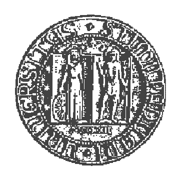

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INSTITUT FÜR EXPERIMENTELLE UND KLINISCHE PHARMAKOLOGIE UND TOXIKOLOGIE

DOCTORAL DISSERTATION
in

"MOLECULAR AND CELLULAR PHARMACOLOGY"
"FARMACOLOGIA MOLECOLARE E CELLULARE"

DOCTORAL PROGRAMME
XXI CYCLE

MOLECULAR ENDOCRINOLOGY APPROACH IN PATHO-PHYSIOLOGICAL CONDITIONS: NEW MARKERS AND ALTERNATIVE BIOLOGICAL MATRICES

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31 December 2008

Index

ABSTRACT……………………………………………………………………...**1**

RIASSUNTO…………………………………………………………………..**3**

LEGEND...**5**

1. INTRODUCTION…………………………………………………….… **7**

1.1 Saliva specimen: a new laboratory tool for diagnostic and basic investigation…………………………………………………………..…**7**

1.1.1 Saliva production……………………………………………….… **8**

1.1.2 Saliva composition………………………………………….…..**10**

1.1.3 Saliva functions…………………………………………………... **12**

1.1.4 Saliva hormones……………………………………………….....**14**

1.1.4a Salivary Free Insulin-like Growth Factor I (sIGF-I)…………**15**

1.1.4b Salivary Cortisol (sF)…………………………………………**19**

1.2 Insulin-like Growth Hormone-I and Cortisol/Cortisone: Clinical Applications, Use and Abuse ……………………………………………….. **24**

1.2.1 Insulin-like Growth Factor I (IGF-I)……………………………... **24**

1.2.1a Clinical Applications………………………………………… **24**

1.2.1b Use……………………………………………………………… 26

1.2.1c Abuse…………………………………………………………… 27

1.2.2 Cortisol (F) and Cortisone (E)………………………………………28

1.2.2a Clinical Applications………………………………………… 28

1.2.2b Use……………………………………………………………… 29

1.2.2c Abuse…………………………………………………..............29

1.3 Physical exercise & training status: physiological conditions to study hormones and saliva……………………………………………………… 31

1.3.1 Exercise and Insulin-like Growth Factor-I………………………….. 32

1.3.2 Exercise and Cortisol………………………………………………… 32

1.3.3 Exercise and Saliva……………………………………………………..32

2. RESERCH AIMS…………………………………………………………...........35

3. Section I: MATERIALS AND METHODS...37

3.1 Specimen collection: saliva and blood...37

3.2 Free sIGF-I assay procedure...38

3.3 Free sIGF-I assay method: evaluation procedure....................................... 38

3.4 Pre-analytical variability tests...39

3.5 Biological variation of sIGF-I...40

3.6 Salivary total protein assay..40

3.7 Plasma hormone measurements..40

3.8 Protocol A - effect of the training condition in females.............................. 41

3.9 Protocol B - effect of an acute physical exercise in male cyclists................41

3.10 Protocol C - effect of an acute physical exercise in male rowers
- effect of the training condition in males................................42

3.11 Statistical analysis...43

4. Section I: RESULTS...45

4.1 Free sIGF-I assay method: evaluation procedure....................................... 45

4.2 Pre-analytical variability tests...46

4.3 Biological variation of sIGF-I..**47**

4.4 Protocol A - effect of the training condition in females.............................. **47**

4.5 Protocol B - effect of an acute physical exercise in male cyclists................**48**

4.6 Protocol C - effect of an acute physical exercise in male rowers.............**50**

- effect of the training condition in males..................................**52**

5. Section II: MATERIALS AND METHODS... **53**

5.1 Specimen collection: saliva and urine...**53**

5.2 Salivary Cortisol and Cortisone assay procedure.. **53**

5.3 Salivary Cortisol and Cortisone assay method: evaluation procedure..........**56**

5.4 Pre-analytical variability tests..**57**

5.5 Urine F and E measurements...**57**

5.6 Urine creatinine measurement... **58**

5.7 Protocol D - effect of an acute physical exercise in male and female rowers..**58**

5.8 Statistical analysis...**59**

6. Section II: RESULTS.. **61**

6.1 Salivary Cortisol and Cortisone assay method: evaluation procedure..........**61**

6.2 Pre-analytical variability tests..**63**

6.3 Protocol D - effect of an acute physical exercise in male and female rowers..**66**

7. DISCUSSION...**69**

7.1 Section I.. **69**

7.2 Section II...**72**

8. REFERENCES...**75**

9. OVERVIEW ON PUBLICATIONS.. **85**

9.1 Papers..**85**

9.2 Abstracts... **92**

Abstract

The study of saliva for laboratory analyses is an increasing area of research with implications for basic and clinical purposes. Although this biological fluid is easy to manipulate and collect, attention must be directed to sample collection and storage, to method development and validation and to variability evaluation.

The analysis of saliva provides important information about the functioning of various organs within the body. In this respect, endocrine research certainly occupies a central role. In effect, some hormones commonly measured in plasma, such as steroids, non-steroid, peptide and protein hormones, can be detected in the oral fluid. The *protein polypeptide hormones* are maybe a new analytical approach of the medicine laboratory but, at present time, there are still too few investigations about protein and polypeptide hormone levels in saliva. Detection of *steroid hormones* is perhaps the most interesting application in salivary hormonal studies. Steroids have often been studied because salivary-free steroid hormones seem to reflect the serum-free levels. Among of these steroids, salivary cortisol measurement is today a widely accepted alternative to the determination in plasma.

In the section I of the present dissertation, a new ELISA method is explained. Research was carried out to study and validate an assay to measure **salivary free IGF-I** (sIGF-I) in human saliva. The detection range, the detection limit, the imprecision, the recovery and the specificity were evaluated. The pre-analytical variation was also studied. After the method validation, sIGF-I levels were measured in sedentary subjects and in athletes (*protocol A* and *protocol C*); moreover two different acute physical exercises in two groups of athletes were investigated to assess a possible effect on sIGF-I (*protocol B* and *protocol C*).

The section II describes an analysis method which used the chromatographic technique. A SPE-HPLC method with UV detection was developed and validated to simultaneously measure **cortisol** (sF) and **cortisone** (sE) **in human saliva**. The analytical performances, in terms of detection range, sensitivity, imprecision, recovery, were evaluated. The pre-analytical variation, with respect to collection strategy and storage conditions, was also examined. After validation, the sF and sE method was applied analyzing specimens collected from athletes, before and after a physical exercise (*protocol C*).

The results suggest further investigation from the laboratory point of view, taking into account the aspects related to the various forms and the specific and unspecific binding proteins (for sIGF-I assay) and to other steroid hormones and related metabolites identified and probably present in human saliva (for sF/sE assay).

Riassunto

Lo studio della saliva nelle analisi di laboratorio è un'area di ricerca in forte crescita, per le sue implicazioni nella ricerca di base ma anche a fini clinici. Sebbene questo fluido biologico sia facile da manipolare e da raccogliere, bisogna porre attenzione ai processi di raccolta e stoccaggio del campione, nonché allo sviluppo e alla validazione di metodi analitici, assieme alla valutazione delle variabilità.

L'analisi della saliva dà importanti informazioni sul funzionamento di vari organi del corpo. In relazione a questo, la ricerca endocrina occupa certamente un ruolo centrale. Infatti, alcuni ormoni normalmente misurati nel plasma, come ormoni steroidei, ma anche ormoni non steroidei, peptidici e proteici, possono essere identificati nel fluido orale. Un nuovo approccio analitico nella medicina di laboratorio è forse rappresentato dagli *ormoni polipeptidici e proteici* ma, tuttora, ci sono ancora troppi pochi studi su questi ormoni salivari. La misura degli *ormoni steroidi*, invece, rappresenta forse l'applicazione più interessante negli studi degli ormoni salivari. Spesso gli steroidi sono studiati perché la concentrazione salivare riflette i livelli sierici. Tra i vari steroidi, la misura del cortisolo salivare è oggi una alternativa alla sua determinazione plasmatici.

Nella I sezione viene spiegato un nuovo metodo ELISA. Sono stati sviluppati test sperimentali per studiare e validare un metodo per la misura dell'**IGF-I libero salivare** (sIGF-I). Sono stati studiati il range di misura, la sensibilità, l'imprecisione, il recupero e la specificità. Inoltre è stata studiata anche la variabilità pre-analitica. Dopo la validazione del metodo, sono stati misurati i livelli di sIGF-I in soggetti sedentari ed in atleti (*protocollo A* e *protocollo C*); inoltre è stato studiato il possibile effetto di due differenti esercizi fisici (in acuto) sulle concentrazioni di sIGF-I (*protocollo B* e *protocollo C*).

La II sezione prende in esame un metodo di analisi che usa la tecnica cromatografia. E' stato sviluppato e valicato un metodo SPE-HPLC con rivelazione UV per la misura contemporanea del **cortisolo** (sF) e del **cortisone** (sE) **nella saliva umana**. Sono state calcolate le performance analitiche (range di misura, sensibilità, imprecisione, recupero). E' stata considerata anche la variabilità pre-analitica con particolare attenzione alle condizioni di raccolta e conservazione del campione. Dopo la

validazione, questo metodo è stato applicato a campioni raccolti da un gruppo di atleti, prima e dopo un esercizio fisico (*protocollo C*).
I risultati ottenuti suggeriscono ulteriori approfondimenti soprattutto da un punto di vista laboratoristico, tenendo presente la possibile presenza di varie forme e di specifiche ed aspecifiche proteine di legame (per sIGF-I) e altri ormoni steroidei e loro metaboliti identificati e probabilmente presenti nella saliva umana (per sF/sE).

Legend

Abbreviation	*Definition*
11 β-HSD	11β-Hydroxysteroid Dehydrogenase
Ab-pox	Peroxidase-conjugated Antibody
ACTH	Adrenocorticotrophic Hormone
Ag	Antigen
ALS	Acid Labile Subunit
AVP	Vasopressin
crea	urine Creatinine
CRH	Corticotrophin-Releasing Hormone
DELFIA	Dissociation-Enhanced Lanthanide Fluorescent Immunoassays
DHEA	Dehydroepiandrosterone
E	Cortisone
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
F	Cortisol

GH	Growth Hormone
GHD	Growth Hormone Deficiency
GR	Glucocorticoid Receptor
HPLC	High Pressure Liquid Chromatography
IGFBP	Insulin-like Growth Factor Binding Protein
IGF-I	Insulin-like Growth Factor-I
IRMA	Immunoradiometric Assay
mAb	Monoclonal Antibody
MR	Mineralcorticoid Receptor
RIA	Radio-Immunoassay
sE	salivary Cortisone
sF	salivary Cortisol
sIGF-I	salivary free Insulin-like Growth Factor-I
SPE	Solid Phase Extraction
sTP	salivary Total Protein
TMB	3,3′,5,5′-Tetramethylbenzidine

1. Introduction

1.1 Saliva specimen: a new laboratory tool for diagnostic and basic investigation

Saliva is a “perfect” analysis medium for health and disease surveillance. Actually, to monitor health status, disease onset and progression and treatment outcome, non-invasively is a most desirable goal in the health care delivery and health research [1-4].
Saliva research and study are highly attractive as non invasive analysis method. In fact oral fluid offers numerous advantages [1, 4-5]:

- with a salivary specimen, one can collect multiple specimens from the same individual at the optimum times for diagnostic information;
- saliva collection without specialized personnel and, with certain devices is usually stable at ambient temperature for several weeks;
- saliva analysis is potentially valuable for children and older adults, since collection of the oral fluid with fewer compliance problems as compared with the collection of blood;
- saliva analysis may provide a cost-effective approach for the screening of large populations.

Saliva samples can be analyzed for: tissue fluid levels of naturally, therapeutically and recreationally introduced substances; emotional status; hormonal status; immunological status; neurological status and nutritional/metabolic influences [6-8].

In spite of all these advantages, interpretation of saliva assays is still difficult [1,5].

Few studies of normal individuals, checking for known variables, such as pH, in relation to biorhythms and medications, have been performed and so absolute ranges show variability in different studies. A factor that could affect the results is the saliva collection procedure, since different devices give different results. Moreover, using the same device, standardization of salivary collection has a great importance in saliva analysis, because several factors may affect salivary flow and composition.

Another crucial aspect in saliva compound measurements is the used assay: to obtain an accurate data, the employed method has to be validated specifically for the saliva matrix; actually, there are a lot of studies based on plasma assays "adapted" for saliva without analytical performances evaluation and validation.

1.1.1 Saliva production

In humans, oral fluid originates mainly from three pairs of major salivary glands (parotid, sublingual and submandibular) and from a large number of minor salivary glands (Von Ebner glands and Blandin-Nühm mucous glands). Parotid glands are entirely serous glands, since their secretion lacks mucins, whereas sublingual and submandibular glands are mixed sero-mucous.

The salivary glands are composed of acini, in which the initial or primary saliva, isotonic compared to plasma, is produced and stored. Through the excretory ducts, secreted saliva is drained to the oral cavity: during this passage, the concentration of several electrolytes changes due to the active ionic transport, which renders the oral fluid its hypotonic character, when compared to plasma (table 1).

Salivation stimulus is regulated by the activity of the autonomic nervous system: the serous part of the glands is under the control of the sympathetic system and the mucous part of both parasympathetic and sympathetic stimuli systems.

Saliva composition varies in relation to the serous or mucous components of the glands. Saliva components have also a non glandular origin, so oral fluid cannot be considered as the only production of salivary glands because it also contains fluids originating from

oropharingeal mucosae, crevicular fluid, food debris and blood-derived compounds (actively or passively transferred).

Table 1: Electrolyte concentrations in whole human oral fluid and plasma

Inorganic compounds (mmol/l)	Whole human *unstimulated* saliva	Whole human *stimulated* saliva	Plasma
Na^{+}	5	20-80	145
K^{+}	22	20	4
Ca^{2+}	1-4	1-4	2.2
Cl^{-}	15	30-100	120
HCO_3^{-}	5	15-80	25
HPO_3^{2-}	6	4	1.2
Mg^{2+}	0.2	0.2	1.2
SCN^{-}	2.5	2	<0.2
NH_3	6	3	0.05

The clearance of compounds from plasma into saliva may involve:

- ultrafiltration through gap junctions between cells of secretory units. Only molecules with MW<1.9kDa are involved;
- transudation of plasma compounds into oral cavity from crevicular fluid or directly from oral mucosa;
- selective transport through cellular membrane by passive diffusion of lipophilic molecules or by active transport through protein channels [1].

Healthy adult subjects normally produce 500-1500 ml of saliva per day, at a rate of approximately 0.5 ml/min [1, 9-11].

1.1.2 Saliva composition

Inorganic compounds: whole saliva contains mainly water, strong and weak ions (Na+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, HCO_3^-, HPO_3^{2-}) which can generate buffer capacity (table 1). Many factors may modify the salivary ionic composition, furthermore, the composition of unstimulated saliva is different from stimulated saliva. Interestingly, ionic composition may also influence the activity of organic components in the saliva [1, 9, 11].

Organic compounds: small amounts of organic *non-protein compounds* can be detected in saliva; uric acid is one of the most important antioxidant salivary components; bilirubin and creatinine are also detectable. Saliva moreover contains also glucose, amino acids, lipids (cholesterol and mono/diglycerides of fatty acids), fatty acids (α-linoleic acid and arachidonic acid), lactate and amines (putrescein, cadaverine and indole) [1, 9, 11]. Saliva contains a large number of *protein/polypeptide compounds* and the recent development of high throughput proteomic approaches has facilitated progress in the cataloguing of the protein composition of saliva (table 2) [12-13]. Many of these proteins contain high levels of proline and are therefore designated proline-rich proteins (PRPs), further divided in acidic, basic and glycosylated basic PRPs. Histatins are small (3-5kDa) basic histidine rich proteins found in both parotid and submandibular/sublingual saliva with anti-candidal effects. Mucins, the major organic component of submandibular/sublingual saliva, are large glycoproteins consisting of two major groups defined MG1 and MG2. Other proteins can be derived from plasma leakage such as albumin, transferrin and IgG [1, 9, 11].

Table 2: Salivary proteins

	Origin	Concentrations
Total proteins		***3 g/l***
α-amylase		476±191 µg/ml
Albumin	Plasma	0.2±0.1 mg/ml
Cystatins	SM>SL	58±25 µg/ml
Hystatin	P	1190±313 µg/ml
sIgA	B lymphocytes	124.3-335.3 µg/ml
Lactoferrin	Mucous>serous	3.7±2.5 µg/ml
Lysozyme	SL>SM,P	21.8±2.5 mg/dl
Mucins	SM,SL	MUC5B:2.4±1.7 U/ml
PRPs	P	Acidic PRPs:456±139 µg/ml Basic PRPs:165±69 µg/ml
Statherin		36±18 µg/ml
Transferrin	Plasma	0.58±0.20 mg/dl

SM=submandibular gland, SL=sublingual gland, P=parotid gland

Moreover there is a large variety of polypeptides in saliva whose functions, in part, remain unknown. However saliva is a rich source of growth factors (table 3). These biologically active proteins include epidermal growth factor (EGF) and nerve growth factor (NGF), which are synthesized by the granular convoluted tubule cells, transforming growth factor -alfa (TGF-α), insulin, insulin-like growth factors I and II (IGF-I and II), transforming growth factor - beta (TGF-β) and fibroblast growth factor (FGF) [14].

Table 3. Growth factors identified in human saliva

Growth Factor	Origin in Human Saliva	Suggested Biological Significance	Reference
EGF	Parotid and submandibular glands	Systemic effect on skin and gastric wound healing processes in vivo	[15]
NGF	Submandibular gland	?	[16]
TGF α	?	Maintaining epithelial cell growth and implication in wound healing	[17]
TGF β	Submandibular gland	Cytoprotective against gastrointestinal tract ulceration by promoting mucosal epithelial cell replacement	[14]
FGF	?	Maintenance of mucosal health in the entire upper digestive tract	[18]
Insulin	Salivary glands or blood transport?	Regulation of amylase synthesis in acinar cells	[14]
IGF-I and IGF-II	?	Regulation of salivary gland homeostasis	[19]

1.1.3 Saliva functions

Saliva function can be organized into 5 major categories that serve to maintain oral health and create an appropriate ecologic balance [7, 20-22]:

1. lubrication and protection: as a seromucous coating, saliva lubricates and protects oral tissues, acting as a barrier against irritants. The best lubricating components of saliva are mucins that are excreted from minor salivary glands. Mucins also perform an antibacterial function by selectively modulating the adhesion of microorganisms to oral tissue surface.
2. buffering action and clearance: saliva behaves as a buffer system to protect the mouth from colonization by potentially pathogenic micro-organisms and neutralizing and cleaning the acids produced by acidogenic microorganisms, preventing enamel demineralization. The carbonic acid-bicarbonate system is the most important buffer in stimulated saliva, while the phosphate system is the prevalent buffer in unstimulated saliva. In saliva fluid is also present urea,

produced by aminoacid and protein metabolism. Urea influences pH releasing ammonia and carbon dioxide when hydrolyzed by bacterial ureases. More than 90% of the non-bicarbonate buffering ability of saliva is attributed to low-molecular weight histidine rich-proteins (HRP).

3. maintenance of tooth integrity: saliva modulates the demineralization and remineralization process. The main factors controlling the stability of tooth enamel are the active concentrations of free ions, namely calcium, phosphate and fluoride in solution and the salivary pH. Statherin, a salivary peptide, contributes to the stabilization of calcium and phosphate salts in solution, serves as a lubricant to protect the tooth from wear and may initiate the formation of the protective pellicle by binding to hydroxyapatite.
4. antibacterial, antiviral and antifungal activities: salivary glands are exocrine glands that secrete fluid containing immunological and nonimmunological agents. Immunological contents of saliva include secretory IgA, IgG and IgM. Secretory IgA is the largest immunological component of saliva: it can neutralize viruses, bacteria and enzyme toxins. Nonimmunological antibacterial salivary contents such as proteins, mucins, peptides and enzymes (lactoferrin, lysozymes and peroxidase) help to protect teeth against physical, chemical and microbial insults. Proteins such as glycoproteins, statherins, agglutinins, histidine-rich proteins and proline-rich proteins work to aggregate bacteria.
5. taste and digestion: the hypotonicity of saliva (low levels of glucose, sodium, chloride and urea) and its capacity to provide the dissolution of substances enhances the tasting capacity due to the presence of proteins and gustin, which binds zinc. Saliva is responsible for the initial digestion of starch, favouring the formation of the food bolus. This action occurs mainly by the presence of the digestive α-amylase (ptyalin). Salivary enzymes also initiate fat digestion. More importantly, saliva serves to lubricate the food bolus, which aids in swallowing.

In figure 1 (Amerongen 2002) the main functions of saliva in relation to its constituents are represented.

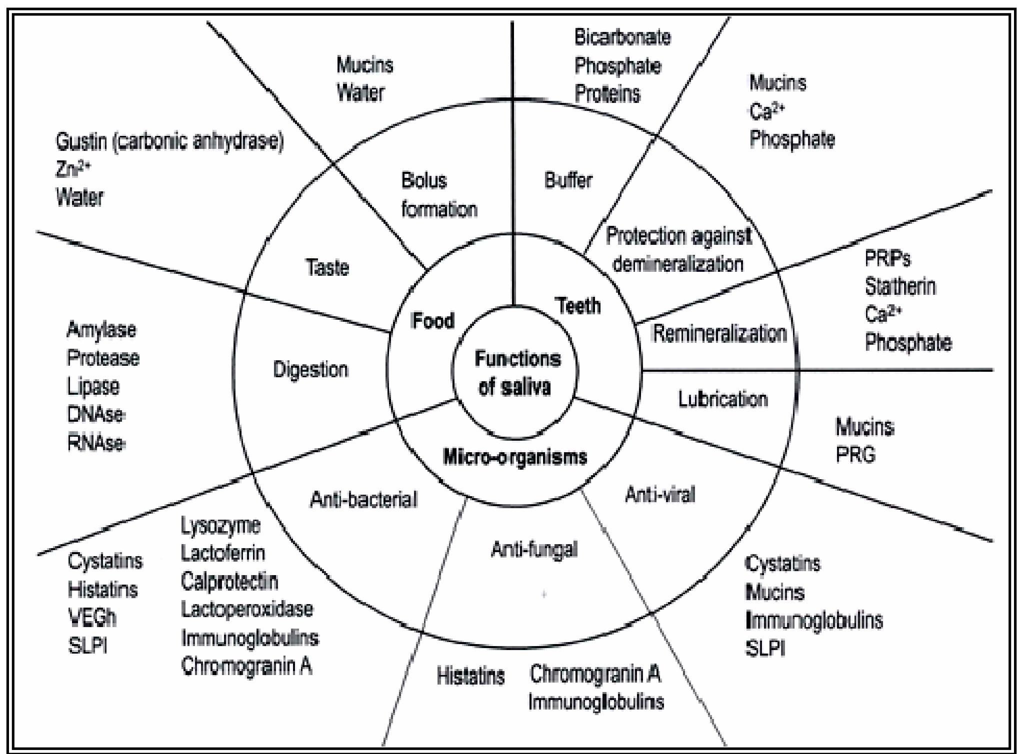

Figure 1. Schematic presentation of the main functions of saliva in relation to its constituents.

1.1.4 Saliva hormones

Some hormones commonly measured in plasma, such as steroids, non-steroid, peptide and protein hormones, can be detected in the oral fluid [1, 23]. *Cathecolamines* can be recognized in saliva ranging from 250 to 800 pg/ml, but their origin is unclear. They seem to originate by diffusion from serum, but there is also an amount of salivary cathecolamines derived by direct release from sympathetic nervous terminations, so their concentration is poorly correlated with that of plasma. [24]. Little is known about *thyroxin* and *triiodothyronine* levels in saliva. In preliminary studies they were detected and their salivary levels seem to correlate with plasma levels [25].

The *protein polypeptide hormones* are maybe a new analytical approach of the medicine laboratory but, at present time, there are still too few investigations about protein and polypeptide hormone levels in saliva. *Growth Hormone* (GH) has been detected using an IRMA method in saliva in 51 healthy individuals. Salivary hGH concentrations (8.6±11.1 μU/l) were 1000-fold lower than the respective values in serum (16.4±23.3 mU/l), but a clear correlation was found between salivary and serum hGH levels, suggesting a passive diffusion of GH from plasma to saliva [26]. Other protein

hormones have been detected in saliva such as prolactin [27], melatonin [28] and Insulin-like Growth Factor-I (for details see below).

Detection of *steroid hormones* is perhaps the most interesting application in salivary hormonal studies. Steroids have often been studied because salivary-free steroid hormones seem to reflect the serum-free levels [29-30]. Table 4 outlines steroid hormones measured in saliva specimens. Among these steroids, salivary cortisol measurement is today a widely accepted alternative to the determination in plasma (for details see below).

Table 4. Steroid hormones detectable in human saliva

	Methods	Concentrations
Androstenedione		
DHEA	ELISA	
DHEAS	ELISA	291.21±294.81 pg/ml
Testosterone	ELISA	140.30±154.15 pg/ml
17α-OH progesterone		
Progesterone	RIA	Luteal phase: 436±34 pmol/l Follicular phase: 22.1±2.7 pmol/l
Oestradiol	RIA	Luteal phase: 20.6±0.4 pmol/l
Aldosterone	RIA	138-475 pmol/l
Cortisol	RIA, EIA, DELFIA, HPLC	

1.1.4A Salivary Free Insulin-like Growth Factor I (sIGF-I)

The IGF family elements consist of three ligands (insulin, IGF-I and IGF-II), three specific receptors (IR, IGF-IR, IGF-II/mannose-6-phosphate receptor) and six specific binding proteins for IGF-I, namely IGFBP1-6 [31].

IGF-I was originally identified as a sulfation factor, stimulating growth of cartilage, and as a hypoglycaemic agent, which could not be neutralized by insulin antibodies and therefore was named “non-suppressible insulin-like activity”. The term “somatomedin”

was coined to reflect the ability of the substance to mediate the effects of GH. Somatomedin C was identified as the GH-responsive form and later IGF-I was shown to be the somatomedin substance. Today, IGF-I is considered as a true multi-potent peptide, affecting cell proliferation, differentiation and apoptosis, tissue growth and organ-specific functions throughout the body. Actually, the ubiquitous expression of IGF-IR indicates that virtually all tissues are responsive to IGF-I. The original somatomedin hypothesis evolved: from an initial proposal in which GH controls somatic growth by stimulating the liver production of IGF-I in 1950's, now it became clear that some local tissue as well as liver produce IGF-I and that there are direct effect of GH, mediated by the GHR and not involving IGF-I. These observations suggested that GH had both direct and indirect (via IGF-I) effects on growth and emphasized local, autocrine/paracrine action by IGF-I [32].

In circulation, IGF-I is present in three different fractions: a free fraction (<1%), binary complexes with IGFBPs and ternary complexes with IGFBP3 and ALS (acid labile subunit) in which more than 95% of circulating IGF-I is bound [33]. Initially, levels of serum IGF-I were determined by bioassays based on cartilage or fat cells, but due to their lack of specificity and/or laborious nature, these methods have been almost completely abandoned following the introduction of specific immunoassays. Serum *total IGF-I* remains the prevailing measurement in most experimental and clinical investigations: the employed immunoassay, usually non competitive immunoassay, necessitated a sample pre-treatment to remove or neutralize the influence of the IGFBPs; the most commonly used treatment is the acid ethanol extraction. Alternatively, acidification (to dissociate the binding between IGF-I and IGFBPs) together to the addition of excess IGF-II (to saturate the IGFBPs) can be employed [31, 33-34].

The development of assays for *free IGF-I* has allowed studies of whether serum free or total IGF-I yields the best picture of IGF-I bioactivity in vivo. There are several studies to indicate the importance of free vs total IGF-I in short-term dynamic metabolic changes but also in long-term steady-state changes of human physiology (like linear growth) [31, 33-34]. In particular it was demonstrated that free IGF-I is responsible for the negative feedback on GH pituitary secretion [35]. Early methods for determination of free IGF-I were based on neutral size-exclusion chromatography, neutral high

pressure liquid chromatography and reverse phase chromatography, although it was widely acknowledged that chromatography distorted the equilibrium between free and bound IGF and grossly overestimated the levels of unbound peptide. At present, there are two approaches to determine free IGF-I. The first includes ultrafiltration by centrifugation, followed by a sensitive sandwich immunoassay (UF free IGF-I). This technique is extremely laborious and not suitable for routine use. Therefore, the second approach foresees a direct sandwich immunoassay based on antibodies said to be specific for unbound IGF-I (IRMA free IGF-I) [31, 33-34]. The comparison of the two assays for free IGF-I has shown that concordant results are obtained in some conditions, whereas in others the difference is striking (table 5). The mentioned studies indirectly show that pituitary GH secretion is feedback regulated by free rather than total IGF-I and this is in agreement with Chen et al. [34-35].

Table 5. Changes in UF free IGF-I, IRMA free IGF-I, total IGF-I and endogenous GH secretion in various physiological and patho-physiological conditions in adult subjects.

Study group	**GH secretion**	**UF free IGF-I**	**IRMA free IGF-I**	**Total IGF-I**
Healthy subjects-increasing age	↓	↓	↓/↑	↓
Healthy subjects - GH treatment	↓	↑	↑	↑
Acromegaly	↑	↑	↑	↑
GHD	↓	↓	↓	↓
Short-term fasting	↑	↓	↓	↔
Obesity	↓	↑	↑	↔
Obesity T2DM	↓	↔	↔/↓	↔
T1DM	↑	↓	↓	↓
Chronic Renal Failure	↑	↓	↔/↑	↔
Anorexia Nervosa	↓/↑	↓	↔	↓

↓ reduced, ↔ unchanged, ↑ increased

Early estimates of free IGF-I levels were given from studies of **salivary content of IGF-I**. Nevertheless, only a few studies have measured sIGF-I providing interesting but not homogeneous results. sIGF-I concentrations, measured by a RIA technique, demonstrated an average of 0.52±0.37 µg/L (70±50 pmol/L) in an equally distributed male and female normal population, from birth to puberty [36]. Whether Ryan's analysis method measured the total or free IGF-I forms, or not, was not discussed. Halimi et al. measured sIGF-I in healthy male and female volunteers (age range 23–54 years); a group of acromegalic patients (29–62 years old) was also investigated. The analysis was carried out by a RIA method and saliva levels ranged from 2.3 to 26.9 µg/L (307 to 3587 pmol/L) and the concentrations were found to be increased in the acromegalic patients [37]. Another study [38], describing the IGF-I analysis in saliva, measured the free fraction of this hormone. A RIA technique was employed. The average concentration in saliva collected from normal individuals was 2.3±0.3 µg/L (307±40 pmol/L); as expected the concentrations in saliva collected from acromegalic and GH deficient patients demonstrated high and low levels respectively. A correlation between salivary and plasma IGF-I levels in all the patients and control subjects was also measured. The above mentioned literature did not demonstrate homogeneous results, but the used methods, in particular disparate antibodies, labelled and/or labelling antigens and experimental conditions, were different in each work. Moreover, the three studies were not comparable in term of subjects, age and other anthropometric data (table 6).

To assess the origin of sIGF-I, investigators have administered ^{125}I-IGF-I intracardially into rats, followed by gel chromatography and SDS-PAGE analyses of plasma and saliva samples [39]. Results obtained showed that IGF-I was unable to cross from the plasma through to saliva, suggesting that IGF-I in rat saliva is produced locally, like the other growth factors [40]. In situ hybridization revealed that IGF-I mRNA was localized primarily in the granular convoluted tubule cells of the gland [41]. Evidence that a functional IGF signalling pathway is present in salivary glands was provided by studies showing that IGF-I receptor is present in rat gland [42]. At present, many issues still have to be resolved, such as the precise molecular mechanisms responsible for the regulatory roles of the IGF system in oral biology and the potential interactions of the IGF system with other signalling systems, including the extracellular matrix and other hormones and growth factors [19]. Recently, Shpitzer et al. demonstrated significantly higher concentrations by 117% (p=0.03) in patients with oral squamous cell carcinoma

(OSCC) compared to healthy age- and gender-matched individuals. The authors suggested IGF-I as the growth factor that plays an important role in OSCC pathogenesis [43].

Table 6. Literature data on sIGF-I

Study group	sIGF-I µg/L	Serum total IGF-I µg/L	Reference
327 normal subjects (1day-20yr)	0.525±0.375	-	[36]
13 healthy adults (23-54 yrs)	5.4±2.6	176±42.9	[37]
17 acromegalics (29-62 yrs)	10.5±5.69	520±98.8	
14 normal subjects (31±5 yr)	2.3±0.3	315.0±26.6	[38]
5 acromegalics (43 yr)	17.2±6.3	494.8±58.0	
15 hypopituitary subjects (13±6)	1.3±0.2	50.3±17.5	

1.1.4B SALIVARY CORTISOL (SF)

Cortisol (F), the principal glucocorticoid in humans, is synthesized from cholesterol in cells of the zona fasciculata of the adrenal cortex. Its release into the systemic circulation is pulsatile and pulse amplitude varies according to a distinct circadian pattern. Serum glucocorticoid concentrations show a 3-5 fold change over 24h, being maximal just waking and declining thereafter to reach a nadir early in the sleep phase. Cortisol is also released in response to physical and/or emotional trauma. The circadian and stress-induced secretion of Cortisol is regulated by the hypothalamo-pituitary axis. The hypothalamus responds to adverse circumstances, be they physical or emotional, and circadian factors by activating the final common pathway to glucocorticoid synthesis. The first step is the release of two hypothalamic neurohormones, corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus to the anterior pituitary gland where they act synergistically via specific receptors to trigger the release of the adrenocorticotrophic hormone (corticotrophin, ACTH) from the pituitary corticotrophs into the systemic circulation. ACTH in turn acts on the adrenal cortex to initiate the synthesis of Cortisol, which is released immediately into the systemic circulation by diffusion. The sensitivity of the hypothalamo-pituitary-adrenocortical (HPA) axis to incoming stimuli is modulated by a negative feedback

system through the sequential release of CRH/AVP and ACTH from the hypothalamus and anterior pituitary gland is suppressed by glucocorticoids themselves [44]. Cortisol exerts its actions via 2 intracellular receptors which belong to the nuclear receptor superfamily and regulate the transcription of target genes, leading to the well known anti-inflammatory actions. The mineralcorticoid receptor (MR) has a high and approximately equal affinity for cortisol and aldosterone; it has a restricted distribution, being localized mainly to the distal renal tubule and other cells/tissues concerned Na^+/K^+ balance (e.g. sweat glands, parotid glands and colon). The glucocorticoid receptor (GR) has a lower affinity for Cortisol, but is glucocorticoid selective and does not bind aldosterone; GRs are widely distributed in the body [44-45]. Probably, the most important factor regulating the access of endogenous glucocorticoids to their receptors (GR or MR) is the local metabolism (pre-receptor metabolism) within the target cells themselves by 11β-hydroxysteroid dehydrogenase (11 β-HSD) enzymes. 11 β-HSD catalyses the interconversion of cortisol (F) and its inactive metabolite cortisone (E). Two distinct isoforms of 11 β-HSD have been characterized, type 1 and type 2 [44,46]. Their properties are summarized in table 7 [47].

Table 7. Comparison of 11 β-HSD type 1 and type 2 enzymes.

	11 β-HSD1	**11 β-HSD2**
Molecular mass	34 kDa	40 kDa
Enzyme kinetics	in vitro bidirectional in vivo mainly reductase	Only Dehydrogenase
Affinity	Low (Km-µM)	High(Km-nM)
Cofactors	NADP(H)	NAD^+
Distribution	Widespread (liver, adipose tissue, mature brain, vasculature)	Discrete (renal tubule, sweat glands, **salivary glands**, colon, developing brain, placenta, vasculature)
Function	supplies cortisol to GR	protects the MR from cortisol

Cortisol analysis can be carried out in serum from blood, urine and saliva specimens [48]. The laboratory analysis procedures carried out to measure cortisol in the

mentioned three different fluids use immunoassays methods and chromatographic techniques. Serum total cortisol measurements are performed by various methods like colorimetric and chromatographic methods, other are ligand assays like radioimmunoassay, competitive protein-binding assay, and non isotopic immunoassays. Non isotopic automated immunoassay procedures for cortisol, employ enzyme, fluorescent, or chemiluminescent-mediated reactions. The plasma cortisol values obtained by an immunoassay should not be directly compared with those obtained by a different assay procedure because total plasma cortisol concentrations are assay dependent. Then, healthy reference range values must be defined for each method. Methods for assaying serum free cortisol are complex, time-consuming, expensive and generally require ultra-filtration, equilibrium dialysis or steady-state gel filtration. No kit for the direct measurement of free serum/plasma cortisol is available at this moment. Free cortisol is usually calculated from measurements of total cortisol and plasma cortisol binding globulin (CBG) binding capacity or total cortisol and CBG plasma levels by Coolens' method [48].

Most laboratories routinely use automated immunoassays and apply the same procedure used for plasma to analyse cortisol urine levels. Assays which measure urine free cortisol directly, without sample pre-treatment, are subjected to interference from other steroids and their conjugates which may be present in urine at high concentrations. The effect of these interfering substances can be significantly reduced, although not completely removed, by organic solvent extraction of the steroids from the urine prior to measure urine free cortisol levels. Therefore the sample pre-treatment is recommended when using commercial immunoassay kits. Due to interferences between steroids and cortisol metabolites present in urine, the results obtained on urine samples, depending on the immunometric method used, are higher than those obtained by more specific methods such as High-Performance Liquid Chromatography (HPLC) techniques. For this reason a more appropriate term for results obtained by automated immunoassay platforms is "urine free corticoids" [48].

sF measurement is today a widely accepted alternative to the determination in plasma or serum: since the adrenal cortex is responsive to stress, venipuncture for blood collection can lead to an iatrogenic increase of plasma glucocorticoid levels. From this perspective, the stress-free salivary collection for F measurement has an advantage compared to plasma, especially when a F measurement has to be achieved in children. Salivary-free F concentrations do not seem to be dependent on salivary flow rate [1].

sF determination is based on the assumption that sF is a reasonable reflection of HPA axis function. Indeed, in the diagnostic setting, sF levels parallel those in plasma following ACTH and CRH stimulation and following exercise induced-stress. However, the correlation of sF with total plasma F is confounded by the presence of corticosteroid-binding globulin (CBG) in plasma and F is largely saturated up to 500-600 nmol/l. Besides sF correlates better with measured plasma free F than total plasma F [29]. The diurnal variation of plasma F corresponds by similar changes [49] in sF and hence timed sF has been used in a diagnostic setting. Early morning sF is useful as a screening tool for adrenal suppression. Recently, there has been considerable interest in the use of night time sF for the initial screening for Cushing's syndrome, even if reported cut-off values differ considerably. *Methodological* and standardisation issues are likely contributors to these differences. Determination of F in saliva has been carried out mainly by immunoassay and different specificities of F antibodies towards cortisone (E) are an important source of result variability. As previously mentioned, the salivary glands have abundant 11 β-HSD2 activity: 11 β-HSD2 is an uni-directional dehydrogenase converting F in E and, as a consequence, saliva, unlike plasma, has up to 3 times the level of E compared to F. Depending on the relative cross-reactivity of F antibodies towards E, there could be quite different values of sF measured by different immunoassays. Other techniques have been carried out to solve this problem: HPLC with UV detection, but also with laser-induced fluorimetric detection and with tandem mass spectrometry have been employed [29, 50].

Over the methodological variation relating to applied laboratory techniques, there are other sources of variation in sF measurements. A crucial factor affecting sF concentrations is the collecting device. The more used collection device is the Salivette®, that has an absorbent material in which saliva is collected. There are three types of absorbent materials, cotton and polyester and polypropylene and studies reported different results in sF measurements according to the different material. Moreover other collecting devices exist, with or without absorbent material and also in this case, investigators described differences in sF measurements. So it is recommended to use the same collecting device throughout a study or studies [50-53].

Above the methodological aspects, *biological* causes for sF variations are reported. Biological factors *independently* of individual choices (diurnal sF profiles, within subject and between subject variation, seasonal variation and effects on age and gender)

and biological factors *dependent* on individual choices (lifestyle choices, diet, medication, smoking, alcohol and physical activity) affect sF levels [50].

Few studies exist on salivary cortisone (sE) and on the simultaneous measure of sF and sE [54-55]. The possibility to determinate E and F in saliva can suggest 11 β-HSD2 activity through the F/E ratio. Jerjes et al. investigated the diurnal patterns of sF and sE in controls and subjects with chronic fatigue syndrome (CFS): sE diurnal rhythm similar to that of F was found. sF and sE levels in CFS syndrome were low compared to healthy controls. The ratio of sF to sE remained constant throughout the day without difference between the groups [55].

1.2 Insulin-like Growth Hormone-I and Cortisol/Cortisone: Clinical Applications, Use and Abuse

1.2.1 INSULIN-LIKE GROWTH FACTOR I (IGF-I)

IGF-I is a growth hormone dependent growth factor produced in various tissues but predominantly in the liver. IGF-I circulates bound to different IGF binding proteins (IGFBP 1-6), which are regulated by GH to varying degrees. IGFBP-3 is the most biologically important of these IGFBPs. IGF-I levels are also dependent on other hormones including sex steroids (which may contribute to the age-dependent fall in IGF-I levels), thyroxine (hypothyroidism is associated with a decrease in IGF-I), glucocorticoids (associated with an increase in IGF-I) and insulin that is the major regulator of the liver's ability to synthesize IGF-I. Moreover other physiological and biochemical variables regulate IGF-I secretion, such as nutritional status (as illustrated below), physical exercise (see next chapter), age, genetic factors and cytokines [56]. IGF-I acts not only as a mediator of the growth hormone action but also possesses a GH independent action. IGF-I plays a pivotal role on postnatal growth (a GH-dependent actions) and on prenatal growth and on a reproductive function (a GH-indipendent action) [32].

1.2.1a Clinical Applications

IGF-I, usually the **total circulating form**, is frequently analysed to assess the clinical impact of disorders of GH secretion and to monitor the response to therapy in patients who have these disorders. A first clinical application is the <u>GHD in children</u>, and, in particular, IGF-I is analysed in short children to select individuals who require a GH stimulation test. IGF-I measurement is not a stand-alone test to diagnose GHD definitively because a level within the normal range does not exclude this diagnosis. Instead, low IGF-I values predict the presence of <u>GHD in adults</u> with 95% accuracy, even if a "normal" value does not exclude the diagnosis. The degree to which a "normal" value will lead to a false-negative result depends on the age of the patient at diagnosis and the chronological age at the time of testing. Another application is the <u>prediction of growth response after GH therapy</u> and a positive correlation between the changes in growth rate and the changes in IGF-I is reported. IGF-I measurements found their greatest usefulness in <u>monitoring GH therapy in adults</u> to avoid side effects. In

general, if side effects develop, the IGF-I levels are usually substantially higher than the upper limit of normal [56, 57]. IGF-I remains the most sensitive and specific test in the diagnosis of acromegaly. However it should not be relied on exclusively for diagnosis or used as the sole indication of disease severity. Measurement of IGF-I is one of the preferred methods for post-operative assessment of cure, remission and long-term monitoring of persisting disease. Nevertheless, discordance between IGF-I and GH is seen more frequently in persistent acromegaly and both tests should be used together to achieve optimal control of disease. An IGF-I alone cannot be used to establish disease remission as it is known that patients with normal IGF-I but an abnormal GH suppression to OGTT are at risk of relapse and knowledge of this may alter the follow-up strategy [56-58].

As it was previously mentioned, in clinical practice serum total IGF-I assay is the most widely used IGF-related measurement in GH disorders, but in certain patients the inclusion of **free IGF-I** may be useful: the measurement of free IGF-I should be limited to cases where the diagnosis is uncertain and/or when treatment does not turn out satisfactory. Inclusion of free IGF-I may be of diagnostic value in patients suspected of GH disorders, who suffer from comorbidities which are known to affect GH/IGF-I axis, for instance obesity and chronic renal failure. Measurement of free IGF-I in patients during somatostatin analog treatment could be considered during dose-titration [33].

Many studies on cirrhotic patients have shown that insulin-like growth factor 1 (IGF-1) plasma levels are related to the severity of liver dysfunction [59-61]. These results suggest that IGF-I analysis is probably useful for monitoring liver function in the perioperative course of orthotopic liver transplantation (OLT). Bassanello et al. demonstrated that the severe GH/IGF-I axis impairment found in patients with end-stage cirrhosis reverted very rapidly in the first days after successful OLT. Postoperative modulation of IGF-I plasma level by the graft suggests that this hormone has the potential to become one of the early indicators of post-OLT liver function recovery [61].

Nutritional status is a key regulator of the circulating and tissue IGF-I. Protein and energy content of the diet influences plasma IGF-I concentrations. Because of its sensitivity to nutrient intake, the nycterohemeral stability of its concentration and its short-half life, IGF-I has been proposed as a marker of nutritional status, both for

screening malnutrition and for monitoring short term variations during nutritional intervention [62]. Low levels of IGF-I, often associated with increased serum GH concentration, have been repeatedly reported in patients with eating disorders such as Anorexia Nervosa and Bulimia Nervosa [63]. Recently Stoving et al. speculated that free or bioactive IGF-I are better indications of the nutritional condition rather than total IGF-I: their study demonstrated that total IGF-I, free IGF-I and bioactive IGF-I were tightly correlated and so total IGF-I level is a suitable marker of IGF-I bioactivity in anorexia nervosa [64].

1.2.1b Use

The IGF system is undoubtedly important both in physiology and pathology. In fact research comprises both body fitness like growth, physical exercise, and many diseases, such as growth disorders, cancer, atherosclerosis, diabetes, osteoporosis and neuromuscular disorders [57].

Growth disorders: growth hormone deficiency is characterized by low GH levels together to low IGF-I levels, usually due to pituitary disorders. In this disease, the GH administration usually is effective, but it became evident that GH therapy will not be effective in all patients with low serum IGF-I concentrations. The children with growth failure and inappropriately low serum IGF-I levels are classified as IGF deficient (IGFD); patients with low serum IGF-I associated with low GH concentrations are considered to have secondary IGFD, reflecting a defect in pituitary production of GH and these patients are typically highly responsive to GH therapy. Children with short stature and low serum IGF-I, despite normal or increased GH levels, are classified as primary IGFD, and generally they show no or marginal responses to GH. The molecular basis of primary IGFD can reflect defects of the GH receptor gene, mutations of the signal transducer and activator of transcription (STAT)-5b gene and mutations or deletions of IG-I gene, including bioinactive IGF-I. In these cases, individuals have elevated GH production and are unlikely to respond to treatment with additional GH. Food and Drug Administration has now approved IGF-I for treatment of primary IGF-I deficiency. Few clinical trials are conducted in patients with primary IGFD, with dosage differences and variations in patient including criteria. All studies showed that growth rates with IGF-I treatment, although significantly greater than pre-treatment values, fail to match the growth rates achieved with GH therapy of naïve GHD patients. The failure of IGF-I to fully duplicate GH’s effect on skeletal growth reflects IGF independent

effects of GH and/or the ability of GH to induce local production of IGFs at the epiphyses [57, 65].

Diabetes: in recent years, a very interesting literature on the role of the IGF system in pancreatic β-cell development and function has evolved. In poorly-controlled diabetic patients circulating IGF-I and IGFBP-3 are decreased. GH levels are increased so that GH resistance underlies the decrease in IGF-I levels. Many of the hypoglycaemic effects of IGF-I are mediated by the IGFR and it was therefore thought that IGF-I might "bypass" the insulin resistance of non-insulin dependent diabetic patients. Short-term studies and some long-term studies confirmed the efficacy of IGF-I treatment [57].

Osteoporosis: the IGF system plays a crucial role in skeletal development. Since IGF-I stimulates mineralization and levels decline with age, IGF-I has been proposed as a treatment for osteoporosis: the role of IGF-I treatment in this disease certainly requires further studies [57].

It is also of note that low serum concentrations of IGF-I in the face of normal or elevated GH levels may be observed in children with nutritional deficiencies, chronic inflammatory processes and some chronic diseases: future clinical studies of the potential efficacy of IGF-I therapy in some of such conditions should be considered [65].

1.2.1c Abuse

Endogenous peptide hormones with potential performance-enhancing properties are listed in World Anti-Doping Agency (WADA)'s List of Prohibited Substances in panel 1 "Substances and methods prohibited in sports at all time" under the section "Hormones and related substances" [66]. GH, IGF-I, together to erythropoietin, insulin, gonadotrophins and corticotrophins, and their releasing factors are prohibited. The misuse of endogenous hormones appears to have increases dramatically since their detection is extremely difficult. These substances are to a significant degree structurally and biochemically identical to the hormones naturally produced by the body and they are usually rapidly degraded and cleared from the body [67].

Nowadays, athletes such as cyclists, swimmers, power lifters and body builders take recombinant GH or IGF-I for their anabolic effects assuming that they will improve their performance, strength and look. Reportedly, it seems that athletes use GH or IGF-I for long periods of time with supra-therapeutic doses or in combination with other

doping substances such as anabolic androgenic steroids (AAS). However, the current clinical evidence of their anabolic effects in healthy adults is not well documented: there is the scientific lack of performance-enhancing effects in short-term studies with normal subjects. On the contrary, severe side effects have been described both in GH treated patients or athletes: intracranial hypertension, visual changes, headache, nausea, vomiting, peripheral edema, carpal tunnel syndrome, arthralgia, myalgia and acromegalic features such as nose and jaw enlargement, hypertension, cardiomegaly, increased cardiovascular risk, arthralgias, insulin resistance and diabetes [67-69].

1.2.2 CORTISOL (F) AND CORTISONE (E)

Cortisol (F), the most important glucocorticoid hormone, plays a pivotal role in the regulation of most essential physiological processes, including energy metabolism, maintenance of electrolyte balance and blood pressure, immune-modulation and stress responses, cell proliferation and differentiation, as well as regulation of memory and cognitive functions [48]. Cortisol exerts widespread actions in the body which are essential for the maintenance of homeostasis and enable the organism to prepare for, respond to and cope with emotional and physical stress (also physical exercise, as successively treated). It promotes the breakdown of carbohydrate and protein and exerts complex effects on lipid deposition and breakdown. Cortisol is also an important regulator of the immune and inflammatory processes and is required for numerous processes associated with host defence [44].

1.2.2a Clinical Applications

Cortisol assay is used in the assessment of adrenal, pituitary and hypothalamic function, and is important in the diagnoses of Cushing’s syndrome and Addison’s disease. Total serum cortisol concentration is the primary measurement following the administration of dexamethasone in the Cushing diagnosis. The diagnostical protocol includes free cortisol measurements in 24 h urine, considering the free form to reflect the integrated, unbound plasma cortisol levels. Urinary free cortisol generally shows good agreement with plasma cortisol levels in hypercortisolemic states. Recently salivary free cortisol has been suggested as a usefulness parameter for this syndrome [48].

1.2.2b Use

Glucocorticoids (GCs) were first introduced for the treatment of chronic inflammatory disease (e.g. rheumatoid arthritis) well over 50 years ago and, despite their multiple unwanted effects, they remain the most effective means of controlling this debilitating disease[44]. Greater understanding of the molecular mechanism whereby GCs suppress inflammation has opened up the potential for improvement in GCs [70]. The most common use of corticosteroids is in the treatment of asthma, where inhaled GCs have become first-line therapy and by far the most effective anti-inflammatory treatment.

There is another disease in which GCs are administrated as hormone replacement therapy (HRT). GC deficiency may occur primarily as a result of disease in the adrenal cortex (usually referred to as Addison disease), secondarily (centrally) due to specific pathology in the hypothalamus and/or the pituitary gland (typically a tumour) or in a tertiary manner due to a suppressed HPA axis after long-term high-dose steroid treatment. For the diseases involving adrenal insufficiency, the main challenge is to achieve adequate replacement therapy. The goals are to mimic the circadian steroid serum-time profile, to respond to the increase need for F during physical and physiological stimulation and to achieve normal well-being, normal metabolism and a favourable long-term outcome. The primary agents used are F and cortisone acetate: despite the anti-inflammatory activity, they have mineralcorticoid effects and are short-acting. Prednisolone has more anti-inflammatory effect than mineralcorticoid activity; dexametasone mainly has anti-inflammatory effect, with no mineralcorticoid activity, and it is longer-acting, with half-life of approximately 36-72 h [71].

1.2.2c Abuse

Glucocorticoids (GCs) are listed in World Anti-Doping Agency (WADA)'s List of Prohibited Substances in panel 2 "Substances prohibited in competition" and their use requires a therapeutic-use exemption [66], even if the maintenance of GCs on this list is a controversial issue. GCs are extensively used in sports medicine and they have not any demonstrated performance-enhancing effect. Anyway, GCs represent the most common substances found during anti-doping tests. Although there is no clear scientific evidence that GCs significantly increase performances in humans, limited data are available. Corticosteroids have anti-inflammatory and psychostimulatory properties: these properties probably explain their frequent prescription to athletes, making it possible to continue sporting activities or to shorten the recovery period after injury [72]. It is

suggested that GCs are taken indiscriminately in ultra-endurance events during competition to induce sense of euphoria and perhaps to mask pain [73].

1.3 Physical exercise & training status: physiological conditions to study hormones and saliva

The term “stress” describes the state of the organism under the influence of external or internal forces, stressors threatening to alter its dynamic equilibrium or homeostasis. Exercise is certainly a stress condition for which the body must find a new dynamic equilibrium. This dynamic process requires, among other things, adaptive responses of the hormonal system [74-75]. Actually, physical exercise affects five major categories of biological functions:

1. Nervous stress responses
2. Availability and utilization of metabolic energy
3. Maintenance of homeostasis or the constancy of internal environment
4. Growth and maintenance of skeletal and cardiac muscle and other components of the lean body mass
5. Reproduction

The effects of exercise are at once immediate and chronically pervasive. Acute adjustments in function to meet the challenges of physical work, homeostatic compensations during and after an exercise bout, as well as longer-lasting structural and functional adaptations, including changes in fertility and growth, are all largely controlled by cooperative actions of hormones and the chemical messengers of the autonomic nervous system (figure 2) [74].

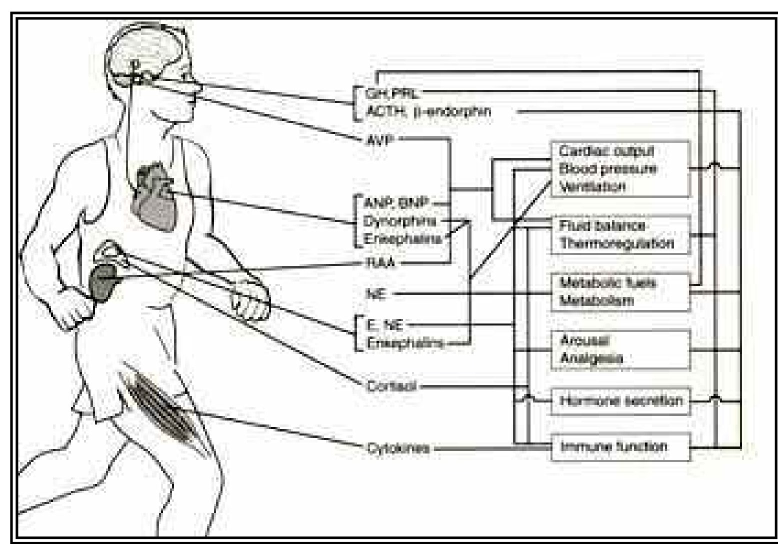

Figure 2. Principal stress hormones and their functions during intense exercise.

1.3.1 EXERCISE AND INSULIN-LIKE GROWTH FACTOR-I

That physical exercise significantly influences and is influenced by GH/IGF axis is well recognized [76]. The GH/IGF-I system, including the binding proteins, plays a key role in the adaptation of the organism to exercise [77-80]. Exercise is the most potent stimulus to GH release. [81] GH levels start to increase 10 to 20 min after the onset of exercise, peak either at the end or shortly after exercise and remain elevated for up to2 h after exercise [82-83].

The literature regarding the connections between circulating IGF-I levels, both as total and free forms, does not demonstrate uniform findings [84-86]. The discrepancies could be related to differences in the investigated physical exercise (type, duration, intensity), but numerous are the factors which might have an influence, such as body composition, training condition, sleep-waking rhythm and nutritional status. Moreover, long and short-term physical exercise, fatigue and energy expenditure/intake equilibrium have not always been taken into account [31, 87-88].

1.3.2 EXERCISE AND CORTISOL

Together to the GH/IGF-I axis, the activation of the HPA axis represents a physiological response to the energy, metabolic, vascular and sometimes neurophysiologic or psychological needs of exercise [72, 75]. Cortisol is the primary catabolic hormone, since it decreases protein synthesis and increases protein degradation. The anti-anabolic properties of this hormone are also related to the attenuation of other anabolic hormones, such as GH and testosterone [80]. Because of this catabolic character, of interest are the methods to reduce the cortisol response to exercise: carbohydrate intake before and during exercise results in lower cortisol responses, even if some reports describe no effect [78-79].

1.3.3 EXERCISE AND SALIVA

Little attention has been directed toward identifying the changes occurring in salivary composition in response to physical exercise [89]. Salivary flow rate appears to be influenced by physical activity; nevertheless, the interpretation of the results is sometimes difficult because of some methodological limitations, mainly concerning exercise protocols, saliva collection procedures and fluid consumption [90-91]. There

are few studies about sF and exercise. Cook reported the effect of an exercise of moderate intensity (heart rates maintained in the range of 130-160 bpm) lasting 40 min in 10 volunteers. Saliva collection procedure was not described and the device was not declared. A significantly increase in sF was demonstrated for all the subjects, but it is not clear the assay method to analyse sF [92]. More recently, Cadore et al. reported correlations between serum and salivary hormonal concentrations in response to resistance exercise in 28 healthy middle-aged males. Unstimulated saliva was collected by passive dribbling into a receptacle tube. sF concentrations were determined using radioimmunoassay kits with the sensitivity adapted for saliva. There were significant increases in response to resistance exercise in serum and salivary F. Serum cortisol was significantly correlated with sF before and after the resistance exercise protocol [93].

2. Research Aims

The present study aims to investigate salivary hormones in relation to a stress condition, such as a physical exercise.
The initial purpose is to develop new analytical methods to quantify hormone levels and subsequently to validate these analytical methods, in order to define sensitivity, limit of detection, precision, accuracy and specificity. Moreover the analytical range of the investigated measurements has to be defined to verify the suitability of the proposed method for the human salivary levels.
The third phase is to apply the studied methods in a group of human subjects, sedentary and athletes, at rest or during/after an exercise, verifying the possible effects of the trained condition and/or the exercising condition on the hormone levels.

In particular, the salivary hormones that are taking into account are:

- **SECTION I**: Insulin-like Growth Factor I (IGF-I) also in relation to the salivary total protein content and to the serum GH and IGF-I levels.
- **SECTION II**: Cortisol (F) and Cortisone (E) also in relation to the urine measurements of F and E.

3. SECTION I: Materials & Methods

3.1 Specimen collection: saliva and blood

Saliva samples were collected, using Salivette tubes containing a cotton swab without citric acid (Sarstedt), in the morning (8.30–9.00 a.m.). Teeth were brushed immediately after breakfast. The mouth was rinsed with tap water at least 2 h after breakfast and 10 min before the saliva collection. Within 10–15 min of collection, each saliva sample (typical volume about 1 ml) was centrifuged at 2000×g for 10 min to remove particulate material. 250 µL saliva aliquots were immediately stored in vials and placed in a freezer (−80 °C). This step is carried out also to break down mucin, until analysis. When frozen saliva samples were defrosted before analysis, each sample was centrifuged again (10 min, 2000×g) to obtain a clear fluid.

Venous blood (10 mL) was obtained by venepuncture of the antecubital vein into Vacutainer tubes containing Kalium EDTA. Blood specimen was centrifuged (15 min at 800×g) at room temperature, within 10–15 min. Suitable aliquots of the plasma specimens were immediately stored at −80 °C in a freezer. When frozen plasma samples were defrosted before the analysis, each sample was centrifuged again (10 min, 2000×g).

3.2 Free sIGF-I assay procedure

The assay was an enzyme-linked immunosorbent assay (ELISA) standardized for measuring the free-IGF-I concentration in human plasma samples by active free IGF-I ELISA (DSL-10-9400 commercial kit). All reagents were stored and used following the indications of the manufacturer.

The analysis procedure was modified with respect to the scheme described in the data sheet. Briefly 20 µl of sample buffer with 50 µl of IGF-I standard (0.05–5.00 µg/l) or unknown saliva sample were distributed in each microplate well (pre-coated with IGF-I monoclonal antibody mAb_1). Various incubation times were tested and 2 h was found to be optimal. The microplate was shaken (600–700 rpm) during incubation at room temperature. An automated microplate washer (ELx50 BioTek) was used for the washing steps. Subsequently, the second antibody, peroxidase conjugated (Ab_2-pox) (100 µl), was added to constitute a trimolecular sandwich complex (mAb_1-Ag-Ab_2-pox). After 30 min the plate was washed again and TMB (3,3′,5,5′-Tetramethylbenzidine) (100 µl) was added. A stopping solution (100 µl) was added (after about 30 min) and absorbance was read at 450 nm (AD340, Beckman Coulter). All steps were carried out at room temperature. Calibrators and samples were analyzed in duplicate.

A calibration curve was constructed using the standards at the pre-defined IGF-I concentrations (namely 0.00; 0.05; 0.10; 0.25; 0.50; 1.00 and 5.00 µg/l). The data points were plotted and a log–log function was calculated. The free IGF-I concentrations of the saliva samples were determined by interpolation from the calibration curve.

3.3 Free sIGF-I assay method: evaluation procedure

The detection limit was determined by the use of a calibrator, IGF-I free (zero standard), and defined as the concentration corresponding to a signal 3 S.D. above the average of the zero standard measured in 8 replicates repeated on three different days. The within and between run imprecision CVs (coefficient of variations) were also measured by the use of saliva specimens.

The within run imprecision was defined as the CV of 8 replicate determinations of 3 human saliva samples at 3 concentrations (low, medium and high) in one run. The between run imprecision was also defined by the coefficient of variation measured with 8 replicate determinations of 3 saliva samples in 8 separate assays. The recovery evaluation was carried out using four human saliva specimens, namely 0.15, 0.18, 0.22

and 0.33 μg/l. A human saliva sample at high IGF-I level was added to the four different samples to achieve saliva concentration increments ranging from 0.11 to 0.31 μg/l of free IGF-I, for a total of three enrichments per sample. The final free IGF-I concentrations were re-estimated taking into account the added quantities and the final volumes. The specificity was tested by spiking zero calibrator with IGF-II and insulin assayed up to 200 μg/l.

3.4 Pre-analytical variability tests

Effects of sample storage time, temperature and preservative solution on free sIGF-I

Fresh morning saliva samples were collected from healthy sedentary female volunteers (n=4). Specimens, after centrifugation, were aliquoted and stored either at 4 °C or at room temperature (25 °C) for 0 h (basal value), 4 h, 8 h and 24 h. After these pre-defined time delays before storage, the samples were deep-frozen and stored at −80 °C until the samples were assayed simultaneously.

The addition of a preservative solution (inhibitor solution - IS) was also studied.

> The IS solution (pH 5.0) was made up of: EDTA 0.27 mmol/L (Sigma ED2SS), PMSF 100 mmol/l (Phenyl-Methyl-Sulfonyl Fluoride), Thimerosal 25 mmol/l (Applichem A1278.0010), Aprotinin 0.5 g/l (Sigma-Aldrich A1153), Leupeptin 0.1 g/l (Sigma-Aldrich L2884), Antipain 0.1 g/l (Sigma-Aldrich A6191) and Pepstatin 0.1 g/l (Sigma-Aldrich P5318).

2 μl of IS were added to 150 μl of different standard solutions (0.0, 0.1, 2.5, 5.0 μg/l) and 10 μl of IS were added to Salivettes before the collection of 4 healthy sedentary female saliva specimens. All samples (standards and saliva) were analysed with and without IS addition (namely time zero). These frozen samples were retested about 9 months after the collection. These results were expressed as the percentage of concentrations measured at time zero.

3.5 Biological variation of sIGF-I

To evaluate the biological variation a day-to-day variation of free sIGF-I was also carried out: 5 saliva samples of 2 subjects for 5 consecutive days were collected and the samples were analysed all in the same batch.

3.6 Salivary total protein (sTP) assay

The assay utilised a modified colorimetric Coomassie BG method (Micro Protein Determination -Sigma Diagnostics cod. A-610). The saliva specimen was diluted with NaCl 9 g/L solution (1:6) and, after centrifugation, 25 µl of the supernatant was added to a spectrophotometer cuvette with 1250 µl of reagent (Coomassie blue). The absorbance was read at 595 nm and the value was measured by a standard curve ranging from 37.5 to 300.0 mg/l. The standard solutions were obtained by dilution of 300 mg/l human albumin with NaCl 9 g/L solution. Each sample (or standard) was analyzed in duplicate. This modified procedure demonstrated a limit of detection of 20 mg/l, the within run and between run imprecision CVs were 4% and 9% respectively.

3.7 Plasma hormone measurements

Plasma free-IGF-I assay was carried out following the “Active free IGF-I ELISA” Kit procedure (DSL-10-9400), an enzymatically amplified “two steps” sandwich-type immunoassay. The measured limit of detection of this assay was 0.09 µg/l. The within-run imprecision CV was 4.0%; the between-runs CV was 9.1%, according to the manufacturer's data.

Plasma total IGF-I was carried out following the “Active IGF-I ELISA” Kit procedure (DSL-10-5600), an enzymatically amplified “one step” sandwich-type immunoassay. The assay included a simple acid–ethanol extraction procedure in which IGF-I was separated from its binding proteins. The limit of detection of this assay was 0.03 µg/l, the within-run and between-runs imprecision CVs were 6.0% and 6.6%, respectively, according to the manufacturer's data.

Plasma GH analysis was carried out following the “Human Growth Hormone ELISA” Kit procedure (DSL-10-1900). This assay was an enzymatically amplified “two steps” sandwich-type immunoassay. The limit of detection of this assay was 0.03 ng/ml, the

within-run and between-runs imprecision CVs were 4.0% and 6.5%, respectively, according to the manufacturer's data.

All ELISA dual wavelength absorbance measurements at 450 and 620 nm were read on an automated microtiter reader (AD340, Beckman Coulter). Calibrators and samples were always analyzed in duplicate.

3.8 PROTOCOL A - effect of the training condition in females

The athletes were recruited from a young female group, well-trained, volleyball players (n=15), aged 22±4 yrs, weight 61±7 kg, height 1.71±0.05 m and BMI 21.0±1.4 kg/m^2. A sedentary females control group (n=14; aged 25±2 yrs, weight 56±8 kg, height 1.66±0.06 m and BMI 20.3±2.1 kg/m^2) was also recruited (table 8). The saliva (with IS included in each Salivette) and blood samples were collected from each subject at rest (in the morning at 9.00 a.m.). The analyses were carried out within 1 week from the specimen collection: free IGF-I and total protein content were measured in saliva as previously described; the ratio between sIGF-I and sTP was also calculated. Free IGF-I, total IGF-I and GH were analysed in plasma, as described in the precedent paragraph. The free IGF-I and total IGF-I ratio percent was also calculated.

3.9 PROTOCOL B - effect of an acute physical exercise in male cyclists

18 male, well-trained cyclists were recruited (aged 19±1 yrs, weight 70±4 kg and height 1.79±0.04 m, BMI 21.8±1.7 kg/m^2) (table 8). Saliva and blood specimens were collected from each cyclist at rest (B; 5-10min before exercise) and at the end of a 45min cycloergometer test at 50-60% of VO_2max (E), and in E about 15min passed between blood and saliva collection. Each plasma and saliva sample was immediately frozen at -80°C until analysis. Salivary free IGF-I and total protein content and plasma free IGF-I were analysed, as previously described. The ratio between sIGF-I and sTP was also calculated. The flow rate of each saliva sample was also calculated. Each salivette device was weighted before and after the collection; considering the saliva density of 1, flow rate was calculated as the ratio between the difference of the obtained

weights and the collection time (2 min). Then the sIGF-I rate of appearance and the sTP rate of appearance were calculated.

3.10 PROTOCOL C- effect of an acute physical exercise in male rowers
- effect of the training condition in males

Salivette saliva samples and blood samples were taken from a male rower group (*n* =8, age 29±8 yrs, weight 81±10 kg, height 1.85±0.08 m, BMI 23.7±1.8 kg/m^2) at Basal (-30min), Pre-Ex (0min), End-Ex (40min), Recovery (100min) undertaking a Concept II Rowing Step-test. This test consisted of six four minute rows, during which each athlete rowed constantly at the rate of 500 m/s. The pace was increased for each step of 25W with a 30 second recovery between each row. The 6th step was at the maximum effort. sIGF-I and sTP were measured as previously described; the ratio between sIGF-I and sTP was also calculated. The flow rate of each saliva samples was calculated, as before described. The sIGF-I rate of appearance and the sTP rate of appearance were calculated. Plasma GH, free-IGF-I and total IGF-I were also measured as previously described. The free IGF-I and total IGF-I ratio percent was also calculated. A group of well-matched male sedentary individuals (*n* =8, age 31±4 yrs, weight 75±10 kg, height 1.81±0.07 m, BMI 23.0±1.9 kg/m^2) was recruited to compare basal steady state levels (table 8).

The Ethic Committee of the Medical School of the University of Padova approved these research protocols. All the subjects were informed of the nature of the investigation before their consent to participate was obtained.

Table 8. Subject's anthropometric data

Protocol	A		B	C	
Subjects	*Athletes*	*Sedentary*	*Athletes*	*Athletes*	*Sedentary*
N	15	14	18	8	8
Sex	F	F	M	M	M
Age yrs	22±4	25±2	19±1	29±8	31±4
Weight kg	61±7	56±8	70±4	81±10	75±10
Height m	1.71±0.05	1.66±0.06	1.79±0.04	1.85±0.08	1.81±0.07
BMI kg/m^2	21.0±1.4	20.3±2.1	21.8±1.7	23.7±1.8	23.0±1.9

3.11 Statistical analysis

Data are expressed as average ± S.D. Wilcoxon signed rank test and Mann–Whitney U-test were used for comparison within groups and between groups, respectively. Friedman ANOVA test for repeated measurements was used. Results with $p<0.05$ were considered statistically significant.

4. SECTION I: Results

4.1 Free sIGF-I assay method: evaluation procedure

The calibration curve extension, for free sIGF-I assay, covered the range from 0.05 to 5.00 µg/l. This range allowed free sIGF-I to be measured in the majority of saliva samples. An example of a dose–response function and curve fit (y=1.18x−0.67; r^2 =0.998) is shown in figure 3. The detection limit was 0.07 µg/l. The within-run coefficients of variation calculated for low (0.23±0.02 µg/l), medium (0.93±0.11 µg/l) and high (2.36±0.19 µg/l) saliva samples were 8, 12 and 10%, respectively. The between-run coefficients of variation of 3 saliva samples (0.46±0.06, 0.59±0.07, 1.32±0.14 µg/l) were 14, 13 and 11% respectively. The average recovery value was 88±11% (n=12). For IGF-II, which has a molecular structure related to that of IGF-I, the cross-reactivity of only 0.1% was detected. Moreover the Insulin the cross-reactivity was not detectable.

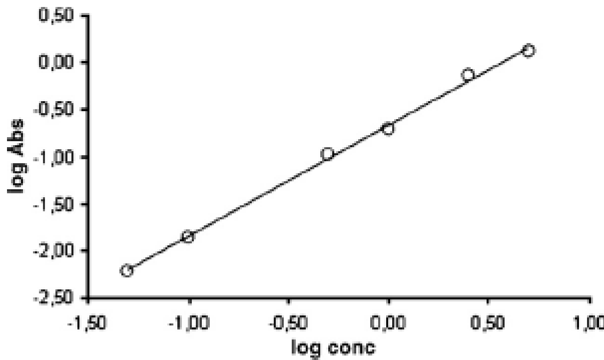

Fig. 3. Example of a standard curve of log of free IGF-I concentration vs. log of absorbance

4.2 Pre-analytical variability tests

Effects of sample storage time, temperature and preservative solution on free sIGF-I

The stability study is shown in table 9. The immunoreactivity detected by free IGF-I assay did not differ from the starting value, which is the measurement carried out in specimens immediately stored at −80 °C without delay, with saliva samples aliquots stored at either 4 °C or 25 °C for up to 8 h. A significant difference was found when saliva samples were stored at either 4 °C or 25 °C for 24 h ($p<0.05$).

The optical density (OD), measured with/without the addition of IS in standard solutions, was equivalent. The saliva samples collected with and witout IS (demonstrated statistically comparable concentrations (0.33±0.04 µg/l and 0.46±0.14 µg/l respectively) at time zero. When these samples (namely 100%) were retested (after 9 months of frozen storage) the saliva samples without and with IS were 28±9% and 71±3%, respectively.

Table 9. Effect of storage of saliva specimens at different times and temperatures

	Mean percentage of initial value (S.D.) n=4	
	4 °C	**25 °C**
4 h	101 (11)	118 (35)
8 h	93 (19)	82 (35)
24 h	76 (7) #	65 (21)#

# $p<0.05$ *vs* initial value.

4.3 Biological variation of sIGF-I

The day-to-day coefficient of variation was 11% for 5 saliva samples of 2 subjects, collected in five consecutive days.

4.4 PROTOCOL A - effect of the training condition in females

Free sIGF-I and sTP levels

Free sIGF-I, measured in 15 athletes and in 14 sedentary females, demonstrated an average concentration of 0.10±0.03 and 0.20±0.05 µg/l respectively. A significant difference ($p<0.001$) was measured comparing the sedentary females with the athlete group.

sTP levels, measured in saliva of the athletes and in the sedentary females, were comparable ($p=0.2$) and their average concentrations were 384±213 and 404±134 mg/l, respectively.

A significant difference ($p<0.05$) between athletes and sedentary females was also measured after the calculation of the ratio of free sIGF-I with sTP levels (0.34±0.15 and 0.58±0.34 µg/g, respectively).

Plasma GH, free and total IGF-I levels

Plasma free IGF-I, measured in 15 athletes and in 14 sedentary females, demonstrated an average concentration of 0.25±0.09 and 0.23±0.17 µg/l respectively ($p=0.2$). Total IGF-I, measured in the plasma of athletes and sedentary females, demonstrated an average concentration of 369±76 and 284±98 µg/l, respectively ($p=0.1$). The free/total IGF-I ratio percent did not demonstrate difference between the groups (0.07±0.02 and 0.07±0.04%, $p=1$). The plasma GH concentrations in the athletes and in the sedentary females were 5.0±6.2 and 2.6±2.9 µg/l respectively, and the GH measured in both groups was not statistically different ($p=0.4$).

4.5 PROTOCOL B - effect of an acute physical exercise in male cyclists

sIGF-I concentrations demonstrated a significant difference before (B) compared with the end (E) of the physical exercise (0.13±0.06 and 0.20±0.13 µg/l, B and E respectively; $p<0.01$). The plasma free IGF-I concentrations did not demonstrate any difference comparing B and E (0.19±0.13 and 0.16±0.06 µg/l respectively). The sTP level was significantly higher comparing B and E conditions (326±156 and 629±345mg/L respectively; $p<0.001$). No difference was observed when the ratio of free sIGF-I with sTP levels was calculated (0.34±0.17 and 0.40±0.36 µg/g, respectively).

A positive correlation between sTP and sIGF-I, both before ($y=0.001x+0.039$, $r^2=0.460$, $p<0.01$- figure 4) and after exercise ($y=0.001x+0.073$, $r^2=0.300$, $p<0.05$- figure 5) was observed. A positive correlation between sIGF-I and plasma free IGF-I only after the exercise ($y=1.448x-0.032$, $r^2=0.523$, $p<0.001$) was found (figure 6).

Taking into account that the flow rate of saliva was comparable in the two collections B and E (0.32±0.18 and 0.28±0.14ml/min respectively), the sIGF-I rate of appearance was significantly increased comparing before with end exercise (0.51±0.35 and 0.82±0.49 ng/min, B and E respectively; $p<0.01$). The sTP rate of appearance was also significantly increased (1.46±1.13 and 2.63±1.74 mg/min, B and E respectively; $p<0.01$).

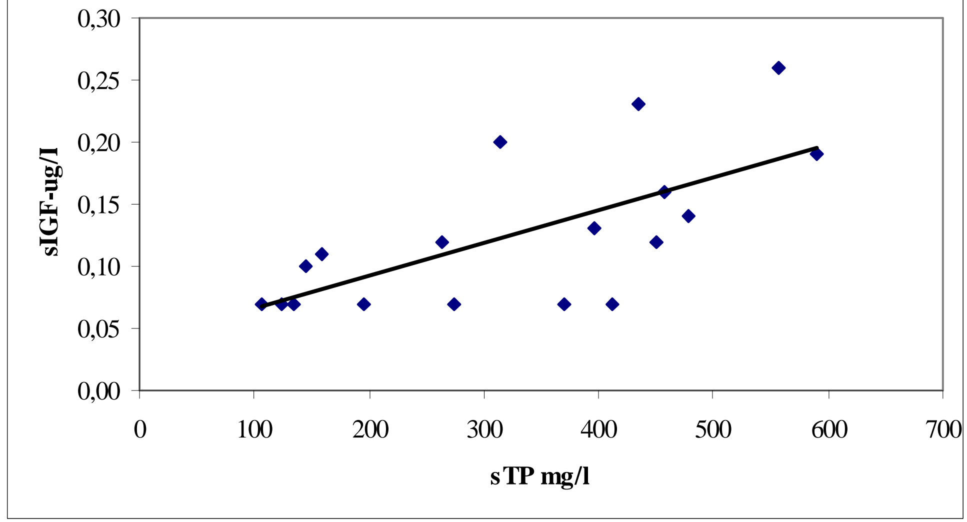

Figure 4. Correlation between sIGF-1 and salivary total proteins in pre-exercise (B).

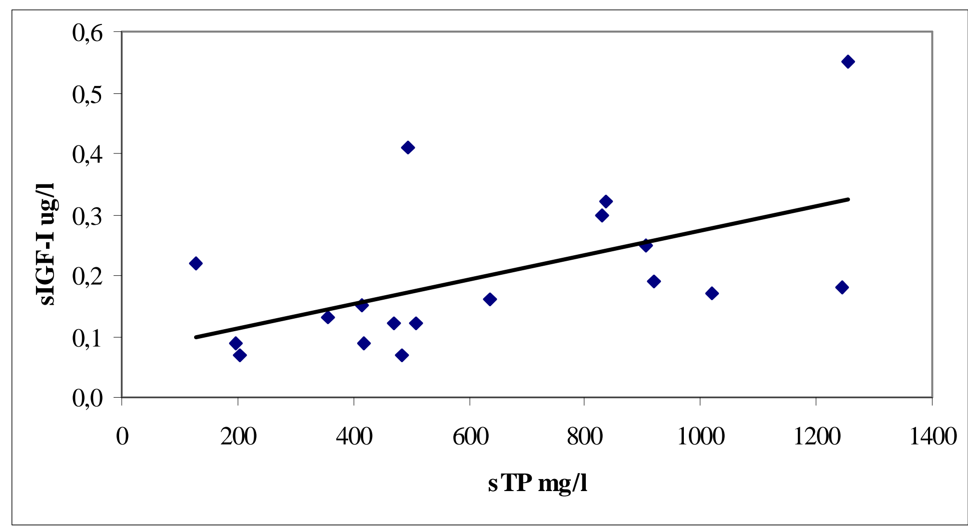

Figure 5. Correlation between sIGF-1 and salivary total proteins in post-exercise (E).

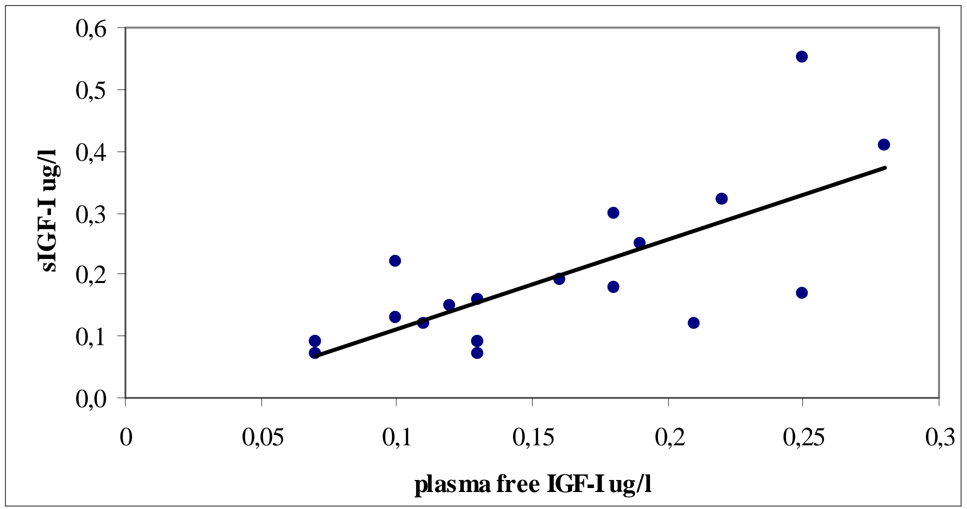

Figure 6. Correlation between sIGF-1 and pIGF-1 in post-exercise (E).

4.6 PROTOCOL C - effect of an acute physical exercise in male rowers
- effect of the training condition in males

Effect of an acute physical exercise in male rowers

Saliva and plasma parameters are reported in table 10 and table 11, respectively.

A positive correlation between sTP and sIGF-I, both in the pre-ex ($y=0.003x+0.040$, $r^2=0.789$, $p<0.001$- figure 7) and in the recovery ($y=0.003x+0.320$, $r^2=0.910$, $p<0.005$- figure 8) was observed.

Table 10. Saliva parameters at Basal (-30min), Pre-Ex (0min), End-Ex (40min), Recovery (100min) of male rowers undertaking a Concept II Rowing Step-test.

	Basal	**Pre-Ex**	**End-Ex**	**Recovery**
sIGF-I µg/l	0.97±0.43	1.23±0.86	1.52±0.57	1.45±0.71
sTP mg/l	383±184	478±306	877±285#	634±241°
sIGF-I/sTP µg/g	2.58±0.91	2.56±1.05	1.82±0.68	2.21±0.43
Flow rate ml/min	0.64±0.02	0.63±0.03	0.62±0.02	0.63±0.04
sIGF-I rate of appearance ng/min	1.52±0.69	1.96±1.36	2.44±0.89	2.32±1.15
sTP rate of appearance mg/min	0.60±0.29	0.75±0.48	1.42±0.46#	1.02±0.39°

# $p<0.05$: end-ex *vs* pre-ex

° $p<0.05$: recovery *vs* pre-ex

Table 11. Plasma parameters at Basal (-30min), Pre-Ex (0min), End-Ex (40min), Recovery (100min) of male rowers undertaking a Concept II Rowing Step-test.

	Basal	**Pre-Ex**	**End-Ex**	**Recovery**
hGH µg/l	0.37±0.56	0.97±1.49	7.56±2.87#	1.02±0.51
Free IGF-I µg/l	0.18±0.04	0.20±0.06	0.27±0.07§	0.23±0.07
Total IGF-I µg/l	262±56	251±53	237±40	240±48
Free/Total IGF-I ratio%	0.07±0.01	0.08±0.02	0.11±0.03§	0.10±0.02

# p<0.005: end-ex *vs* pre-ex

§ p<0.001: end-ex *vs* pre-ex

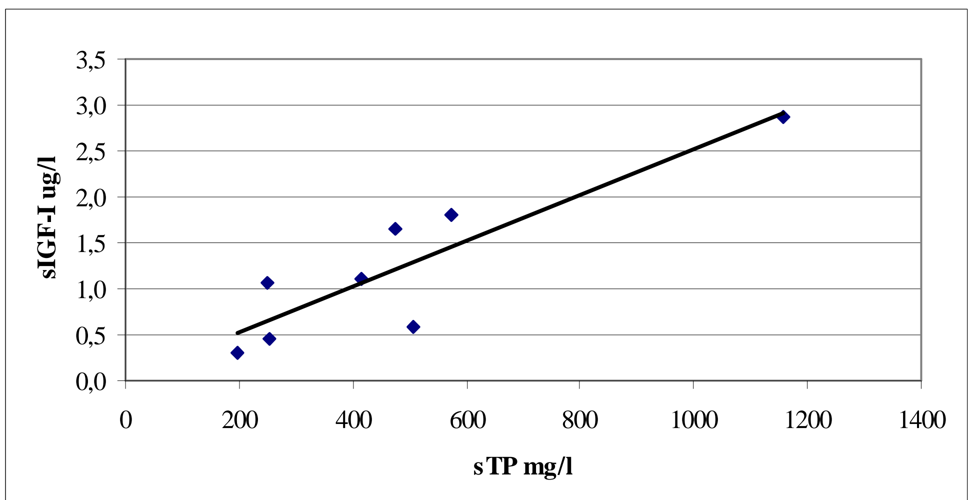

Figure 7. Correlation between sIGF-1 and salivary total proteins at pre-exercise (Pre-Ex).

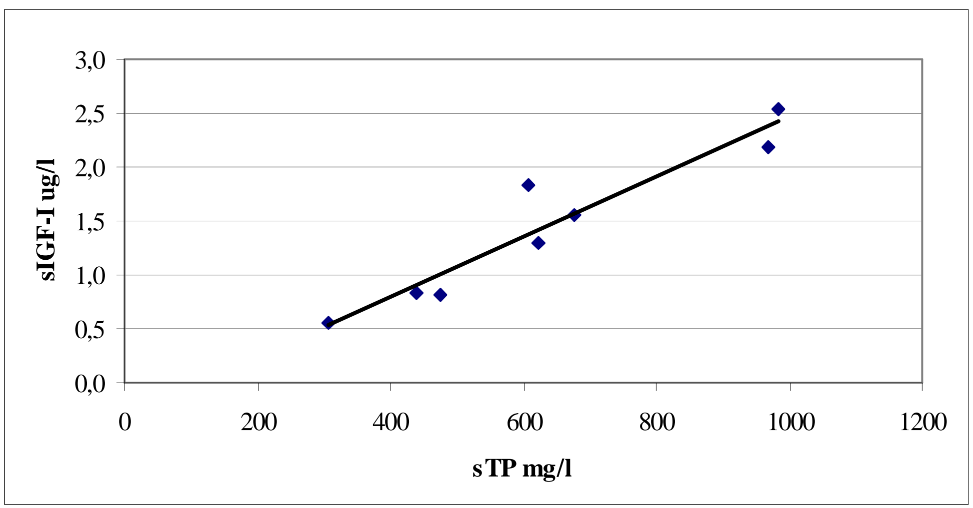

Figure 8. Correlation between sIGF-1 and salivary total proteins at the Recovery.

Effect of the training condition in males

sIGF-I levels in rowers were significantly higher compared to sedentary males (0.97±0.43 and 0.18±0.11 μg/l, respectively; $p<0.001$). No difference was observed in sTP of the athletes compared to the sedentary (383±184 and 475±179 mg/l, respectively; $p=0.3$). A significant difference was observed when the sIGF-I and sTP ratio was calculated (rowers: 2.58±0.91 ng/mg, sedentary: 0.35±0.14 ng/mg; $p<0.001$).

Plasma free IGF-I, measured in 8 rowers and 8 sedentary males, demonstrated an average concentration of 0.18±0.04 and 0.23±0.11 μg/l respectively ($p=0.3$). Total IGF-I, measured in the plasma of the athletes and the sedentary males, demonstrated an average concentration of 262±56 and 357±167 μg/l, respectively ($p=0.1$). The free/total IGF-I ratio percent did not demonstrate difference between the groups (0.07±0.01 and 0.07±0.01 %; $p=0.2$). The plasma GH concentrations in the athletes and in the sedentary males were 0.37±0.56 and 0.43±0.97 μg/l respectively, and the GH measured in both groups was not statistically different ($p=0.9$).

5. SECTION II: Materials & Methods

5.1 Specimen collection: saliva and urine

Urine samples were collected as detailed in protocol D. Suitable aliquots of the collected urine specimens were immediately stored at –80 °C in a freezer. When frozen urine samples were thawed before the analyses, each sample was centrifuged again (10 min, 2000×g).

Saliva samples were also collected from the investigated subjects at the same time of urine collection; the saliva specimens were also collected and processed following the procedure already described in the chapter Material & Methods-section I.

5.2 Salivary Cortisol and Cortisone assay procedure

Stock solutions of 1 mg/ml of cortisol (2.77 mmol/l), cortisone (2.75 mmol/l) and Internal Standard (IS) (2.67 mmol/l) were prepared separately by dissolution in methanol. All solutions were stable for up to 4 months when stored at –80 °C. The standard solutions were prepared from the stock solutions by dilution with 20mM phosphate buffer pH 7.4.

Extraction procedure. Each saliva specimen was extracted using the solid phase extraction (SPE) technique. The SPE Discovery DSC 18 columns (endcapped - 100

mg/ml Supelco – Bellefonte, PA, USA) were used for saliva sample clean-up and enrichment. Each sample (500 µl), spiked with IS 53 nmol/l, was applied to the SPE column, which was previously equilibrated with 3.0 ml methanol followed by 1.5 ml water. The successive steps were: washing with 0.25 ml of water followed by 0.5 ml of acetone: water solution 1:4 and 0.25 ml of hexane, and elution with 1.5 ml of diethyl ether. Each sample was dried and successively re-suspended with 50 µl of HPLC mobile phase.

The standard solutions were also extracted following the same procedure to obtain the calibration curve.

HPLC-procedure. After the SPE, each extracted sample (saliva and standard) was separated by HPLC technique. The chromatographic equipment consisted of a solvent delivery system mod.126 (Beckman Coulter, Fullerton, CA, USA), an injector mod.7725 Rheodyne (Beckman Coulter, Fullerton, CA, USA), a detector UV mod. 166 (Beckman Coulter, Fullerton, CA, USA) and a column oven Thermosphere TS-130 (Phenomenex, Torrance, USA). The analytical column was a stainless steel Discovery HS-F5 column (15cm x 2.1 mm, 5µm), purchased from Supelco (Bellefonte, PA, USA). The chromatography separation was carried out by isocratic elution, with 27% acetonitrile and 73% water at 35°C and the process of each chromatographic analysis ended in 14 min. The mobile phase flow rate was 0.25 ml/min. The sample injection volume was 10 µl. A System Gold (Beckman, V 8.1) software integration was employed for F and E quantitative analysis measuring the absorbance at 254 nm wavelength. The chromatographic conditions were chosen in terms of peak shape, chromatographic analysis time, column efficiency (N), capacity (k), selectivity (α) and resolution (R). These experimental parameters are summarised in table 12. An example of an extracted standard chromatogram is reported in figure 9. An example of an extracted saliva sample is reported in figure 10.

The range of 5.5-55.0 and 11.0-110.0 nmol/l for F and E, respectively, were used for the calibration curves, defined by the peak height ratios of F and E against IS (53 nmol/l). The concentrations of saliva F and E were calculated from these height ratios using the calibration curves. The linearity of the calibration curves was also calculated, and a determnation coefficient (r^2) of 0.998 and of 1.0 was deemed satisfactory for F and E, respectively.

Table 12. Chromatographic parameters related to the experimental conditions utilizing HS-F5 column.

	Cortisol (F)	**Cortisone (E)**	**IS**
Retention time (*t*) min	6.82	8.23	10.41
Efficiency (*N*)	1323	1781	3082
Capacity (*k*)	5.50	6.84	8.91

	F-E	**E-IS**
Selectivity (α)	1.24	1.30
Resolution (*R*)	1.84	2.85

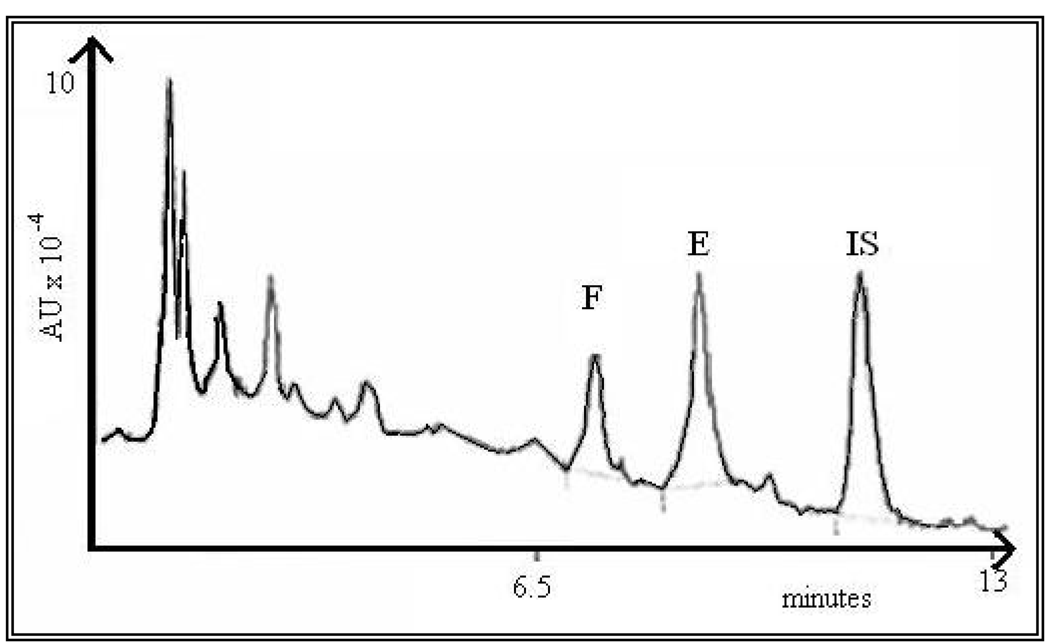

Figure 9. HPLC separation of an extracted standard solution containing 13.9 nmol/l F, 27.5 nmol/l E and 53 nmol/l IS.

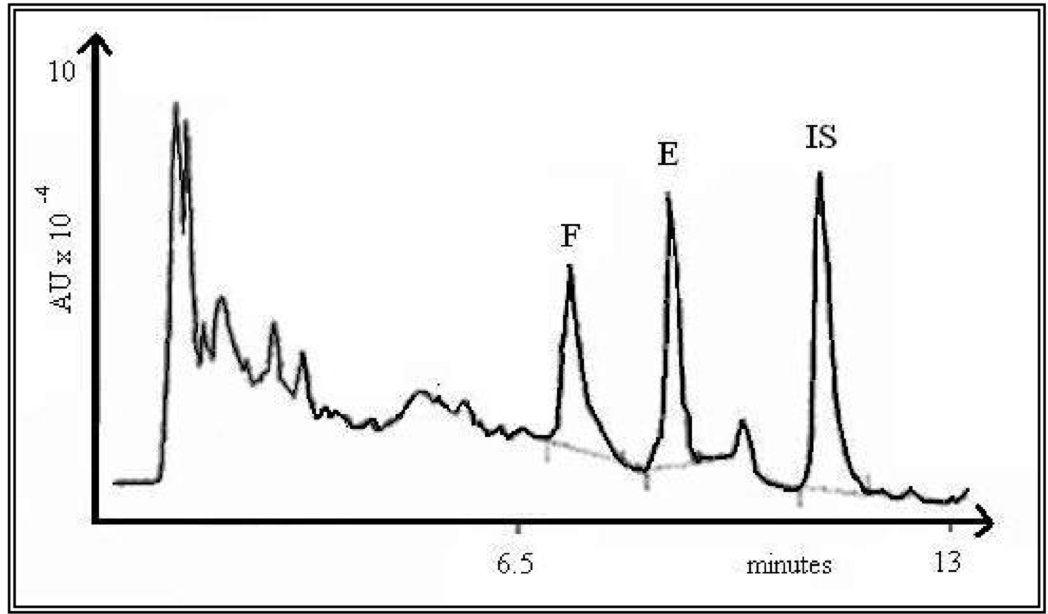

Figure 10. HPLC separation of an extracted saliva sample (16.3 nmol/l F, 32.4 nmol/l E).

5.3 Salivary Cortisol and Cortisone assay method: evaluation procedure

The detection limit was calculated by measuring the noise of the chromatography signal in three zones (1–2 min interval) after the F, E and IS peaks using saliva extracted samples (n = 10). The average of the various peaks (in total thirty peaks) in these windows plus three standard deviations was calculated and the F and E LODs were obtained from the calibration curves.

The within- and between-runs imprecision CVs (coefficient of variations) were also measured by the use of saliva specimens. The within-run imprecision was defined as the CV of 10 replicate determinations of 3 human saliva samples extracted in one run. The between-run imprecision was also defined by the coefficient of variation measured analysing three saliva samples extracted for five consecutive days.

The dilution test was carried out with four different saliva samples previously spiked with 40 and 79 nmol/l of F and E, respectively, then diluted 1:2, 1:4 and 1:8 using 20 mmol/l phosphate buffer pH 7.4 and finally extracted and analysed for F and E measurements.

The recovery evaluations were carried out using four different saliva samples spiked with two different levels of standards: 13.9 and 27.5 nmol/l and 27.7 and 55.0 nmol/l for F and E, respectively. The recovery % was expressed as ratio of (found concentration - endogenous concentration) / spiked concentration x 100.

5.4 Pre-analytical variability tests

Effects of sample storage time, temperature, cycles of freeze/thaw on sF and sE.

Salivary stability before centrifugation. Three stimulated saliva samples were collected with three Salivette tubes from each subject constituting a group of five volunteers. The first Salivette collection was immediately centrifuged and the saliva sample was stored at -80 °C (basal); the second and third Salivette specimens were stored at +4 °C and at room temperature, for 24h for each subject, respectively. Then both were centrifuged and each saliva was stored at -80 °C until analyses. The results were expressed as percentage of its related basal values.

Salivary stability after centrifugation. Stability at different temperatures: the saliva stability, obtained after centrifugation, was determined by keeping aliquots of three saliva samples either at room temperature and at 4 °C for 0 h (basal), 4 h, 8 h and 24 h before storage at -80 °C, and analyzing these samples simultaneously. The results were expressed as percentage its basal values.

Salivary stability stored at -80°C. The stability of saliva samples stored at -80°C was tested: three saliva samples were stored at -80 °C for 1 day (basal) and for 1, 2 and 3 months before extraction and separation. The results were expressed as percentage of basal values.

Stability after freezing-thawing procedure. The salivary stability of F and E was also assessed after a freezing-thawing various procedures: one aliquot of three saliva samples was stored at –80 °C (basal sample), another aliquot of the same sample underwent freezing and thawing for four cycles and then stored at –80 °C until the analysis. The results were expressed as percentage with respect to its basal values.

5.5 Urine F and E measurements

Urine cortisol (uF) and cortisone (uE) were analysed with a previously described SPE-HPLC method [94]. Summing up, 3.0 ml of each urine or standard sample (F: 69-690 nmol/l, E: 139-1387 nmol/l) spiked with IS 133 nmol/l, was applied to a SPE column DSC 18 (endcapped- 500mg/ 3 ml), which had previously been equilibrated with 10 ml of methanol followed by 5ml of water. After sample injection the washing steps were: 1ml of water followed by 5ml of acetone/water solution (1/5, v/v) and 1ml of hexane.

Two millilitres of diethyl ether were then added and the eluate was collected and desiccated. Each dried sample was successively re-suspended with 200µl of HPLC mobile phase. Chromatography separation was carried out by isocratic elution, with methanol–water (63/37, v/v) at 30 °C and the process of each chromatographic analysis ended in 11 min. The mobile phase flow rate was 0.8 ml/min. The sample injection volume was 20 µl and the absorbance detection was monitored at 254 nm. The calculated detection limits were 9 and 82 nmol/l for F and E, respectively. The intra-assay CVs were 0.9% and 2.2% for F and E, respectively, with inter-assay CVs of 7.1% and 5.0% for F and E, respectively. The average analytical recoveries of urine samples were 95% for F and 96% for E. The dilution test demonstrated good correlations between the measured and the expected values for F (r^2= 1.000) and E (r^2= 0.999).

5.6 Urine creatinine measurement

Urinary creatinine (crea) was measured by a previously described capillary electrophoresis (CE) method [95]. Briefly, the CE method was carried out in a fused-silica capillary of 47cm length x 50 µm internal diameter. The running buffer was 100mmol/l acetate, pH 4.4. The constant voltage was 30 kV and the samples (40-200 µmol/l standards or urine diluted 1:80) were electrokinetic mode injected, 10 kV for 10s. UV absorbance detection was monitored at 254 nm and the creatinine migration time was 3.19 min. The calculated detection limit was 23.1 µmol/l, the intra-assay and the inter-assay CVs ranged from 2.9 and 3.7 % and from 8.0 and 8.4 %, respectively. The average analytical recovery of urine creatinine samples was 99% and the dilution test demonstrated a good correlation (r^2=0.988, p=0.001) between the measured and the expected values.

5.7 PROTOCOL D - effect of an acute physical exercise in male and female rowers

Salivette saliva samples were taken from male rowers (table 13) (*n* =8, age 29±8 yrs, weight 81±10 kg, height 1.85±0.08 m, BMI 23.7±1.8 kg/m^2) and from female rowers (*n* =5, age 20.2±0.4 yrs, weight 62.2±0.4 kg, height 1.68±0.45 m, BMI 22.0±0.4 kg/m^2) at Basal (-30min), Pre-Ex (0min), End-Ex (40min), Recovery (100min) undertaking a

Concept II Rowing Step-test, described in detail in the chapter Material & Methods-section I. Urine samples were taken at Basal, End-Ex and Recovery. sF and sE, together to uF and uE and uCr were measured as previously described.
The Ethics Committee of the Medical School of the University of Padova approved this research protocol. The subjects were informed of the nature of the investigation before their consent to participate was obtained.

Table 13. Subject's anthropometric data

Protocol	D	
Athletes	Rowers	
N	8	5
Sex	M	F
Age yrs	29±8	20.2±0.4
Weight kg	81±10	62.2±0.4
Height m	1.85±0.08	1.68±0.45
BMI kg/m^2	23.7±1.8	22.0±0.4

5.8 Statistical analysis

Data are expressed as mean ± S.D. Wilcoxon signed rank test and Mann–Whitney U-test were used for comparison within groups and between groups, respectively. Friedman ANOVA test for repeated measurements was used. Differences with $p<0.05$ were considered statistically significant.

6. SECTION II: Results

6.1 Salivary Cortisol and Cortisone assay method: evaluation procedure

The calibration curves were linear in the range of 5.5-55.0 and 11.0-110.0 nmol/l for F ($y = 0.024x + 0.124$; $r^2 = 0.998$; $p<0.001$) and E ($y = 0.022x + 0.073$; $r^2 = 0.999$; $p<0.001$), respectively (figure 11 and figure 12). The calculated LOD were 0.1 and 0.2 nmol/l for F and E, respectively. The intra-day CV of the method ranged from 5.8 % to 7.0 % for F and from 2.7 % to 6.6 % for E, for the three saliva samples. The inter-day CV ranged from 11.7 % to 13.1 % for F and from 5.6 % to 7.0 % for E, for the four saliva samples. Figure 13 and figure 14 show the correlation between the expected and measured concentrations of F ($y = 0.989x - 0.348$; $r^2 = 0.973$; $p<0.0001$) and E ($y = 1.015x - 2.154$; $r^2 = 0.996$; $p<0.0001$), respectively, for the dilution test. The recoveries for the first and the second level of enrichment were 88±5 and 92±14 % (for F) and 103±12 and 107±12 % (for E), respectively.

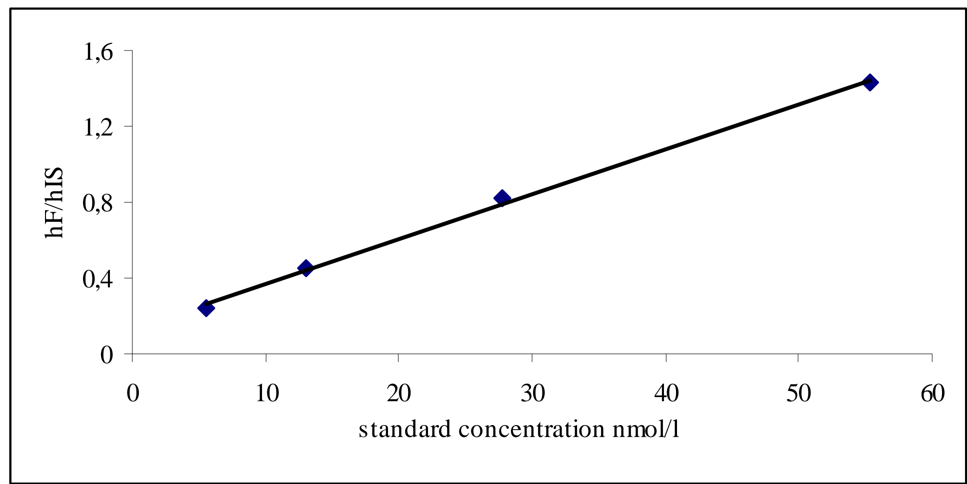

Figure 11. F calibration curve ($y = 0.024x + 0.124$; $r^2 = 0.998$; $p<0.001$).

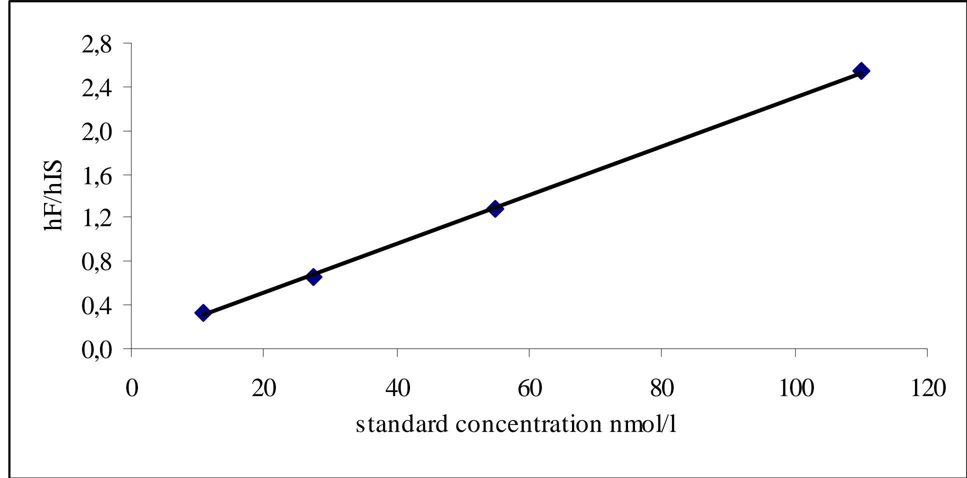

Figure 12. E calibration curve ($y = 0.022x + 0.073$; $r^2 = 0.999$; $p<0.001$).

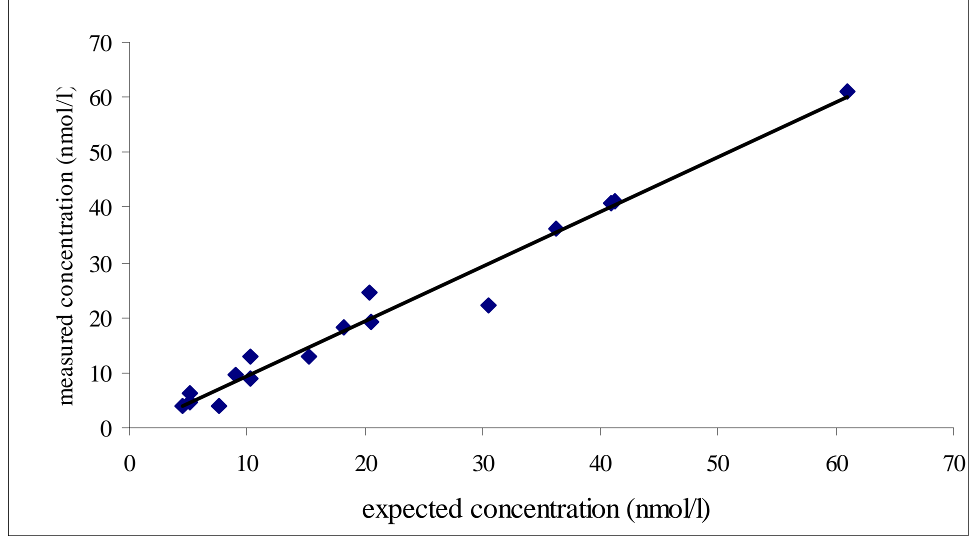

Figure 13. F dilution curve.

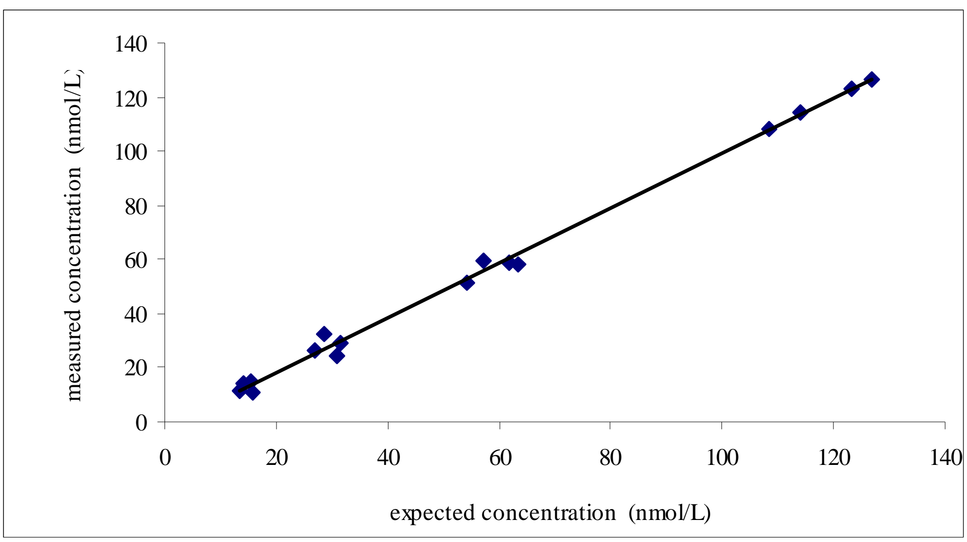

Figure 14. E dilution curve.

6.2 Pre-analytical variability tests

Effects of sample storage time, temperature, cycles of freeze/thaw on sF and sE.

Salivary stability before centrifugation. F and E values measured in stimulated saliva samples, from five volunteers, collected in Salivette tubes, kept at 4°C and RT for 24h and then centrifuged and stored at -80°C, did not demonstrate significant differences in respect to the values measured in the samples collected and immediately centrifuged and stored at -80°C (figure 15).

Salivary stability after centrifugation. No significant differences were observed in F and E values measured in three saliva samples collected and kept at RT and at +4°C for 0 h (basal), 4h, 8h and 24h before separation and storage at -80°C (figure 16 and figure 17).

Salivary stability stored at -80°C. The F and E concentrations in three saliva samples collected and stored, after separation, at -80°C until three months before extraction and separation, did not demonstrate any significant difference (figure 18).

Stability after freezing-thawing procedure. The F and E values, obtained in three saliva samples after separation and four cycles of freezing and thawing, did not differ significantly in respect to the basal measured values (figure 19).

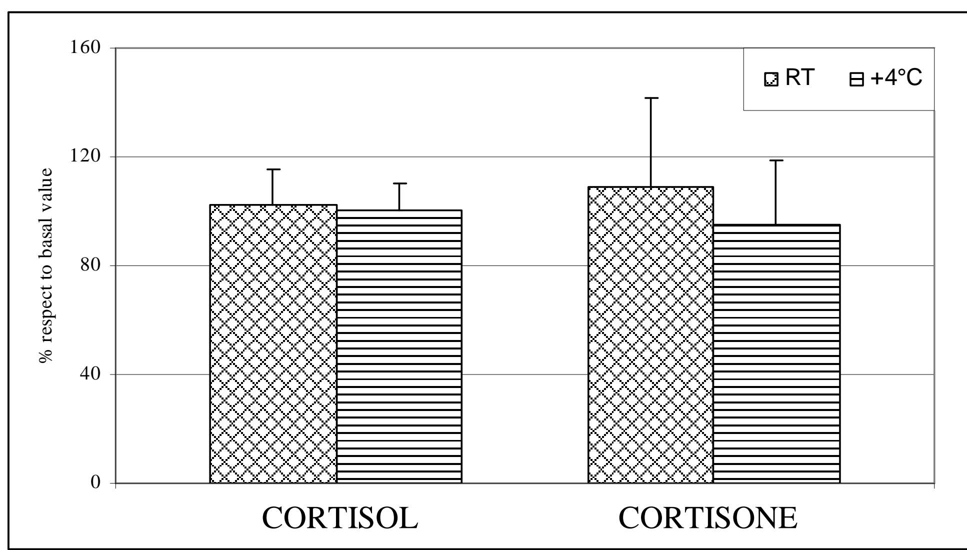

Figure 15. Salivary F and E stability at room temperature (RT) and at 4°C for 24h before Salivette centrifugation and separation (n = 5). The results (average ± SD) are expressed as percentage of the concentration obtained analysing each saliva sample after its separation and directly stored at -80°C (basal value, 100%).

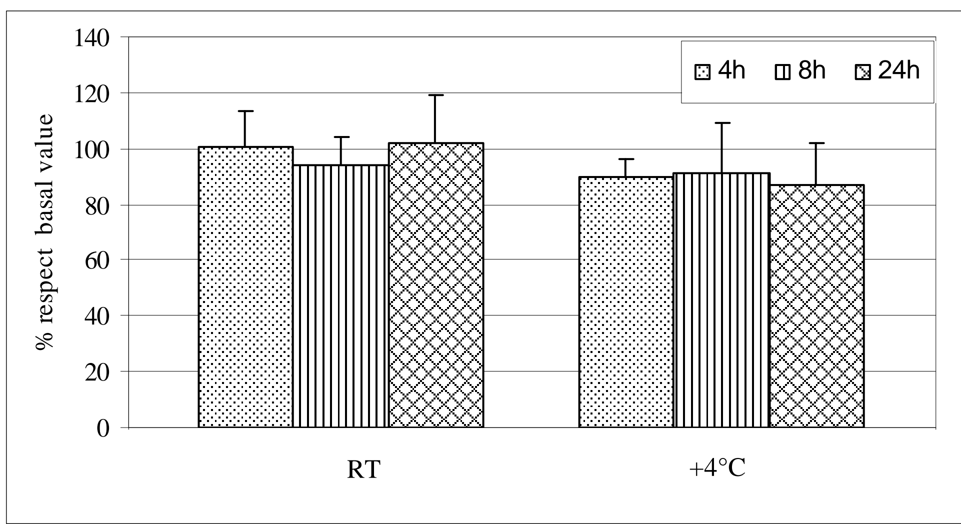

Figure 16. Salivary F stability at different times and temperatures after saliva Salivette centrifugation and separation (n = 3). The results (average ± SD) are expressed as percentage of the concentration obtained analysing each saliva sample after its separation and directly stored at -80°C (basal value, 100%).

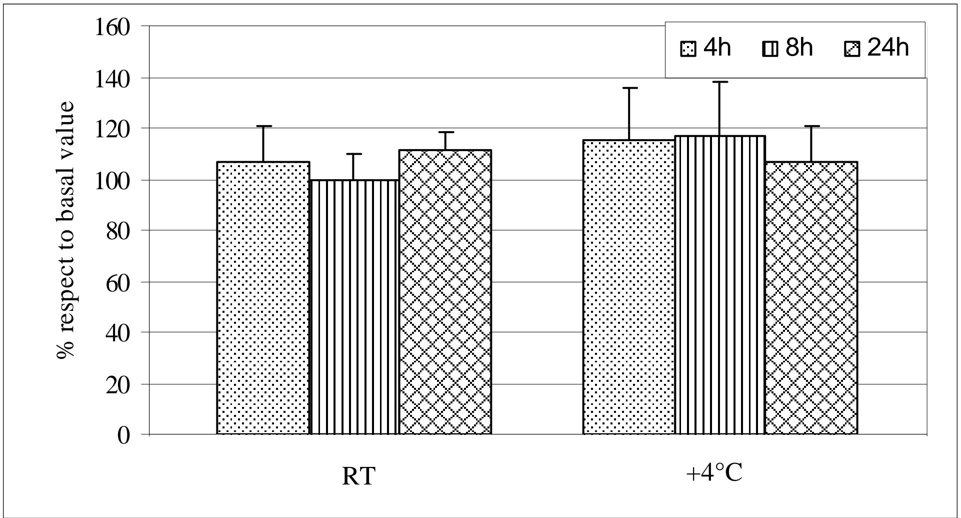

Figure 17. Salivary E stability at different times and temperatures after saliva Salivette centrifugation and separation (n = 3). The results (average ± SD) are expressed as percentage of the concentration obtained analysing each saliva sample after its separation and directly stored at -80°C (basal value, 100%).

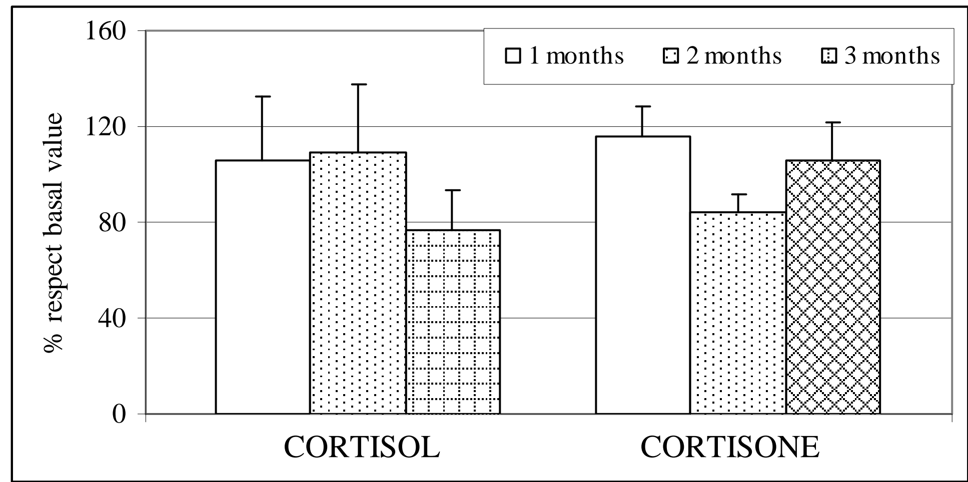

Figure 18. Salivary F and E stability at -80°C up to three months (n=3). The results (average ± SD) are expressed as percentage of the concentration obtained analysing each saliva sample after 1 day of storage at -80°C (basal value, 100%).

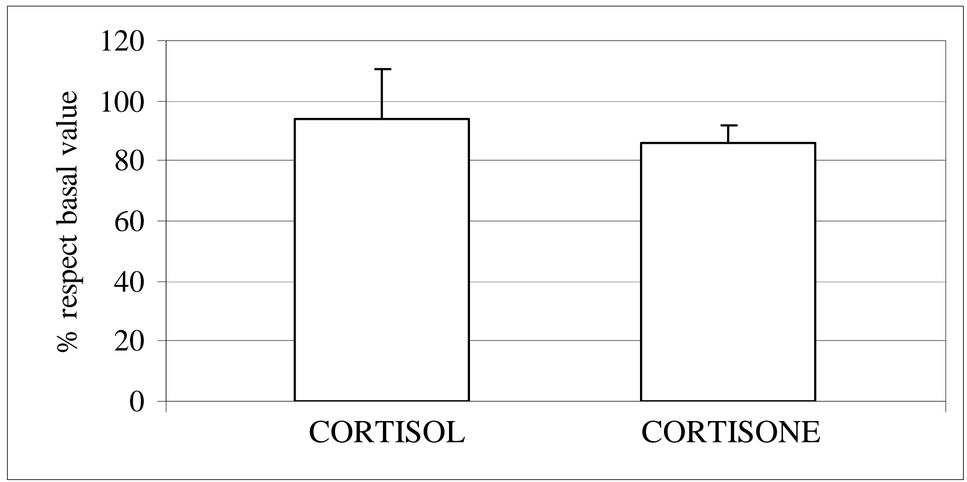

Figure 19. Salivary F and E stability after four cycles of freezing and thawing and then storage at −80°C (n = 3). The results (average ± SD) are expressed as percentage of the concentration obtained analysing each saliva sample after its separation and direct storage at -80°C (basal value, 100%).

6.3 PROTOCOL D - effect of an acute physical exercise in male and female rowers

Salivary cortisol, cortisone and cortisol/cortisone ratio levels in male and female rowers are reported in table 14 and in table 15, respectively.

Table 14. Saliva parameters at Basal (-30min), Pre-Ex (0min), End-Ex (40min), Recovery (100min) of *male* rowers undertaking a Concept II Rowing Step-test.

	Basal	**Pre-Ex**	**End-Ex**	**Recovery**
sF nmol/l	45±26	45±41	49±17	42±20
sE nmol/l	46±11	51±20	62±17$^{\#,\S}$	59±17
sF/sE	1.0±0.5	0.9±0.6	0.8±0.3	0.7±0.2

# p<0.05: end-ex *vs* basal

§ p<0.01: end-ex *vs* pre-ex

Table 15. Saliva parameters at Basal (-30min), Pre-Ex (0min), End-Ex (40min), Recovery (100min) of *female* rowers undertaking a Concept II Rowing Step-test.

	Basal	**Pre-Ex**	**End-Ex**	**Recovery**
sF nmol/l	23±15	21±8	28±11	16±5
sE nmol/l	35±9	41±5	46±14	49±11
sF/sE	0.6±0.3	0.5±0.2	0.7±0.3	0.3±0.1*

* p<0.05: recovery *vs* basal

Gender differences. Significantly higher levels of salivary cortisol at basal (p<0.05), pre-ex (p<0.03) and recovery (p<0.004) were observed in males compared to females, together to a significant higher level of cortisol/cortisone ratio (p<0.04) at recovery in males compared to females.

Urine cortisol, cortisone, cortisol/cortisone ratio, creatinine levels in male and female rowers are reported in table 16 and in table 17, respectively.

Table 16. Urine parameters at Basal (-30min), End-Ex (40min), Recovery (100min) of *male* rowers undertaking a Concept II Rowing Step-test.

	Basal	**End-Ex**	**Recovery**
uF nmol/l	226±158	265±326	388±341
uE nmol/l	305±185	360±265#	445±262
uF/uE	0.7±0.2	0.6±0.3	0.8±0.5
uCr mmol/l	15±5	12±10	18±9
uF/uCr nmol/mmol	17±12	22±16	21±12
uE/uCr nmol/mmol	22±13	38±17	26±10

# p<0.01: end-ex vs basal

Table 17. Urine parameters at Basal (-30min), End-Ex (40min), Recovery (100min) of *female* rowers undertaking a Concept II Rowing Step-test.

	Basal	**End-Ex**	**Recovery**
uF nmol/l	156±72	232±112	343±167*
uE nmol/l	372±163	460±185	545±204*
uF/uE	0.4±0.1	0.5±0.2	0.6±0.2
uCr mmol/l	7±6	9±11	10±7*
uF/uCr nmol/mmol	39±34	54±44	44±21
uE/uCr nmol/mmol	85±57	100±63	72±34

*p<0.04: recovery vs basal

Gender differences. Significant lower levels of urine cortisone/creatinine ratio at basal ($p<0.03$) and recovery ($p<0.03$) and of urine cortisol/creatinine ratio at recovery ($p<0.03$) were observed in males compared to females, together to a significant higher level of cortisol/cortisone ratio ($p<0.03$) at basal in males compared to females.

A positive correlation was observed between salivary cortisol and urine cortisol/creatinine ratio in female rowers ($y=0.233x+11.723$, $r^2=0.4304$, $p<0.007$) (figure 20).

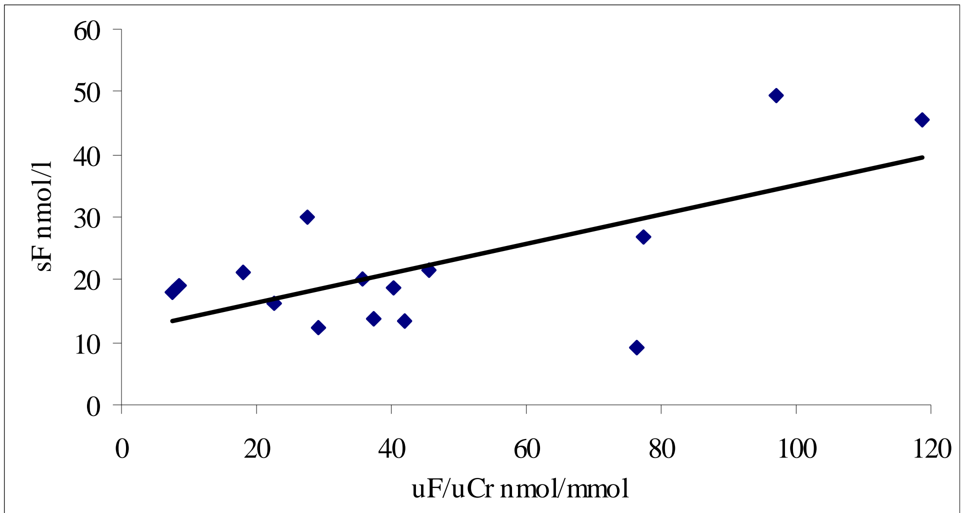

Figure 20. Correlation between salivary cortisol (sF) and urine cortisol/creatinine ratio (uF/uCr) in female rowers.

7. Discussion

7.1 Section I

To our knowledge this is the first time that the level of free IGF-I has been measured in human saliva in relation to training condition and physical exercise.

Scentific literature demonstrates very limited research in the salivary IGF-I, furthermore these few studies used a RIA method and investigated well characterized GH related clinical conditions. In these papers, the authors observed high and low levels of salivary IGF-I in acromegalic and GH-deficient patients, respectively [36-38], suggesting that salivary IGF-I reflects the systemic GH status [19], even if there are no literature data about r-hGH administration effect on sIGF-I levels. Consequently, the evidence of GH-dependence of salivary IGF-I is presently not consistent.

In 2007, Shpitzer et al. reported significantly higher concentration of IGF-I in saliva of patients with oral squamous cell carcinoma (OCSS) compared to healthy, age- and gender-matched individuals, suggestive of an important role in OSCC pathogenesis [43].

We studied and validated an ELISA method to analyze sIGF-I: the sensitivity, specificity, precision and accuracy of this method, taking into account the analytical performance parameters measured in the experimental validation tests, are suitable for the measurements of the concentration of free IGF-I in human saliva. The present research demonstrates the importance of the pre-analytical variability, and so the effects of time intervals, from collection to analysis, and temperature of storage before freezing

at −80 °C are also relevant. Moreover when the analysis is carried out after several months' storage, the use of the inhibitors' preservative solution (IS) has been demonstrated to be crucial.
The free sIGF-I levels, measured in the female (n=14, 25±2 yrs, 56±8 kg, 1.66±0.06 m, 20.3±2.1 kg/m^2) and male (n=8, 31±4 yrs, 75±10 kg, 1.81±0.07 m, 23.0±1.9 kg/m^2) sedentary groups, were 0.20±0.05 μg/l (*protocol A*) and 0.18±0.11 μg/l (*protocol C*). Taking into account the literature data, even if they are limited, the present results are comparable with Ryan's results (0.52±0.37 μg/l) [36]. The latter and our methods are certainly different but their sensitivities are similar (0.05 vs. 0.07 μg/l respectively), when compared with the other two literature methods demonstrating 10-fold higher values (0.5 and 0.7 μg/l Halimi et al. and Costigan et al. respectively [37,38]).
The measured levels in female and male sedentary did not demonstrate significant difference even if the anthropometrical data are different and consequently the comparison is not completely correct.

The free sIGF-I levels in saliva specimens collected from well-trained athletes and sedentary subjects demonstrated significantly different concentrations. This variation could be related to the difference in a sedentary *vs* exercise training condition. In particular in *protocol A* we found significantly lower ($p<0.001$) sIGF-I levels in female athletes compared to female sedentary subjects (0.10±0.05 μg/l and 0.20±0.05 μg/l, respectively), but in *protocol C* significantly higher ($p<0.001$) sIGF-I levels in male athletes compared to male sedentary subjects (0.97±0.43 μg/l and 0.18±0.11 μg/l, respectively). The scientific bases of these results are not easy since the data are not so numerous but actually the difference in the athletes (volleyball players and rowers) has to be taken into account.
The sIGF-I levels studied in a steady state, stimulated our interest to investigate the effect of an acute condition, like a physical exercise, on salivary free IGF-I, as in *protocol B* and in *protocol C*.

In *protocol B* sIGF-I was significantly increased at the end of the exercise ($p<0.01$), the free IGF-I rate of appearance was increased ($p<0.01$) in agreement also with sTP ($p<0.001$) and sTP rate of appearance ($p<0.01$); a correlation between saliva and plasma free IGF-I was found only in post-exercise. This could be explained by local synthesis of IGF-I in the basal condition. In the literature, to our knowledge, there are no studies

on the origin of human salivary IGF-I, but Ryan et al. demonstrated a local synthesis of IGF-I in Sprague – Dawley rats [39]. After the acute exercise a correlation with the plasma value was found in the present study. One possible hypothesis to explain this result is that exercise, like a stress condition, may lead to a free diffusion from plasma to saliva.

In *protocol C* a significantly increase only in sTP ($p<0.05$) was demonstrated, even when we calculated the rate of appearance ($p<0.05$) at the end of the exercise, maintained also in the recovery (sTP $p<0.05$, sTP rate of appearance $p<0.05$). The correlation between sIGF-I and sTP in pre-ex is in agreement with the results obtained in protocol B.
The increase on sTP levels obtained in both protocols agrees with Ljungberg et al. who demonstrated an increased total protein concentration after a marathon in well trained subjects [96]. Moreover the sTP rate of appearance was significantly increased at the end of exercise. Therefore, the observed phenomenon suggests that the increase in sTP level might not be induced by fluid losses from the saliva.

The obtained differences between these two protocols on sIGF-I were expected since the two protocols were different in many aspects: subjects' anthropometrical data, athlete (cyclists vs rowers), exercise (in terms of type, intensity, duration). Moreover the saliva sample collection at the end of the exercise was a bit different: in protocol C we collected saliva simultaneously to blood, instead in protocol B saliva was collected about 15 min after blood. This may explain why in protocol B we found a correlation between saliva and plasma free IGF-I at the end of the exercise, but not in protocol C.
However, the observed differences in the effect of the exercise on sIGF-I are in agreement with the literature data describing plasma IGF-I. In literature, there are mixed results, either increased, decreased or no variation, in studying the effect of physical exercise and/or exercise training in the circulating IGF-I levels, mainly total IGF-I [84-86]. The discrepancies could be related to differences in the investigated physical exercises (type, duration, intensity), but the influencing factors, like age, body composition, sleep-waking rhythm, nutritional status and energy balance are numerous.

7.2 Section II

This is the first time, to our knowledge, that the human saliva levels of cortisol and cortisone, in relation to a physical exercise, have been measured with a cromatographic method.

In the present study a SPE-HPLC method was utilized for simultaneous salivary F and E assay. This method is recommended to substitute the immunoassays, the most common analysis methods for urinary, plasma and salivary F concentration measurements. In effect, considering that F concentration in saliva is generally less than in plasma, RIA methods are usually suggested as sensitive methods. However, inherent differences between immunoassays may influence the data analysis limits and ranges, therefore, the diagnostic criteria. Many steroid immunoassays are sensitive, although the presence of F cross-reactivity with other steroid isomers/metabolites caused relatively high measured concentration. Undoubtedly, given the specificity of the LC-MS/MS and HPLC and UV detector techniques, salivary F assay with this type of methods should be preferred to RIA or ELISA [48]. Moreover saliva has a high viscosity and contains some floating insoluble substances such as dead cells and food residues: a SPE pre-treatment of saliva sample was utilized to eliminate interferences and to enrich ten-fold the salivary F and E concentrations. The sensitivity, specificity, selectivity, precision and accuracy of this method, taking into account the analytical performance parameters measured in the experimental validation tests, are suitable for the measurements of the concentration of cortisol and cortisone in human saliva. In agreement with the literature, salivary F and E did not demonstrate significant variations comparing before and after centrifugation, moreover the saliva samples did not demonstrate differences after three months at –80°C [97].

The basal levels of sF and sE, measured in the male (n=8, 29±8 yrs, 81±10 kg, 1.85±0.08 m, 23.7±1.8 kg/m^2) and female (n=5, 20.2±0.4 yrs, 62.2±0.4 kg, 1.68±0.45 m, 22.0±0.4 kg/m^2) rowers, were 45±26 and 23±15 nmol/l (for sF) and 46±11 and 35±9 nmol/l (for sE), respectively (*protocol D*). The calculated cortisol and cortisone molar ratios were 1.0±0.5 and 0.6±0.3 for males and females, respectively. Even if very limited, these levels are in agreement with the Morineau and Jerjes' results with RIA [54] and immunofluorimetric methods [55].

It is well known that exercise (as a physical stressor) activates the HPA axis with a circulating cortisol increase [78-80]. Anyway, several factors have been identified as

influencing the adrenocortical response to acute exercise, notably timing of exercise (fort the cortisol circadian rhythm), exercise intensity and training status [98]. Limited data exist on salivary cortisol and exercise, but it is clear that the results are influenced by many factors (as for plasma cortisol) [92-93,99]. Maybe the more interesting finding is the gender difference: a less HPA activation was found in female compared to male rowers with a higher level of cortisol/cortisone ratio at recovery in males compared to females. These differences might represent a diversity training status between males and females. Certainly further investigations are necessary to confirm these data and to better understand if saliva analysis can be an alternative to plasma o urine in athlete training status evaluation. The urine cortisol and cortisone ratio is proposed by many authors as a marker of training condition, in particular to assess the overtraining syndrome [94, 100-102].

The results of the study on salivary free IGF-I have been submitted and accepted for oral and poster presentations during high level international scientific congresses. Moreover manuscripts were submitted and published in peer-reviewed scientific journals (described in the chapter 9 "Overview on publications").
The results of the study on salivary cortisol and cortisone have also been subjected to poster presentations at international congresses and they are now submitted and under evaluation for the publication in peer-reviewed scientific journals (described in the chapter 9 "Overview on publications").

In conclusion, these two methods are able to measure free IGF-I and cortisol and cortisone in human saliva with appropriate analytical performances. These results suggest further investigation from the laboratory point of view, taking into account the aspects related to the various forms and the specific and unspecific binding proteins (for sIGF-I assay) and to the other steroid metabolites identified in saliva (for sF/sE assay).
Certainly these analysis offer numerous perspectives. Undoubtedly, the measurement of salivary IGF-I levels would allow new perspectives in GH/IGF axis study in athletes and sedentary subjects but also training state evaluation and doping purposes could be proposed. Moreover clinical application could be proposed in GH/IGF disease: in particular it would be interesting to verify sIGF-I levels in GH deficiency, before,

during and after r-hGH administration. Surely, the salivary cortisol and cortisone analysis with a chromatographic method would consent to investigate many physio-pathological conditions in which the activation/inactivation of HPA axis is known; in addition, the simultaneous assessment of cortisol and its inactive metabolite cortisone could enhance the knowledge on 11β-HSD activity in a local district as mouth.

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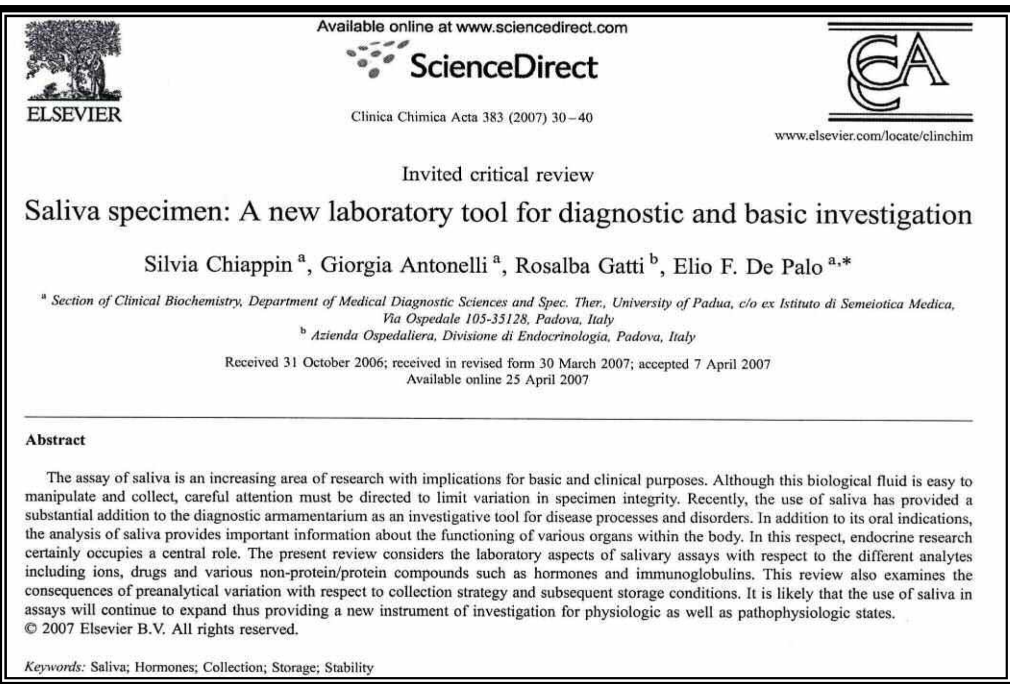
9. Overview on publications

9.1. Papers

- Chiappin S, **Antonelli G**, Gatti R and De Palo EF. Saliva specimen: a new laboratory tool for diagnostic and basic investigation. Clin Chim Acta **2007**; 383:30-40.



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Invited critical review

Saliva specimen: A new laboratory tool for diagnostic and basic investigation

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Received 31 October 2006; received in revised form 30 March 2007; accepted 7 April 2007
Available online 25 April 2007

Abstract

The assay of saliva is an increasing area of research with implications for basic and clinical purposes. Although this biological fluid is easy to manipulate and collect, careful attention must be directed to limit variation in specimen integrity. Recently, the use of saliva has provided a substantial addition to the diagnostic armamentarium as an investigative tool for disease processes and disorders. In addition to its oral indications, the analysis of saliva provides important information about the functioning of various organs within the body. In this respect, endocrine research certainly occupies a central role. The present review considers the laboratory aspects of salivary assays with respect to the different analytes including ions, drugs and various non-protein/protein compounds such as hormones and immunoglobulins. This review also examines the consequences of preanalytical variation with respect to collection strategy and subsequent storage conditions. It is likely that the use of saliva in assays will continue to expand thus providing a new instrument of investigation for physiologic as well as pathophysiologic states.
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Keywords: Saliva; Hormones; Collection; Storage; Stability

• **Antonelli G**, Cappellin E, Gatti R, Chiappin S, Spinella P and De Palo EF. Measurement of free IGF-I saliva levels: perspectives in the detection of GH/IGF axis in athletes. Clin Biochem **2007**; 40:545-550.

Available online at www.sciencedirect.com

Clinical Biochemistry 40 (2007) 545–550

CLINICAL BIOCHEMISTRY

Measurement of free IGF-I saliva levels: Perspectives in the detection of GH/IGF axis in athletes

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Received 11 September 2006; received in revised form 18 January 2007; accepted 18 January 2007
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Abstract

Objectives: To examine an immunoassay for measuring free IGF-I in a saliva specimen (free sIGF-I) and to study the levels in relation to the training conditions comparing young athletes and sedentary females.
Design and methods: The analysis was carried out by modifying a commercial kit for plasma matrix to measure the free sIGF-I. The plasma free and total IGF-I fractions, hGH and salivary total proteins were also measured. Saliva and blood specimens were collected from 15 well-trained young female volleyball athletes and from a control group of 14 young sedentary females.
Results: The calibration curve to assay free sIGF-I covered the range 0.05–5.00 μg/L. The detection limit was 0.07 μg/L. The within-run and between-run imprecision CVs were 10% and 13% respectively. The average recovery was 88%. Free sIGF-I, measured in 15 athletes and in 14 young sedentary females, was 0.10 ± 0.03 and 0.20 ± 0.05 μg/L respectively ($p<0.001$).
Conclusions: There were decreased levels of free sIGF-I in well-trained athletes, compared with sedentary females. This decrease could be related to a greater tissue requirement by the active muscle subjected to intense exercise for several days.
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Keywords: IGF-I; Saliva; Immunoassay; Physical exercise; Training

Introduction

The positive effects of the GH/IGF system on muscle function, performance capacity and health status of physically active people have been proposed by numerous Authors [1–4]. It has been verified that a healthy status has reciprocal positive effects on the GH/IGF system [5–7]. The literature regarding the connections between circulating IGF-I levels, both as total and free forms, does not demonstrate uniform findings. However, nutritional state and training condition seem to be responsible for these contradictory results. Moreover, long and short-term physical exercise, fatigue and energy expenditure/intake equilibrium have not always been taken into account. Further findings and investigation could help in a better understanding of these various effects and different conditions.

The complex interplay in the metabolic actions is important for this GH/IGF system. Protein anabolic aspects have principally been studied in the circulation, and little is known at saliva level. The fact that IGF-I stimulates protein synthesis with no effects on proteolysis must be taken into account in this matrix of the body. Moreover IGF-I stimulates growth and has been proposed as acting both as an endocrine hormone via the blood and as a paracrine and autocrine growth factor locally [8]. The repair of oral tissue has been reported to be in relation to the abundance of growth factors in the oral cavity. IGF-I together with IGF-II certainly occupy a crucial role among these growth factors [9].

Another important aspect is that the saliva is a rich deposit of proteins of which immunoglobulins are typically an important component. Moreover human saliva proteins are different from

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doi:10.1016/j.clinbiochem.2007.01.014

• De Palo EF, **Antonelli G**, Gatti R, Chiappin S, Spinella P and Cappellin E. Effects of two different types of exercise on GH/IGF axis in athletes. Is the free/total IGF-I ratio a new investigative approach? Clin Chim Acta **2008**;387:71-74

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Clinica Chimica Acta 387 (2008) 71 – 74

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Effects of two different types of exercise on GH/IGF axis in athletes. Is the free/total IGF-I ratio a new investigative approach?

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Received 18 December 2006; received in revised form 11 September 2007; accepted 11 September 2007
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Abstract

Background: Human growth hormone (hGH) responds to bouts of exercise by increasing, while the insulin-like growth factor-I (IGF-I) responses are conflicting.
Methods: Twenty well-trained male cyclists completed a brief duration exercise (A: warm up+increasing workload until exhaustion, lasting 25 min) and a medium duration exercise (B: warm up+70–80%$VO_{2\ max}$+increasing workload until exhaustion, lasting 40 min). The immunoreactivity of plasma hGH, the IGF-I in its total and free fraction were measured before and at the end of the exercise, and the free/total IGF-I ratio response to the two cycling exercise bouts was examined.
Results: Both A and B demonstrated increased hGH (from 77±122 to 544±327 and 28±68 to 369±276 pmol/l respectively) and total IGF-I (from 67±10 to 70±10 and 55±14 to 61±15 nmol/l respectively). The free IGF-I was decreased only in A (from 0.38±0.16 to 0.32±0.14 nmol/l). Both A and B demonstrated a decreased free/total IGF-I ratio (from 0.57±0.30 to 0.46±0.22 and 0.61±0.37 to 0.52±0.29).
Conclusion: Brief and medium duration physical exercise influences the hGH, the total and free IGF-I concentrations. The free/total IGF-I ratio was also influenced and it might be related to the GH/IGF system. Its investigation might be a way of studying the training condition.
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Keywords: GH/IGF axis; IGF-I free/total ratio; Physical exercise; Physical training

1. Introduction

The GH/IGF-I (Growth Hormone/Insulin-like Growth Factor-I) system, together with binding proteins, plays a key role in the adaptation of the organism to exercise. While the GH response to exercise is established [1,2], the IGF-I research demonstrated mixed, non-homogeneous results [3–5]. The discrepancies could be related to differences in the investigated physical exercise (type, duration, intensity), but there are numerous factors which might have an influence, such as body composition, sleep-waking rhythm, nutritional status and energy balance [6–8]. Recently Izquierdo et al. [9] studied the effects of 11 weeks of resistance training leading to failure (RF) versus not to failure (NRF), followed by 5 weeks of maximal strength and power training. The total IGF-I levels were reduced after 11 weeks and after 16 weeks in RF group. In NRF group only the serum IGFBP-3 was significantly increased after the 16 weeks of training. This Author suggested that IGFBP-3 increment was a compensatory phenomenon preserving IGF-I bioavailability [9]. IGFBP-3 was proposed as a marker of overtraining [10]. Furthermore the IGFBP-3 area under the curve (AUC, representing the total amount of IGFBP-3 measured, therefore the presence and bioavailability of this binding protein in the circulation during the investigated time)

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0009-8981/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2007.09.005

• Gatti R, **Antonelli G**, Zecchin B, Spinella P, Mantero F and EF De Palo. Urine cortisol and cortisone and water intake in athletes. J Sports Med Phys Fitness **2008**;48:404-408.

J SPORTS MED PHYS FITNESS 2008;48:404-8

Urine cortisol and cortisone and water intake in athletes

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Aim. **The aim of this study was to investigate the urine cortisol (F) and cortisone (E) relation, having a well-defined water intake.**

Methods. **Urine specimens were collected from 10 male trained cyclists (19±1 year, 70±4 kg, 179±4 cm), at rest just before the test (pre-exe) and until 45 min after the cycle ergometer exercise test (45 min at 50-60% $\dot{V}O_{2max}$) (post-exe) in the morning. This investigation measured the diuresis in the pre-exe and post-exe after each athlete had drunk 1 L of water from waking-up, after bladder emptying, to the start of the test (pre-exe) and 1 L during the 45 min after the exercise (post-exe).**

Results. **Urinary F and E concentrations demonstrated a significant decrease comparing pre-exe with post-exe (177±134 *vs* 64±21 and 706±475 *vs* 372±178 nmol·L^{-1} respectively, $p<0.05$). This significant decrease was verified when diuresis and urinary creatinine were taken into account and the ratio measured.**

Conclusion. **One litre of water intake after exercise seemed to have no effect on urine F and E excretion. Moreover the urine F/E ratio was not statistically different comparing pre-exe with post-exe.**

KEY WORDS: Drinking - Hydrocortisone - Cortisone - Urine - Exercise.

The hypothalamo-pituitary-adrenal axis (HPA) activation represents a physiological response to the strain of muscle exercise. Numerous factors interact with the HPA axis like stress, exercise, etc.[1-7] The HPA response to exercise may depend on training status, intensity and duration of exercise.[8] The urinary cortisol/cortisone ratio (F/E ratio) has been proposed as a marker of the catabolic status.[9, 10] It has also been suggested that factors namely type, duration and intensity of physical exercise, influence the anabolic and catabolic turnover processes.

The F response to exercise depends on several factors including the previously mentioned ones, mode of exercise and the training status of the athletes.[7, 11] But the meaning of urinary F and/or E values in relation to exercise may be difficult to standardize, not only from the physical exercise point of view, but also from other aspects such as water intake and urine volume collection. Previous studies showed that the urinary F and E excretion depends on urine volume and the reason is that F and E excretion changes with the times and intervals of the collection.[12] In relation to the diuresis (804.4 - 823.3 mL·day^{-1} in female and male, respectively), Atlaoui *et al.*[9] reconsidered the initial data of

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Acknowledgements.—We are indebted: to Mrs. Julia Stevens for her excellent and patient help with the English grammar.

Received on April 27, 2007.
Accepted for publication on April 10, 2008.

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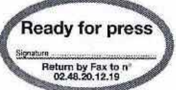
• **Antonelli G**, Gatti R, Prearo M and De Palo EF. Salivary Free IGF-I Levels: effects of an Acute Physical Exercise in Athletes. J Endocrinol Invest **2008;** 31.



J. Endocrinol. Invest. 31: ??-??, 2008

Salivary free insulin-like growth factor-I levels: Effects of an acute physical exercise in athletes

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ABSTRACT. ***Background/aims:*** **The offer of human saliva IGF-I (sIGF-I) measurement in athletes investigation is a new proposal. The aim was to investigate the physical exercise effect on sIGF-I and explore plasma free IGF-I relation.** ***Materials and methods:*** **Saliva and blood were collected from well-trained athletes, investigated immediately before and at the end of a physical exercise test.** ***Results:*** **sIGF-I was significantly increased at the end of the physical exercise. The plasma free IGF-I concentrations did not demonstrate any difference. The saliva total protein level (sTP) was also significantly increased. A positive correlation between sTP and sIGF-I, was observed, both before and after physical exercise, and between salivary and plasma free IGF-I only after physical exercise. The salivary free IGF-I level significantly increased after physical exercise, moreover a correlation with the plasma levels exists in post-exercise condition.** ***Conclusion:*** **In conclusion, the physical exercise affects sIGF-I as well as the sTP. The correlation between plasma and salivary free IGF-I levels only in post-exercise condition suggests further studies to investigate the effects of different type and duration of physical exercise. The comparison with other salivary biochemical parameter investigation would also further increase comprehension on the role of salivary IGF-I.**
(J. Endocrinol. Invest. 31: ??-??, 2008)
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INTRODUCTION

IGF-I is a peptide hormone which plays a crucial role in the GH/IGF axis (1). While the GH response to exercise is well known (2, 3), the literature data about the effects of physical exercise on the IGF-I system does not give the same uniform results. In any case, it is commonly accepted that IGF-I influences and is influenced by physical exercise and training condition (3-5). Some studies have shown an acute increase in serum IGF-I levels following physical exercise (6-8), others have observed a decrease in circulating IGF-I in response to exercise training (9, 10), while others have failed to observe any changes after resistance training and acute heavy resistance exercise (11, 12). Some of these discrepancies could be related to the different exercise protocols (type, duration, intensity), but numerous other factors such as gender, age, body composition, level of training, nutritional status, and energy balance may also be involved (6-13).
However, IGF-I is not only the endocrine mediator of GH-induced metabolic and anabolic actions but, taking into account the IGF-I receptor ubiquity (14), this hormone also acts in a paracrine and autocrine manner; in particular, it is thought to play important roles in stimulation of DNA synthesis, cellular proliferation and differentiation, amino acid uptake, protein synthesis, and glucose transport in a variety of tissues (15).
IGF-I in the blood circulates in free and bound forms: the former is the bio-active form, the latter, namely the bio-inactive form, is bound to a family of 6 specific proteins, namely IGF binding proteins (IGFBP). IGFBP prolong the half-life of circulating IGF-I and regulate its biological actions by modulating its bioavailability for the receptor (16).
We recently demonstrated that free IGF-I is also present and measurable in saliva (17). The saliva laboratory analyses represent an upcoming research area for basic and clinical application purposes (18). The salivary components are of different origins, locally from the mouth cells, mainly from the salivary glands, and/or by diffusion, mainly from the circulation. Among the numerous compounds, proteins play an important role, having immunological, enzymatic, and other activities (19). Between proteins, also growth factors play an important role in the mouth and their analysis may help in understanding hormonal involvement both in oral and systemic biological processes (20, 21). IGF-I is actually involved in many physiological and pathological oral processes, in particular in growth regulation of salivary gland cells, tooth growth and development, promoting periodontal wound healing (22).
Moreover other non-peptide hormones have been studied in saliva, in particular steroid hormones represent an investigated endocrinology topic (23). Their relationship with training conditions has been reported (24). Furthermore IGF-I, like GH (25, 26), is potentially abused for doping (27, 28) and the finding of a reliable IGF-I analysis method in saliva specimens could be practical in sports doping investigation.
The aim of the present study was to investigate the effect of an acute physical exercise on the salivary free IGF-I levels (sIGF-I) and to explore if a relation exists with the plasma free IGF-I levels (pIGF-I). The purpose of the present work was also to examine the salivary total proteins (sTP) to verify if a relation with the sIGF-I exists and if physical exercise has an effect on the salivary proteins.

MATERIALS AND METHODS

Subjects

Eighteen male, well-trained cyclists were recruited [aged 19±1 yr, weight 70±4 kg and height 179±4 cm, body mass index 21.9±1.7 kg/m²]. About a month before participating in the

Key-words: IGF-I, physical exercise, saliva, saliva rate appearance, total proteins.

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Accepted June 12, 2008.

- Gatti R, **Antonelli G**, Prearo M, Spinella P, Cappellin E and De Palo EF. Cortisol assays and diagnostic laboratory procedures in the different human biological fluids. *Submitted at Clin Biochem.*

Elsevier Editorial System(tm) for Clinical Biochemistry
Manuscript Draft

Manuscript Number: CLB-D-08-00571

Title: Cortisol assays and diagnostic laboratory procedures in the different human biological fluids.

Article Type: Critical Review

Section/Category: Clinical Investigation

Keywords: Steroids; Interferences; Reference range; Analytical performance; Diagnostic performance.

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Abstract: The overview of cortisol physiology and actions is achieved as well as the pathologies in relation to the hypothalamic-pituitary-adrenal axis alterations. Blood, urine and saliva compound measurements used to study the physio-pathological cortisol involvement, are critically reviewed. The immunoassay and chromatographic methods for cortisol measurement in different biological fluids are compared in relation to the analytical performances, the references ranges and the diagnostic specificity and sensibility.

Suggested Reviewers: JM Queraltó
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• De Palo EF, **Antonelli G**, Benetazzo A, Prearo M and Gatti R. Human saliva cortisol and cortisone analysis using reverse phase HPLC technique. *Submitted at Clin Chem.*

Clinical Chemistry

Clinical Chemistry

Manuscript Title: Human saliva cortisol and cortisone analysis using reverse phase HPLC technique.

Manuscript No: CLINCHEM/2008/122549

Manuscript Type: Article

Date Submitted by the Author: 18 Dec 2008

Complete List of Authors: Elio F De Palo, Giorgia Antonelli, Arianna Benetazzo, Maddalena Prearo, and Rosalba Gatti

Keywords: Cushing's syndrome; Solid Phase Extraction; chromatography; corticosteroid ; hydroxysteroid dehydrogenase; salivary

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