, for, for example, muscle function is still a matter of debate because exercise may lead to shifts of compounds between compartments and tissues. As such a low level, measured in blood, will not necessarily reflect a low level in muscle tissue.

The production of free radicals is, due to their extremely short life time, very difficult to measure. Current methods include amongst others the detection of free

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radicals by time resolved optical spectroscopy and by electron spin resonance. These methods are complex and can only be applied under strict controlled laboratory conditions. Other measurements concern indirect markers of free radical production, for example the release of myoglobin and CPK from damaged muscle or the release of lipid peroxides.

The complexity of this issue makes an appropriate description of the types of antioxidants, their bio-availability, distribution and metabolism as well as the methods to measure antioxidant status and free radical production methods within the scope of this paper impossible. For that reason the interested reader is referred to several recent review publications that give details in great depth [188–192].

Immune function

■ Claims

- Improves immune function
- Attenuates immunosuppression during or after exercise
- Reduces incidence of infection
- Reduces severity (duration) of infection

■ Definitions, physical background and relevance

Athletes engaged in heavy training programmes, particularly those involved in endurance events, appear to be more susceptible than normal to infection. For example, according to some surveys (e. g. [193–195]) sore throats and flu-like symptoms are more common in athletes than in the general population, and once infected, colds may last for longer in athletes [196]. There is some convincing evidence that this increased susceptibility to infection arises due to a depression of immune system function (for detailed reviews see [197–199]). The immune system protects against, recognises, attacks and destroys elements that are foreign to the body. This statement succinctly defines the functions of this homeostatic system, which nevertheless are far more complex than the above remark initially indicates. It involves the precise co-ordination of many different cell types and molecular messengers yet, like any other homeostatic system, the immune system is composed of redundant mechanisms to ensure that essential processes are carried out.

The immune system can be divided into two broad functions: innate (natural or non-specific) and adaptive (acquired or specific) immunity which work together synergistically [200, 201]. The attempt of an infectious agent to enter the body immediately activates the innate system. This so-called 'first-line of defence' comprises three general mechanisms with the common goal of restricting micro-organism entry into the body: i) physical/structural barriers (skin, epithelial linings, mucosal secretions); ii) chemical barriers (pH of bodily fluids and soluble factors); and iii) phagocytic cells (e. g. neutrophils, monocytes/macrophages). Failure of the innate system and the resulting infection activates the adaptive system, which aids recovery from infection. This is helped greatly by T- and B-lymphocyte acquisition of receptors that recognise the antigen, engendering specificity and 'memory' that enable the immune system to mount an augmented response when the host is reinfected by the same pathogen.

The components of the immune system comprise cellular and soluble elements. The immune cells (leukocytes) have diverse functions despite their common origin: the hematopoietic stem cell of the bone marrow. Leukocytes consist of the granulocytes (60–70 % of circulating leukocytes), monocytes (10–15 %) and lymphocytes (20–25 %). Various subsets of the latter can be quantified through the use of monoclonal antibodies which are used to identify specific proteins (known as clusters of differentiation or cluster designators, CD) that are expressed on the cell surface of a particular cell type [201]. For example, T-lymphocytes all express the protein CD3 on the cell surface. This distinguishes them from B-lymphocytes which do not express CD3, but express CD19, CD20 and CD22. A particular sub-set of T-lymphocytes called T-helper cells specifically express the CD4 protein, whereas the T-cytotoxic cells express CD8. T-cells recognise short peptide sequences from antigens only if they are held on the cell's surface and complexed with a major histocompatibility complex (MHC) molecule. The ability of the immune system to distinguish self from non-self depends largely on the MHC protein markers. These are present on the surface of every cell and are slightly different in each individual.

Soluble factors of the immune system act in several ways: i) to activate leukocytes, ii) as neutralisers (killers) of foreign agents, iii) as regulators of the immune system. Such factors include the cytokines. These polypeptide messenger substances stimulate the growth, differentiation and functional development of immunocytes via specific receptor sites on either secretory cells (autocrine function) or immediately adjacent immunocytes (paracrine function). Cytokine action is not confined to the immune system; they also influence the central nervous system and the neuroendocrine system. Other soluble factors include complement, lysozyme and the specific antibodies produced by activated B-lymphocytes.

The introduction of an infectious agent to the body initiates an inflammatory response which augments that of the immune system. Acute inflammation increases local blood flow in the infected area, and this coupled with augmented vascular permeability facilitates the entry of

leukocytes and plasma proteins into the infected tissue. The immune response itself varies according to the nature of the infectious agent (parasitic, bacterial, fungal, viral) but a general response pattern is evident. The ingestion of the invading micro-organism by the phagocytic macrophage initiates a chain of events. Enzymes and oxidising agents are released from within the macrophage. The foreign proteins normally found on the micro-organism's surface are ingested and processed by the macrophage and incorporated into its own cell surface and are presented alongside MHCII proteins. The antigen can now be presented to the other cellular immune components. T-helper cells (CD4+) coordinate the response via cytokine release to activate other immune cells. Mature B-cell stimulation results in proliferation and differentiation into immunoglobulin (antibody)-secreting plasma cells. Reaction of the immunoglobulin with a specific antigen forms an antibody-antigen complex. This represents the humoral (fluid) immune response and is an effective defence against extracellular pathogens present in the body fluids. T-cytotoxic cells and non-specific Natural Killer (NK) cells are responsible for cell-mediated immunity which is effective in the elimination of intracellular pathogens.

■ Markers of immune system status

There is no single marker of immune system status. Circulating numbers and functions of cells involved in innate immunity can be measured (e. g. neutrophils, monocytes and NK cells). The circulating numbers of cells involved in acquired immunity (B and T lymphocytes) and some functions (e. g. proliferative responses) or markers of activation (e. g. CD45RO and CD38 expression) can be measured. Normal healthy ranges are established for circulating numbers of the major leukocytes and some lymphocyte subsets. However, absolute values for most functional measures (e. g. neutrophil and monocyte respiratory burst, monocyte MHCII expression, lymphocyte proliferation) cannot be used to indicate abnormal immune function, unless simultaneous comparison with a control group is made or previous healthy baseline measures have been established.

Size of the effect and physiological importance

Small (< 10 %) decreases or increases in selected indices of immune function may not be clinically important, especially if values are within the normal range [202]. A significant improvement in one or more aspects of immune function is likely to reduce infection risk although, of course, infection risk also depends on the degree of exposure to pathogens and the experience of previous exposure. Improving several aspects of immune function will convey a more effective immune protection than an improvement in just one aspect of immune function.

Acute bouts of exercise cause a temporary depression of various aspects of immune function (e. g. neutrophil respiratory burst, lymphocyte proliferation, monocyte MHCII expression) that lasts ~3–24 hours depending on the intensity and duration of the exercise bout [203–209]. Periods of intensified training (overreaching) lasting 7 days or more result in chronically depressed immune function [210–215]. Claims for improvements in immune function in athletes as a result of consumption of a functional food could therefore be linked to 1) an attenuation of the temporary immunosuppression following a standardised bout of exercise, 2) an improvement in one or more aspects of immune function in the resting state, or 3) both 1) and 2).

Study criteria

Several immune cell functions and circulating numbers are affected by hormones such as cortisol and adrenaline, so blood sampling for assessment of immune function should be conducted at the same time of day [199, 202]. Ageing, weight loss, recent exercise, race, gender, pregnancy, nutritional status and infection may influence immune variables, so control and experimental subject groups must be appropriately matched, free of infection for at least 3 weeks, and recent exercise/food intake controlled. Most functional measures of immune function use chemical stimulants (e. g. lipopolysaccharide [LPS], fMLP, pokeweed mitogen, concanavalin A) to activate the immune cells in vitro (simulating the way they would respond to an infectious agent). Because of this, standardisation of procedures (e. g. chemical nature and dose of stimulant used; incubation time; isolated cells or whole blood assays) is important and results from different laboratories may not be directly comparable.

For studies evaluating the effect of an intervention (e. g. functional food) on immune responses to exercise, the exercise bout should be of sufficient intensity (> 55 % VO$_2$max) and duration (> 1.5 hours) to induce a temporary period of immunosuppression. The degree of exercise-induced immunosuppression is influenced more by the duration of exercise than the intensity [216], so the most suitable protocols are continuous cycling or running at 55–75 % VO$_2$max for 1.5–2.5 hours.

■ Blood measures of immune status

Some of the more common measures of immune system status are listed below and given in Table 16. Details of most of the methods can be found in the textbook by Fernandez-Botran & Vetvicka [217].

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Table 16 Markers of immune function

Method	Reproducibility (CV %)	Precision	Advantages	Disadvantages	Reference
Cell counts and lymphocyte subsets	2–5 %	Very good	Rapid automated measurement	Does not give information about cell function Requires FACS to measure subsets	[219]
Neutrophil phagocytosis	5–10 %	Moderate	Simple assay	Not necessarily related to killing capacity Only measures % of activated cells	[220]
Neutrophil or Monocyte respiratory (oxidative) burst	5–10 %	Very good	Measure is related to killing capacity	Activity depends on dose and type of stimulant used Requires FACS or luminometer	[212] [213] [221]
Neutrophil degranulation	About 10 %	Good	Possibly the best measure of function as it represents the end stage of neutrophil killing	Time consuming assay	[216] [213]
Monocyte MHCII surface expression (mean fluorescence intensity)	Not known	Good	Related to antigen presentation activity of monocytes	None	[222] [223]
Monocyte or Lymphocyte cytokine production	5–10 %	Moderate	None	Time consuming assay Requires FACS or expensive ELISA kits	[219] [225] [228]
Lymphocyte proliferation	About 10 %	Moderate	None	Assay requires at least several days of incubation Cell harvester and gamma counter required	[207] [208]
Lymphocyte antibody production	10–20 %	Moderate	None	Time consuming assay Requires FACS or expensive ELISA kits	[211]
NK cell cytolytic activity	5–10 %	Good	None	Requires source of ^{51}Cr labelled target cells	[204] [207]
Serum complement	2–5 %	Very good	Simple turbidometric assay	None	[229]
Serum immunoglobulins (total IgA, IgM, IgG)	2–5 %	Very good	Simple turbidometric assay	Does not give information about antigen-specific antibody concentration	[210]
Saliva IgA	About 10 %	Moderate	Simple ELISA assay Some evidence that low levels of IgA are related to infection incidence in athletes	IgA concentration affected by saliva flow rate and swab collection	[233]
Specific antibody response to vaccination	5–10 %	Good	Measure is related to *in vivo* humoral immunity	Responses only specific to the antigen tested Test cannot be repeated on same individual Large subject numbers required	[209] [232]
DTH response to skin injection of antigens	Not known	Moderate	Measure is related to *in vivo* cell-mediated immunity	Measurements must be made 24–48 hours post-injection Large subject numbers required	[209]
Infection incidence by self-reporting of symptoms of URTI	Not known	Poor	Simple and inexpensive Only requires questionnaires	Possible response bias and numerous confounding factors	[193] [194] [234]

Cell counts

White blood cell counts can be reliably measured on the basis of size and granularity using automated cell sorters with a CV of < 2 % [216, 218]. These give data on total leukocyte count and differential counts (neutrophils, monocytes, lymphocytes, eosinophils, basophils). A guide to normal adult ranges (x 10^9 cells/l) is given below as the 5th and 95th percentile [219]:

Total leukocytes   4.0–11.0

Neutrophils   2.0–7.5
Monocytes   0.2–0.8
Lymphocytes   1.0–3.4
Eosinophils   0.0–0.4
Basophils   0.0–0.1

Lymphocyte subsets

Lymphocyte subsets (B, T, NK cells; T-Helper CD4 + /T-Suppressor CD8 + ratio) and activation markers (e. g.

CD45RO, CD38) can be reliably measured using fluorescent-labelled antibodies and an automated Fluorescence Activated Cell Sorter (FACS) with a CV of < 5 % [217, 218]. The percentage of cells in the lymphocyte gate that stain for the CD marker are multiplied by the lymphocyte count obtained on full blood count analysis and divided by 100 to obtain absolute numbers of NK cells, T and B cell subsets. A guide to normal adult ranges (x 10^9 cells/l) is given below as the 5th and 95th percentile [219]:

Total lymphocytes   1.0–3.4
CD3+ all T cells   0.60–2.50
CD4+ T cells   0.35–1.50
CD8+ T cells   0.23–1.10
CD4/CD8 ratio   0.66–3.50
CD56+ NK cells   0.20–0.70
CD19+ B cells   0.04–0.70

Activation markers (CD38, CD45RA and CD45RO) can be measured in combination with CD4 or CD8 to give percentages of activated (memory) and naïve T-Helper (T-H) and T-Cytotoxic (T-C) cells.

Neutrophil function

Several aspects of neutrophil function can be assessed including chemotaxis, phagocytosis, respiratory (oxidative) burst and degranulation stimulated by LPS, phorbol 12-myristate 13-acetate (PMA; an activator of protein kinase C), fMLP (a chemotactic peptide), or bacteria (e. g. *E. coli; Candida albicans*) [213]).

Neutrophil phagocytosis can be measured in whole blood *in vitro* by the ingestion by neutrophils of nitroblue tetrazolium dye [220] or bacteria (e. g. *Candida albicans*).

Neutrophil respiratory burst can be measured by FACS. Upon activation, neutrophils assemble the NADPH oxidase system. This complex forms the superoxide radical and other reactive oxygen species (ROS) which can be used to destroy ingested bacteria. Dihydrorhodamine is added which upon contact with ROS is oxidised to the fluorescent compound rhodamine 123. The fluorescence of the sample, measured in a FACS system, is proportional to the amount of ROS generated. Hence, respiratory burst activity can be assessed on a per cell basis. Respiratory burst can also be measured by a manual luminometric procedure using luminescent compounds such as lucigenin, luminol and pholasin [220].

Neutrophil degranulation response (enzyme release) to LPS can be measured by measuring the amount of myeloperoxidase or elastase released following *in vitro* incubation of whole blood with LPS [216].

Monocyte/macrophage function

Monocyte respiratory burst can be measured in the same way as for neutrophils. Monocyte cell surface expression of MHCII protein can be measured by FACS [222, 223].

Cytokine (e. g. IL-1, IL-6) production in whole blood can be measured in vitro following incubation with PMA or LPS [224] or can be measured as intracellular cytokine production by monocytes using FACS [225]. The main problem with evaluating blood monocyte function is that it may not be representative of tissue macrophage activity [223]. Most studies on the effects of exercise on tissue macrophage function have used animal models (e. g. [226]).

Lymphocyte function

Lymphocyte proliferation in response to mitogens (e. g. Pokeweed mitogen, Phytohemaglutinin, Concanavalin-A) is usually measured by extent of incorporation of ^{3}H-thymidine into dividing cells following 2–7 days of culture incubation [205, 208]. Results may be expressed as absolute counts per minute (cpm) or as the "Stimulation index" which is the stimulated value expressed as ratio to the unstimulated (no mitogen) value. Lymphocyte proliferation is sensitive to the number of T cells that are present, so it is common practice to express the absolute cpm or the Stimulation index per T cell.

Cytokine production by stimulated T cells (e. g. IL-2, TNF-β and IFN-γ production by TH-1 cells and IL-4, IL-5, IL-6, IL-10 and IL-13 production by TH-2 cells) can be measured in whole blood [224] or can be measured as intracellular cytokine production by CD4 + and CD8 + T cells using FACS [227, 228].

Immunoglobulin M and G production by mitogen-stimulated lymphocytes can be measured *in vitro* [211].

NK cell function

NK cell function can be assessed *in vitro* as NK cell cytotoxicity against tumours or animal erythrocytes preloaded with ^{51}Cr [204, 207].

Soluble factors

Serum concentrations of complement proteins [229] and total immunoglobulins (IgA, IgG and IgM subclasses) [210] can be measured by turbidometric spectrophotometry.

Specific study design and methodological aspects

There are several methodological issues that need to be considered when examining apparent changes in immune function in response to acute exercise and the possible modifying effects of nutritional supplements on such responses. Firstly, the reported response of various components of the immune system to exercise is transient and quite variable, depending upon the type of

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exercise, the immunological methodology used, the intensity of effort relative to the fitness of the individual and the timing of the observations [202, 230]. Effects disappear usually within six hours post-exercise [231]. Many findings are method dependent; blood analyses are time consuming and so some investigators collect few blood samples. Delaying taking samples by thirty minutes post-exercise may lead to quite different results and all blood measures will be affected by haemoconcentration. Secondly, responses may also be influenced by natural circadian variations in circulating lymphocyte numbers and plasma hormone (e. g. cortisol) concentrations [202], yet many studies do not include non-exercising time-controls in their protocols. Thirdly, overall and differential leukocyte counts can also be modified by changes in blood volume, margination and demargination of cells, modification of leukocyte/endothelial interactions, sympathetic and parasympathetic neural activity and cell redistribution with the release of granulocytes from bone marrow [230]. Thus, changes in populations of cells may be responsible for some apparent changes in leukocyte functions.

Numerous studies report effects of exercise on functions of isolated leukocytes when these cells are stimulated *in vitro* by added antigens or mitogens. However, it is difficult to extrapolate from the *ex vivo* stimulated response of isolated cells to how these same cells would respond in the far more complex *in vivo* environment. In addition to the presence of antigens, leukocyte function is also influenced by endogenous chemicals including hormones, neurotransmitters and cytokines and the plasma concentration of these may change during exercise. The pH and temperature of the blood also change during exercise, but these factors are often ignored in experiments on isolated cell types. Thus, separating cells from their *in vivo* environment is somewhat artificial and to a large degree excludes the effects of exercise-induced chemical changes in the blood that will undoubtedly modify leukocyte function. The closest one can get to the *in vivo* condition is by performing measurements on whole blood, in which the proximity between the leukocytes and the extracellular milieu is retained.

Finally, only 0.2 % of the total leukocyte mass is circulating at any moment [202]; the remainder is in lymphoid tissue, the bone marrow and other tissues. It may thus be more important to assess the status of leukocytes in the skin, mucosa, and lymph nodes rather than in the blood, though this is not usually possible in human studies.

Response to injection of antigen

Another way of demonstrating altered immunity is to measure the specific antibody (immunoglobulin) response to vaccination (an indicator of humoral immu-

nity). This cannot be repeated in the same individual (due to the nature of the adaptive response) but can be compared between two separate groups (control and experimental). In such studies, sample size needs to be quite large (n > 50) because of the wide inter-individual variation in antibody response [209]. This measure can be of real clinical relevance [232]. Examples of suitable antigens include hepatitis B, influenza and pneumococcal vaccines. Specific antibody titres in serum are measured by ELISA, usually 2–4 weeks after injection of the antigen.

A similar approach can be used to measure cell-mediated immunity using superficial skin injection of a number of antigens. The diameter of skin swellings that develop over a 24–48 hour period gives a measure of this delayed-type hypersensitivity (DTH) response which is mediated by T-cytotoxic cells [209].

Saliva measures of immune status

The concentration of immunoglobulin A (IgA) in saliva is decreased during periods of chronic physical or psychological stress. Saliva IgA can be measured by an ELISA method with a CV of about 10 %. There is some evidence that low levels of saliva IgA are associated with increased incidence of upper respiratory tract infection (URTI) in endurance athletes [198, 233]. The use of cotton swabs to collect saliva is not recommended as IgA and other proteins are adsorbed by the cotton fibres. Saliva samples should be obtained in a tube without stimulation as chewing increases saliva flow rate and decreases IgA concentration.

Incidence of infection

Claims for a beneficial effect of functional foods could be based on reducing the incidence of infection or on reducing the severity (duration) of symptoms of illness. As stated in section Study criteria, actual incidence of infection is determined both by immune system status and the degree of exposure to pathogens. Upper respiratory tract infection (URTI) is the most common infection in athletes [196, 231]. Self-reporting of symptoms of URTI using questionnaires has been used in a number of studies involving exercise with or without nutritional supplement intervention [193–195, 234, 235]. However, this approach leaves such studies open to the criticism that the reporting of respiratory symptoms was increased by increased body awareness, respiratory muscle fatigue or inhalation of air pollutants in regularly exercising individuals rather than by a specific infection of the upper respiratory tract. Furthermore, if the reported incidence of respiratory symptoms is compared with anticipated infection rates for the general population,

there may be a response bias. For example, in the study by Nieman et al. [194], only 47 % of questionnaires were returned, and the respondents may have been mainly those who developed symptoms. Thus, it is preferable that infections are clinically confirmed rather than self-reported.

Other confounding factors that could give rise to an increased incidence of infection in athletes other than through an exercise-induced depression of immune function include exposure to contaminated air or water; increased lung ventilation and breathing through the mouth as well as the nose; a reduction of tracheal ciliary or mucosal secretory activity by the inhalation of cold air; inadequate diet (e. g. in athletes attempting to lose weight by restricting food intake); physical contact and skin abrasions; and psychological stress.

If consumption of a functional food supplement can be shown to be associated with a reduction in URTI frequency or severity together with a measured improvement in one or more aspects of immune function, this would be a very good association: a global effect and an explanatory mechanism.

Summary of immune function measurements

There is no single biomarker of immune system status. Several numbers and functions of cells involved in innate immunity can be measured. However, absolute values for most functional measures cannot be used to indicate abnormal immune function unless simultaneous comparison with a control group is made or previous healthy baseline measures have been established. This makes the interpretation of the immune status on the basis of the available immune biomarkers very difficult and complicated. A more functional approach is the measurement of altered immunity by measure of specific antibody response to vaccination or by measure of immoglobulin status. The most direct indicator of immune function is the change in incidence of infection in particular upper respiratory tract infections (URTI) as the most common infection in athletes. However, this second set of methods is generally labour-intensive to use due to the larger number of subjects to be included.

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