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# Salivary IGF-1 Detection in Growing Patients with a Lateral Flow Immunochromatographic Assay. A proof-of-concept study


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**Dedication**

I dedicate this thesis to all those who have fought the evil of this century and to those who are still seeking the truth

*Quid es veritas?*
*"…omnis, qui est ex veritate, audit meam vocem."*
*Giovanni (18:38)*

**Abstract**

This study investigated the potential of a lateral flow immunochromatographic assay (LFIA) for the detection of salivary IGF-1 as a noninvasive biomarker of growth in a sample of 35 orthodontic patients. The patients consisted of 22 females and 13 males, aged between 8 and 16 years. In addition to saliva sampling, patients underwent body mass index (BMI) analysis and a frequently used method for assessing skeletal growth, i.e., the radiographic assessment of skeletal maturation using the third finger Middle Phalanx Maturation method (MPM), which assesses the degree of skeletal growth based on the degree of ossification of the middle phalanx of the third finger on an x-ray. Salivary IGF-1, involved in tissue and skeletal development, was measured using the ELISA method and a prototype of LFIA, the results of which were compared with the MPM method. The following reagents were used to develop the LFIA: anti-IGF1 monoclonal antibody, anti-IGF1 polyclonal antibody, human IGF-1 standard, and protein A. The monoclonal antibody was used to develop the Test line, protein A to develop the Control line, and the polyclonal antibody bound with colloidal gold particles to develop the Conjugate Pad. Sample collection was carried out at the Orthognathodontics clinic at Careggi, Florence, while ELISA analyses and the development of the LFIA prototype were performed at the Department of Clinical and Experimental Medicine at Careggi, Florence. MPM analysis revealed that 9 of 35 patients were at the peak of growth (MPS3), 9 patients were pre-peak (3 in MPS1 and 6 in MPS2), and 17 were post-peak (5 in MPS4 and 12 in MPS5). ELISA analysis revealed mean salivary IGF-1 levels of 3.79 ± 1.94 ng/mL, with higher mean levels observed in MPS3 (4.39 ± 2.01 ng/mL), i.e., at the peak of growth, and lower levels in MPS1 (3.44 ± 1.39 ng/mL), pre-peak of growth, and MPS5 (3.25 ± 1.62 ng/mL), post-peak of growth. The BMI test showed four patients with excess weight problems.

Among these, a patient classified as MPS2, whose class had a mean IGF1 value of 3.95 ± 1.77 ng/mL, showed a higher value, 6.44 ng/mL, while another in MPS5 phase (with a mean value of 3.25 ± 1.55 ng/mL), showed a lower IGF1 value, 1.76 ng/mL. The LFIA demonstrated a visual limit of detection between 40 and 80 ng/mL, but positively detected the presence of salivary IGF1 in some MPS3 samples, even with analyte concentrations detected by ELISA above 3.12 ng/mL. Issues related to the matrix effect of saliva and the sensitivity of the test were identified, which indicate the need for further optimization. Although with pending improvements in sensitivity and reproducibility, our preliminary results are promising for assessing the LFIA as a useful tool for a rapid chairside test to optimize the timing of orthodontic procedures.

**Introduction**

Insulin-like Growth Factor-1 (IGF-1), originally called Somatomedin C, is a 70 amino-acids peptide produced under the control of the Growth Hormone (GH). [1] IGF-1 contains three disulphide bridges, between amino acids 6 and 48, 18 and 61 and 47 and 52, which create the tertiary structure critical for optimum binding to the IGF-1R. As its name suggests, IGF-1 is structurally similar to insulin and is capable of binding to the insulin receptor (I-R), but with a lower affinity than that towards IGF-1R. IGF-1 is for 75% produced by the liver while the other 25% is produced mainly by muscle, adipose tissues and other tissues[2]. After secretion, almost all of IGF-1 binds to six different binding proteins (IGFBP-1, -2, -3, -4, -5, -6, -7) forming binary complexes of 30-40 kDa that extent its half-life from 10 minutes to 30-90 minutes, leaving the unbounded IGF-1 in a minimal percentage. When IGFBP-3 and IGFBP-5 bound to IGF-1, they also bind to a further glycoprotein, the acid-labile subunit (ALS), forming a ternary complex of 150kDa. IGFBP3-ALS is the most abundant ternary complex, figuring as 80-90% of the total. This complex is retained and accumulates in the circulation, extending the half-life of IGFs to between 12 and 20 h[3]. The production of IGF1, its transport proteins IGFBPs and GH itself are regulated by a complex inhibitory and stimulatory feedback mechanism (Fig.1).

IGF-1 is a multi-potent growth factor, controlling cell proliferation, differentiation and apoptosis, tissue growth and organ-specific functions throughout the body. IGF-1 also promotes bone growth with stimulation of mesenchimal and stem cells differentiation into osteoblasts and chondrocytes, promoting also their proliferation[4]. Indeed, a deficiency in GH or the GH receptor causes severe postnatal growth retardation, while IGF-1 deficiency causes severe intrauterine and postnatal growth retardation and developmental defects in bone, muscle and reproductive systems. In embryonic development, IGF1 stimulates the mandibular growth acting on

the Merck cartilage[5], but it exerts an anabolic effect also during the growth spurt[6]. During embryonic development endothelial cells provide an essential source of IGF1 that is secreted in a paracrine manner to instruct proliferation of Meckel's cartilage chondrocytes. Given that Meckel's cartilage directly serves as the scaffold for mandible bone ossification, this proliferation supports correct jaw lengthening and extension during embryonic development. In experimental conditions, local administration of IGF1 shows endochondral bone formation in 3- to 12-week-old growing rats, while an increase in thickness of condylar cartilage and decrease in bony tissue is observed in 12-week-old rats, meaning a reduced growing effect in later stages of development. During human growing phases both GH and IGF-1 are produced at high level reaching in the human the peak at 10-12 years for female and 12-14 years for males.

Besides serum, IGF-1 was found also in saliva[7], although near 100 level lower than in serum. Salivary IGF-I level mirrored plasma IGF-I level and reflected the GH level of the patient. IGF-1 and IGBP3 are also produced directly in the salivary gland[8,9], in part exerting an autocrine and paracrine action over the glandular tissue[10]. Indeed, it was postulated that IGF-I system participates in the maintenance of viability and the functions of salivary gland cells.

IGF1 salivary and serum level may also change in some clinical conditions. Some eating disorder and related clinical conditions may contribute to modify these levels. Adult obese subjects show serum IGF1 levels higher than norm weight subjects[11], whereas anorexic patients have lower serum and salivary IGF1 levels compared to controls[12]. Moreover, younger obese subjects show a skeletal and dental maturation higher than norm weight subjects[13]. The evaluation of IGF1 levels in subjects with eating disorders may be necessary for the correct timing of therapy, especially due to

the high number of these clinical situations.

In dentistry, the assessment of the circumpubertal growth status is fundamental for the appropriate timing of some orthodontic treatments. In fact, some skeletal disharmonies, such as mandibular class III and maxillary transverse discrepancy, benefit from treatment during the prepubertal phase[14], while for class II the best time to start treatment is at the growth peak[15].

Numerous methods have been developed to assess skeletal growth changes. Some of these, such as changes in cervical vertebrae morphology[16] or skeletal maturation of the middle phalanx of the third finger (MPM)[17,18], are radiographic and therefore invasive methods. Between them, the changes in cervical vertebrae morphology method is the most invasive. It evaluates on a two-dimensional skull radiograph the mutation of morphology of the cervical vertebrae during growth and compare these results with the growth of the mandibular condyle. The MPM is the less invasive and evaluates the maturation of the mid phalange of the middle finger of the right hand. As in the case of the first method, it compares as well the mutation in shape of the phalange with the mandibular condyle. But the first method needs to make a full skull radiograph instead of a little radiograph for the middle finger. Anyway, both methods are subjective and depend on the diagnostic ability of the researchers to evaluate the radiographs. Other methods are based on the quantification of some biomarkers through urine or blood sampling[19]: they are certainly less invasive but not applicable during daily clinical practice. Saliva sampling to quantify IGF-1 is instead a less invasive and more practical method to evaluate growth changes. Furthermore, GH has a pulsatile release pattern, unlike IGF-1 which has a continuous release pattern, thus allowing reliable measurements[20].Some authors have already found a correspondence between quantification of salivary IGF1 and pubertal growth status assessed

both with the method of changes in cervical vertebrae morphology[21] and with the method of skeletal maturation of the middle phalanx of the third finger[22]. However, the reliability of assessing salivary IGF-1 levels as indicative of a precise timing of circumpubertal growth in patients undergoing orthodontic therapy needs to be definitively demonstrated. Between the methods to measure IGF1, Enzyme-Linked Immunosorbent Assay (ELISA) represents the Gold Standard. This is a versatile immunological analysis method used in biochemistry to detect the presence of a substance using one or more antibodies to one of which an enzyme is linked: this investigation method falls into the category of immunoenzymatic tests. Nevertheless, ELISA is a method that requires properly trained laboratory personnel and high costs.

Lateral Flow Immuno-Assay method (LFIA) represents instead a paper-based analytical chromatographic technique for the on-site detection of target substances[23]. This device is composed by an adsorbed pad made of paper divided in some parts (Fig. 2). The first part is the sample pad where the target substance is placed. The target substance flows over the adsorbent paper to the conjugate pad containing antibodies labeled with colored particles, usually colloidal gold. If the target analyte is present in the sample, it will bind to the labeled antibodies in the conjugate pad. The sample, along with the labeled conjugates, flows along the adsorbent paper by capillary action until a test line where antibodies specific to the target analyte are immobilized (Fig.3). If the target analyte is present and binds to the immobilized antibodies at the test line, a visible red line will appear, indicating a positive result. A control line is also present on the adsorbent paper. This line is designed to bind to the excess labeled conjugate, regardless of the presence of the target analyte. The control line has other immobilized antibodies specific to the target and ensures that the test has functioned correctly and that the sample has migrated through the device as

intended. The presence of two lines (test and control) indicates a positive result, while the presence of only the control line indicates a negative result (Fig.4). This description refers to a non-competitive LFIA. The competitive LFIA differ from the non-competitive LFIA in the results displayed on the adsorbent paper. In the first case the absence of the test line indicates a positive result. The experimental section of this paper refers to the non-competitive LFIA. The device is closed with a plastic cover .

LFIA provides rapid results quickly determining the presence or absence of a substance, often within minutes, and requires minimal training or equipment, making them suitable for point-of-care testing and is relatively inexpensive in comparison to ELISA[24]. However, it provides a qualitative or semi-quantitative result, which can be less informative and have lower sensitivity and specificity compared to more sophisticated laboratory techniques like ELISA, potentially leading to false positives or negatives. Moreover, visual interpretation of results can lead to inter-operator variability, especially in the case of weak antibody signals.

The present study was designed with the main aim of establishing the feasibility and applicability of a rapid test able to fulfill the necessary requirements for evaluating the presence of salivary IGF-1 levels indicative of different growth stages in orthodontic patients. Saliva samples were analyzed with both ELISA and a prototype of LFIA. These data were statistically compared with the results obtained from the radiographic analysis performed with the MPM method.

## Material and Methods

A proof-of-concept monocentric cross-sectional observational study was designed in accordance with the Helsinki declaration and the STROBE statement and approved by the Regional Ethics Committee of Tuscany – Pediatric c/o Meyer IRCCS University Hospital, V.le Pieraccini 28-30, 50139 Florence (CET_57/2023). The clinical procedures were performed at the Orthognathodontics Clinic, SOD Odontostomatology, AOU Careggi, Via del Ponte di Mezzo 46-48, 50127, Florence, while the laboratory procedures were performed both at the Department of Experimental and Clinical Medicine (DMSC), University of Florence, Viale Pieraccini 6, Florence, 50139, Italy for the specimens processing and ELISA testing and at the Department of Chemistry, University of Turin, Via Pietro Giuria 7 – 10125 Turin (Turin) and for the research and development of the LFIA.

This project was also selected from the University of Florence Service Center for the Promotion of Research and Management of the University Incubator (CsaVRI), (Decree rep n. 11204/2023 prot. n. 246007 of 17/10/2023) as an entrepreneurial project deemed suitable for access to IUF pre-incubation services under the code name SLFIA-IGF1 (Salivary Lateral Flow Immuno-Assay-IGF1).

*Patients selection*

35 patients were selected for this study (22 female, 13 male) aged between 8 and 16 years. Patients with xerostomia, confirmed or suspected pregnancy, breastfeeding, growth defects or diseases, systemic diseases, taking medications one month before saliva sampling, were excluded from the study. All patients selected were both seeking for an orthodontic consultation or were under control after ending an orthodontic therapy (Table

1). All participants and parents of subjects signed an informed consent form before being enrolled.

*Patients data collection*

All patients were tested for weight imbalance measuring weight and height (SECA, GmbH & Co. KG., Hamburg, Germany) and for Body Mass Index (BMI) (BMI Calculator for Child and Teen, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA).

Then, an X-ray of the middle phalanx of the middle finger of the right hand was performed to establish the growth stage. This X-ray was performed on a phosphorous plate by an X-ray machine (X-70 Timer S, Castellini, Bologna, Italy) for intraoral X-rays with the exposure parameters 70 kV, 8 mA, exposure time 0.03 seconds, and developed with a digital x-ray scanner (GS PS-500, Gendex, Tuusula, Finland). All patients were staged according to Perinetti e coll. MPM radiographic method for the growth stage determination[25]. Briefly, this method stages a patient growth status according to the middle phalange stage of ossification of the middle phalanx of the middle finger of the right hand. Stages of maturation are divided as MPS1, MPS2, MPS3, MPS4, MPS5, regarding as MPS1 and 2 as non ossified phalange stages and MPS4 and 5 as almost full ossified phalange stages. MPS1 are pre-pubertal stages, MPS2 and MPS3 are pubertal stages with MPS3 related to the peak of growth, whereas MPS4 and 5 are post-pubertal stages (Table 2).

Regarding saliva collection, patients will be asked not to eat, chew gums, drink fruit juice or carbonated drinks and to brush their teeth 90 minutes before collection. Sampling was performed between the 9 and 11 am following the protocol of the Human Microbiome Project Procedures Manual, Version 12.0, Sampling Protocol "A" of July 29, 2010, Section 7.3.1.5. Saliva

collection was obtained with a dedicated collection kit (Salivabio oral swab, Salimetrics® LLC, PA, USA).

Saliva was collected as follow (Fig. 5). Patients were asked being seated in an upright position. A swab, part of the collection kit, was placed under the tongue for 1-2 minutes. Following, the swab was insert into the tube insert ("swab basket") of the swab storage tube (SST), which was sealed, marked with the patient code and the collection date and placed in a freezer at 4° C.

*ELISA salivary testing*

In less than two hours, collected samples were centrifuged at 3000 RPM for 20 min to remove particles (Precision, Napco 2028R, Winchester, VA, USA). Upon removing the swab basket from the SST, the clear supernatant was collected in some aliquots of not less of 250 μL. Ten of these samples were added with protease inhibitors diluted at 1:100 (P8340; Sigma-Aldrich, St Louis, MO, USA) to test if salivary proteases could reduce the amount of detectable IGF-1. One of these samples was added with a known amount of IGF1 to test whether saliva could generate a matrix effect reducing the analyte reading. All were stored at -20° C until the testing. Four samples staged as MPS3, which are therefore assumed to have the highest level of IGF1, were used to develop the new LFIA at the Department of Chemistry in Turin. All collected samples were tested with a commercially available ELISA kits purchased from Cloud-Clone Corp Katy, TX, USA for salivary IGF1 (Product No. SEA050Hu). According to the manufacturer's instructions, serial dilutions of IGF1 standard protein provided by the kit, have been prepared to obtain a standard curve of known IGF1 concentrations. Then, one hundred microliters of the standard dilutions and samples were pipetted into each of the wells of the ELISA plates pre-coated with the anti-IGF1antibody. The plates were incubated at 37 C for 1 h. The clear liquid was removed from each

well. Then, 100 μL of "prepared detection reagent A" from the kit was added. This solution was mixed, incubated at 37° C for 1 hour, further aspirated and washed 3 times. Later, 100 μL of prepared detection reagent B was added, incubated for 30 min at 37 C, then aspirated and washed 5 times. To this, 90 μL of substrate solution was added and incubated for 10–20 min at 37 C. Finally, 50 μL of stop solution was added, and the optical density (OD) was immediately read using an ELISA reader at 450 nm (MultiSkan FC, V1.00.79, Thermo Fisher Scientific K.K., Tokyo, Japan). The OD value of the standard (X-axis) was plotted against the log of the concentration of the standard (Y-axis). The best-fit straight line was drawn through the standard points, as determined by regression analysis. The concentration of the biochemical constituents in the saliva samples was determined from the standards curve. The detection range of IGF-1 was 0.78–50 ng/mL. The sensitivity of this assay is 0.31 ng/mL.

*LFIA development*

Antibodies and reagents used were anti-IGF1 monoclonal antibody (mAb) 1 mL with a concentration of 0.5 mg/mL (Gibco, ThermoFisher Scientific, Carlsbad, California, USA), anti-IGF1 polyclonal antibody (pAb) 0.2 mL with a concentration of 0.5 mg/mL (Invitrogen, ThermoFisher Scientific, Waltham, Massachusetts USA) and standard human IGF-1 with a concentration of 0,2 mg (Gibco, ThermoFisher Scientific, Carlsbad, California, USA).

*Preparation and Characterization of AuNPs*

A standard conjugation procedure was performed with 5 μL (0.5 mg/mL) of antibody per 1 mL of gold nanoparticles of diameter approximately 35 nm (AuNP) with optical density equal to 1. AuNPs were synthesized through the tetrachloroauric acid reduction with sodium citrate (Sigma–Aldrich). A 1 mL of 1% w/v sodium citrate was added to 100 mL of boiling 0.01% w/v tetrachloroauric acid (Sigma–Aldrich) under vigorous stirring. Finally, the AuNPs were cooled down to room temperature and stored at 4 °C for successive conjugation to anti-IGF1 pAb. AuNPs were characterized by UV-vis spectroscopy on a Spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA, USA) and by an high resolution transmission electron microscopy (Jeol 3010-UHR, Jeol Ltd, Tokyo, Japan) equipped with a LaB6 filament operating at 300 kV and analyzed with an Energy Dispersive Spectrometer (EDS, Inca Energy TEM 300 X-ray, Oxford Instruments NanoAnalysis, High Wycombe, UK). For TEM imaging, a drop of the Gold Nano Particles (GNPs) aqueous suspension was put on a copper grid covered with a lacey carbon film for the analysis. The GNP resulted almost spherical in shape, with a sharp Surface Plasmon Resonance band (SPR) centered at 525 nm.

*Preparation and characterization of anti-IGF1 Ab-AuNPs conjugate*

The AuNPs-Ab anti-IGF1 (AuNPs-Ab) conjugate was prepared by passive adsorption of the anti-IGF1 pAb on the surface of the citrate-capped AuNPs. The AuNPs pH was adjusted to ~8.5 with carbonate buffer (0.05 M, pH 9.6). Then, for each mL of AuNPs with optical density (OD) 1, the appropriate amount of the pAb was added and gently mixed for 30 min at 37 °C. Subsequently, 100 μL of blocking solution (20 mM borate buffer pH 8

supplemented with 1% w/v BSA) were added (for each mL of AuNPs) for 10 min at 37 °C to block the unbound sites. Finally, the AuNPs–pAb conjugate was recovered by centrifugation (10 min at 7100×*g*), washed twice with borate buffer supplemented with 0.1% Bovine Serum Albumin (BSA, Sigma–Aldrich), and reconstituted in borate buffer supplemented with 2% (w/v) sucrose 1% (w/v) BSA, 0.25% (v/v) Tween® 20, and 0.02% (w/v) $NaN_3$. AuNPs-pAb conjugates were stored at 4 °C until use.

*LFIA strip manufacturing*

The mAb anti IGF1 (0.5 mg/mL) and protein A (Sigma–Aldrich) (0.2 mg/mL) diluted in phosphate buffer (20 mM, pH 7.4) were spotted onto nitrocellulose membranes (CNPC-SS12, 15 μm pores, Advanced Microdevices Pvt. Ltd., Ambala, India) at 1 μL/cm by means of a robotic dispensing platform (XYZ3050, Biodot, Irvine, CA, USA) to form the Test and Control lines, respectively. The glass fiber conjugate pad (GFCP103000, Merck Millipore, Billerica, MA, USA) was pre-saturated with the 20 mM borate buffer supplemented with 1% BSA, 2% saccarose (Sigma–Aldrich), 0,25% Tween 20 (Sigma–Aldrich) and 0,02% sodium azide (Sigma–Aldrich) and dried at 60 °C for 1 h. Subsequently, AuNPs-Ab conjugate solution at OD 3 (80 μL/cm) was used to saturate the Conjugate pad. Then, it was dried at room temperature for 3 h. The nitrocellulose membranes were dried for 60 minutes at 37°C under vacuum, then layered with sample and conjugate pads. Finally, the membranes were cut into 3.5mm wide strips using a fully automatic guillotine cutting module (CM4000, Biodot Irvine, CA, USA), and placed in plastic cassettes (Kinbio Tech.Co., Ltd., Shanghai, China) to produce the LFIA device ready to use.

*LFIA testing*

To develop the LFIA, we performed the antibody pair evaluation, i.e.,

we evaluated which antibody anti-IGF1 to use as capture Ab (on the Test line) and which as detection Ab (labelled with AuNPs). This evaluation has been performed through a spot test: 0.8 μL of 0.5 mg/mL Ab anti IGF1 (pAb or mAb) were deposited as a dot on the nitrocellulose membrane and the AuNPs-Ab conjugate (obtained using 2.5 μg of mAb or pAb during the conjugation process) at OD = 2 was used. Four IgF1 standard solutions (0, 0.01, 1 and 10 μg/mL prepared in phosphate buffer (20 mM, pH 7.4) + 1% BSA + 0.1% Tween 20 as the running buffer) have been tested to observe in which condition a coloured dot was formed.

Once decided the capture and the detection Ab, a complete strip configuration (0.5 mg/mL of mAb and 0.2 mg/mL of protein A as Test and Control line, respectively) has been used to evaluate which optical density (3, 4, 5 and 6) of the AuNPs-pAb produced the best colorimetric signal analyzing 0.1 μg/mL IGF1 standard solution.

Finally, we assessed the visual limit of detection (vLOD) intended as the lowest concentration of IGF1 that showed a visible Test line signal. Starting from 10 μg/mL IGF1 we performed serial dilutions to evaluate up to which IGF1 concentration we observed the Test line signal. In this evaluation we used pAb-AuNP (obtained using 7.5 μg of pAb during the conjugation process) at OD3 .

Standard solutions of IGF1 (0, 0.01, 0.02, 0.04, 0.08, 0.16, 0.3, 0.6, 1.3, 2.5, 5 and 10 μg/mL) were prepared in the running buffer (phosphate 20 mM buffer, pH 7.4 supplemented with 1% BSA, 0.1% Tween® 20); 70 μL of the sample to be analyzed were applied to the sample well of the LFIA cassette and the test results were qualitatively evaluated by the naked eye after 10 min. To obtain quantitative and more objective information, the strip images were acquired and processed with a dedicate software (QuantiScan 3.0, Biosoft, Cambridge, UK).

*LFIA salivary testing*

The objective of this experimental session was to evaluate the system performance by analyzing the real matrix (saliva). Under the same conditions in which we evaluated the vLOD (Test line: mAb 0.5 mg/mL, Control line: protein A 0.2 mg/mL, pAb-AuNP at OD3), we analyzed a pool of saliva from adult subjects as controls, i.e. supposed with low level of salivary IGF1. The saliva pool was analyzed both without any additions and with addition of IGF1 in order to have a concentration 2-times higher than the vLOD measured in buffer solution (0,16 μg/ml). Under the same conditions, were also analyzed 4 salivary samples collected from 4 MPS3 subjects, i.e. supposed with the highest level of salivary IGF1. These samples were analyzed both without any dilution and diluted 1:2 with an appropriate buffer.

**Statistical Analysis**

The statistical design used a linear model (general linear model) with salivary IGF-1 concentration as the response variable and MPS (5 groups), Gender, BMI and Age as explanatory variables. A t-test with a p-value set at 0.05 was use for Age, BMI and IGF-1, while a Fisher exact test was used for Gender. In the original experimental design of the study, to estimate the number of participants, a power of 80% was used, with a standard deviation of 0.82 ng/ml, a clinically significant difference of delta = 0.82 (1 effect size), alpha = 0.0083 (Bonferroni correction for 4 groups, i.e., 6 comparisons). With these parameters, 104 subjects would had been needed, divided into 52 males and 52 females. Patients would be enrolled into four groups of 26 subjects each (13 males and 13 females) with the following specifications: group 1 (pre-pubertal, MPS1, 26 subjects, of which 13 males and 13 females), group 2 (pubertal, MPS2, 26 subjects, of which 13 males and 13 females), group 3 (pubertal, MPS3, 26 subjects, of which 13 males and 13 females), group 4 (post-pubertal, MPS4, 26 subjects, of which 13 males and 13 females). However, owing to the importance of the experiment it was decided to organize this exploratory study in a form of a Proof of Concept.

## Results

*Patients data collection*

Of the 35 patients enrolled in this study 22 (63%) were female and 13 (37%) were male. Medium age of the population was 12.3 ± 2.06 (years, months) minimum 6.5 and maximum 15.8, with 12.1 ± 2.04 for female and 12.6 ± 2.10 for male. Based on BMI calculation (which was on average 18.9 ± 2.7), 4 patients of 35 (11%) suffered of weight imbalance, of which 3 (8%) were overweight and 1 (3%) was obese. According to the radiographic MPM method, 3 patients were classified as MPS1 (8%), 6 were MPS2 (17%), 9 were MPS3 (25%), 5 were MPS4 (14%) and 12 were MPS5 (34%).

*ELISA salivary testing*

The average level of salivary IGF1 in all patients was 3.79 ± 1.94 ng/ml. Patients classified with MPM showed the following IGF-1 mean values: MPS1 3.44 ± 1.39 ng/ml, MPS2 3.62 ± 1.76 ng/ml, MPS3 4.39 ± 2.01 ng/ml, MPS4 4.43 ± 3.06 ng/ml and MPS5 3.25 ± 1.62 ng/ml. (Table 3). Patients classified and grouped according to the peak of growth showed a level of IGF-1 higher at the MPS2 and MPS3 stages in comparison with the MPS1 and MPS4-5 (Table 4). The 4 patients with weight imbalance have an average level of IGF1 of 3.79 ± 1.72 ng/ml with one patient reported a value of 6.44 ng/ml and an MPS2 classification. Protease inhibitor cocktail addiction to the samples displayed an average value of 3.28 ± 2.14 ng/ml not resulting in an improvement of IGF1 levels detected, especially if compared with the value of 3.88 ± 1.64 ng/ml related to the samples not addicted with proteases inhibitor. The sample added with a known amount of standard IGF1 revealed a salivary matrix effect, since the result was lower than that expected.

*LFIA testing*

*Preparation of the Ab-AuNPs conjugate:* both the monoclonal and polyclonal Ab anti IGF1 showed effective conjugation with AuNPs. pAb-AuNPs showed some agglomeration during the conjugation process but the sonication of the solution resulted in effective resuspension. The expected red shift of the $\lambda_{SPR}$ (from 525 nm to 531.5 nm and 532.0 nm for mAb and pAb, respectively) measured by means of a UV-Vis spectrophotometer showed the success of the conjugation.

*Ab pair evaluation*

Ab pair combinations (mAb-AuNPs and pAb as Test line, and pAb-AuNps and mAb as Test line) were evaluated through the ppot tests (Fig.6). In both the combinations we did not observe any not-specific signal. When using the pAb on the Test line and the mAb labelled to the AuNPs we were not able to observe any signal in the presence of IGF1. On the contrary, using the pAb as detection Ab and the mAb as the capture Ab we were able to observe a specific signal for IGF1 at 1 and 10 μg/mL. Therefore, this configuration was used for further fine-tuning of the system.

*Ab-AuNPS optical density evaluation*

The Ab-AuNPs concentration (expressed as the optical density) can modulate the sensitivity of the LFIA and therefore must be evaluated in the fine-tuning of the system. As shown in fig. 7, a not-specific signal was clearly visible from OD4 onwards, whereas specific signals in the presence of IGF1 100 ng/mL were clearly distinguishable from the buffer. Therefore, Ab-AuNPs OD 3 was chosen for the vLOD evaluation.

*vLOD evaluation*

IGF1 standard solutions prepared in the running buffer (from 0 to 10 μg/mL) were analysed with the develop LFIA. The calibration curve (fig. 8) was obtained fitting data with a four-parameter logistic equation (SigmaPlt v 14.0, Systat Software, Inc.) and the images of the strips are reported in fig. 9. The four parameters of the fitting are reported in table 3.

A visual LOD was observed between 0.08 and 0.04 μg/mL of IGF1 (Fig.9)

Even if the vLOD is not good enough to detect the IGF1 at the expected concentration in real samples, we decided to evaluate the developed LFIA analysing salivary samples,

*LFIA salivary testing*

The pool of saliva from adult subjects as controls analyzed both without any additions and with addition of IGF1 showed that only the control line appeared, while the test line signal was not observable in the presence of 0.16 μg/mL IGF1 (Fig.10).

This would seem to demonstrate the absence of not-specific signals due to the matrix, but also a poor ability of the system to detect the presence of IGF1 (the standard solution of 0.16 μg/ml IGF1 prepared in buffer was visible). The effect of the matrix is not completely absent: in fact it is possible to observe very low signals of the Control lines and of a non-bright red color (as is classically observed).

The four salivary samples collected from 4 MPS3 subjects analyzed under the same conditions, without any dilution and diluted 1:2 with an appropriate buffer showed test line signals for samples pz3, pz4, and pz12 (Fig.11). Sample pz5, analyzed without any treatment, gave an invalid response (absence of the control line), demonstrating a strong matrix effect. The control

line for pz5 appeared when the sample was diluted 1:2, but the signal was significantly lower than that of the other samples (both without any treatment and diluted). From these results (Tab.4), it would appear that pz3 and pz12 contain similar IGF1 concentrations while pz4 contains a higher IGF1 concentration than pz3 and pz12, , even if more analyses are needed to confirm the result (the samples were analyzed in single due to the low amount of the samples themselves and due to the small amount of LFIA strips produced). Moreover, we cannot exclude that the observed signal are not-specific signals.

**Statistical results**

The inferential statistics were conducted by comparing the “Peak” group with the “Post-peak” group since the “Pre-peak” group consisted of only 3 subjects (Table 5). The statistical tests used were the t-test for Age, BMI, and IGF-1 and the Fisher exact test for Gender. Only Age was significant: obviously, age was greater on average for Post-peak subjects than for Peak subjects.

IGF-1 was higher for Peak subjects, with a difference of 0.49 ng/ml compared to Post-peak (95% CI -0.97 to 1.94). However, the difference was not statistically significant (P = 0.5009).

A sensitivity analysis for IGF-1 was also conducted, considering Gender, Age, and BMI in a general linear model. Again, the difference between Peak and Post-peak was not statistically significant (P = 0.2802).

Ultimately, although the numbers are low and therefore the power is low, no significant difference in salivary IGF-1 concentration was found between Peak and Post-peak subjects. The difference may be more marked than in Pre-peak subjects. Nevertheless, this study included only three Pre-peak subjects, so it was not possible to compare them with the other groups.

Analyzing Table 6, we observe that mean salivary IGF-1 levels tend to increase progressively from prepubertal stage (MPS1) to the most advanced pubertal stages (MPS4), and then decrease dramatically in the postpubertal stage (MPS5). The highest IGF-1 level is recorded in MPS4, but the value in MPS3 is almost identical. These data suggest that the peak of salivary IGF-1 levels really occurs during puberty.

This trend is further confirmed in Table 7, which aggregates patients based on their growth spurt. The "Peak" group (combining MPS2 and MPS3) had a mean IGF-1 concentration of 4.08 ± 1.89 ng/ml, a value higher than both the "Pre-peak" group (3.44 ± 1.39 ng/ml) and the "Post-peak" group (3.60 ± 2.11

ng/ml). Although the numbers are small and conclusions must be cautious, the descriptive data trend is scientifically plausible and in line with what would be expected from a larger and more robust sample. These results appear consistent and support the biological hypothesis underlying the study.

Inferential statistical analysis was limited to comparing the "Peak" group (N=15) with the "Post-Peak" group (N=17) due to the insufficient sample size of the "Pre-Peak" group. As reported in Table 5, the only statistically significant result was Age ($P < 0.0001$), which was expected and confirms the correct separation of the groups.

For the variable of interest, IGF-1 salivary level, the P value was 0.5009, well above the conventional significance threshold of 0.05. The mean difference of 0.49 ng/ml between the two groups was not statistically significant. The 95% confidence interval for this difference, which ranges from -0.97 to 1.94 ng/ml, is particularly wide and, containing the value zero, indicates that, based on this sample, the true difference could be zero, positive, or negative. The width of this range is direct evidence of the study's low statistical power. Although the difference of 0.49 ng/mL is not significant, the descriptive data is consistent with the expected trend; therefore, the non-significance may be due to the limited sample size rather than a true absence of difference. This result is also confirmed by the sensitivity analysis.

The rapid LFIA test was performed on only four subjects, all belonging to the MPS3 group, i.e., at peak. Of these, three tested positive and one tested negative. To evaluate the efficacy of a diagnostic test, it is essential to compare results between distinct and clinically relevant groups (e.g., pre-peak vs. peak or peak vs. post-peak). Since the LFIA test was applied to only one group in this study, no conclusions can be drawn about its ability to distinguish between different growth phases, though LFIA may be a promising device to detect the highest level of salivary IGF-1. Nevertheless, from a statistical point

of view it is impossible to generalize the results.

All analyses were carried out with JMP SAS Institute Inc., version 13.0 (JMP Statistical Discovery LLC 920 SAS Campus Drive Cary, NC 27513).

**Discussion**

This study showed the preliminary steps and relative drawbacks to develop a Lateral Flow Immunochromatographic Assay aimed to detect salivary IGF1 of growing patients at the peak of growth for orthodontic treatments. To our knowledge this is the first study designed and conducted with this purpose. LFIA represents a simpler analysis method than ELISA to be used during daily clinical practice. However, it requires sophisticated technological approaches before reaching the assessment of a reliable and useful test system. One of the major challenge in LFIA development is matrix effect reduction to increase sensitivity. Matrix effect or more correctly *Interference effect* is considered as a sum of effects of all components of a system, excluding the analyte to be measured[26]. Many methods, more or less complex, were proposed to enhance the sensitivity and reduce the matrix effect in LFIA[27,28]. Combining LFIA elements of which this device is composed may contributes in improving the sensitivity[29]. As an example, nitrocellulose membrane where the capture antibodies are coated is selected based on the size of analyte to be detected. In particular, pores size of the membrane is inversely proportional to sensitivity. As the pore size increases also the flow rate over the membrane increases and diminishes the time to see the result of the test, but at the same time diminish the sensitivity because of reducing the time for an adequate antigen-antibody interaction. Following the same principle, Tsai and coll.[30] reduced the flow rate across the nitrocellulose developing a stacking pad while maintaining the same pore size. They added an additional layer of membrane between the Sample pad and the Conjugation pad thus increasing the time of antigen/antibody interaction and increased the sensitivity. Sample pad, the platform where the analyte is placed, needs sometimes to be pre-treated with surfactants like Tween-20 (< 0,05%) or Triton X-100 (< 0,05%) to reduce background noise and non-specific

interaction. In the Conjugate Pad nanoparticles gold-coated interact with antibody-antigen. The kinetic of this interaction and the pH need to be carefully controlled to enhance sensitivity. Not optimal pH, which should be set at 8, results in aggregation of nanoparticles and reduction of line color intensity. Also the nature and the concentration of the capture antibodies contributes to the sensitivity enhancement of the LFIA. Monoclonal antibodies are a good choice to develop this method. They have far distant placed epitopes as capture and detector antibodies, whereas polyclonal antibodies can create large structures resulting in inadequate migration through nitrocellulose membrane and retained not-specifically. Comparing to other immunoassays, the concentration of capture antibodies in LFIA needs to be higher, ranging between 50-500 ng per strip. This is the case of this study, where first LFIA sensitivity testing actually showed a good catching ability of monoclonal antibody, while the labeled polyclonal antibody showed some non-specific reactions, mainly at high concentrations. Using the same monoclonal but at a higher concentration or two monoclonal antibodies could likely solve this drawback. In the latter case, capture antibodies and labeled antibodies should be different since IGF1 may not simultaneously bind to the same epitope[31]. However, saliva remains a complex fluid because of its composition rich in proteins and salts[32] and this may cause a strong matrix effect in LFIA. Beyond the modification of the constitutional elements of the LFIA, sample pretreatment need to be performed in order to improve this method. In this study adult salivary testing showed a low red colored Control Line, which could be explained by not-specific interferences, or by some matrix effects. Same results were obtained for saliva of MPS3 patients, tested with and without surfactant dilution. In this specific case, all tests but one showed a higher red colored Control Line compared with the latter cases, both with and without surfactant dilution, because of the higher

concentration of IGF1 in the sample. In one case the Control Line appeared only with 1:2 sample dilution, showing a strong matrix effect. Because the dilution of the sample reduces the quantity of analyte to be bound with the labeled antibody, a higher concentration of antibodies to be placed on the lines is mandatory.

LFIA qualitative results of this study referring to the MPS3 patients agreed with the ELISA salivary IGF1 results in the same patients. Nevertheless, only the values over 3.12 ng/ml displayed the positive results, suggesting that LFIA sensitivity may be identified with this analyte concentration. Furthermore, quantitative results of ELISA were consistent with MPS average values of this study. Indeed, the higher value met the MPS3 class while the lower values met the MPS1 and MPS5 classes, indicating a positive correlation of salivary IGF1 between MPS and ELISA methods. Moreover, ELISA results are partially consistent with some studies reported in the scientific literature. In this regard, a recent literature review reported that ELISA test protocols are often tailor-made for each experimental procedure and differ significantly from one another[33]. As an example, Almalki group published four studies in 2022[22, 34, 35, 36] where they reported in patients at the peak of growth an IGF1 average level between 1.80 and 2.57 ng/ml, lower than the average value reported in our study (4.39 ± 1.89 ng/ml for MPS3 class). Of note, in all of these papers, differently from the present study, they do not follow the manufacture instructions but doubled the amount of kit reagents, based on the fact that the ELISA kit that they used is produced for most of the biological fluids, but not for saliva, where the IGF1 concentration is 100-fold lower than serum. In addition, saliva was collected with the spitting method resulting in 5 ml of fluid, differently from this study in which 2 ml of unstimulated saliva was the maximum volume obtained per patient. Furthermore, to collect unstimulated saliva it was chosen the swab

method instead of the spitting method; but the latter may produce more easily blood contamination, particularly in patients with gingival inflammation, which can lead to an increase in salivary IGF1 values[37]. On the other end, the lower level of salivary IGF1 detected in these studies could be referred to the unbound form, which is part of the total IGF1. Indeed, further assessments are required to verify if the antibodies used are able to detect also the bound form of IGF1. To measure the full bioavailability of salivary IGF1, IGFBP3 would be specifically measured or removed. For instance, one of the older method proposed to dissociate the IGFBP3 was based on lowering the pH of the solution combined with addition of IGF2, which has a higher affinity for IGFBPs. However, there was no total agreement with this method, since it does not represent the total IGF1 amount in the solution[38]. Pre-treating the Sample pad with some acidic solution may be another way to detect a higher level of IGF1, enough to determine a sound red-colored signal on nitrocellulose. Nevertheless, care should be taken to not introduce a low pH in the adjacent Conjugated pad, which could impair the AuNPs bindings with the Ab.

Differently, Veena et al.[39] found a salivary pubertal IGF1 level of 5.19 ± 0.96 ng/ml, higher than in our study. They proposed 3.96 ng/ml as a value below of which the patients are not-pubertal. Nevertheless, they did not describe in detail the ELISA protocol used. However, the IGF1 value detected in MPS3 patients in our study showed a positive LFIA only for values above 3.12 ng/ml, thus below the value of 3.96 ± 0.96 ng/ml reported by Veena et al. (Fig. 12).

Referring to the BMI results resulting from our study, and in agreement with other studies[40,41], we did not find any difference in salivary IGF1 level of patients with weight imbalance in comparison with the average level of the totality of the patients population (3.79 ± 1.72 and 3.85 ± 1.93,

respectively). However, an overweight patient classified as MPS2 showed an IGF1 level of 6.44 ng/ml, higher than the average calculated for this class, which was 3.95 ± 1.77 ng/ml. At the opposite, another overweight patient classified as MPS5 showed an IGF1 level of 1.76 ng/ml, lower than the average calculated for this class, which was 3.25 ± 1.55 ng/ml. We could speculate that weight imbalance may in some way interferes with IGF1 levels, at least in patients not near the peak of growth. Nonetheless, a large number of this class of patients will be necessary to support this hypothesis.

Salivary proteolytic activity is an intrinsic function of oral cavity, partly related to the initial digestion of foods before the introduction into the stomach. However, proteases activity may represent a challenge when using saliva withdrawal as a method to test protein analytes [42,43]. To avoid these problems some authors added protease inhibitors to reduce and retard this enzymatic activity naturally present in saliva [44,45]. In the present study we used protease inhibitors for some of the salivary samples, but we did not obtain a significant improvement of IGF-1 level detection, compared with samples without protease inhibitors. Nonetheless, because of the difficulty in the management of saliva as a complex body fluid, the use of protease inhibitors should be considered in the assessment of experimental procedures.

**Conclusions**

Various authors had asked for a simple, rapid, chair-side and not invasive diagnostic tool to evaluate the peak of growth [33,38]. The CVM method, one of the most widespread diagnostic methods used in orthodontics, was in the past criticized about the subjectivity in assessment, reproducibility issues and predictive validity[46,47]. In a similar fashion, the third Finger Middle Phalanx Maturation method (MPM) faced some controversy regarding its reliability and accuracy[48]. Nonetheless, MPM exposed the patient to a minimal radiation exposure and it is easier in interpretation comparing to the CVM method. In addition, there is an overall agreement about the comparison of diagnostic efficacy between the CVM and the MPM methods[49]. In this context, although preliminary, our study adds promising perspectives to the assessment of a novel and not invasive diagnostic method for identifying orthodontic patients at the peak of growth. However, several bias need to be addressed before obtaining a reliable diagnostic tool.

The most important limitation of the study stems from the marked discrepancy between the planned sample size and the actual one. Calculating the sample size during the study design phase is a crucial step in ensuring that the research has sufficient statistical power to detect a clinically or biologically significant difference. For this study, the original calculation called for the inclusion of 104 subjects. Here we reported the analysis of only 35 subjects collected, less than a third of the expected number, thus drastically reducing the statistical power well below the acceptable threshold.

Low power reduces the probability of detecting a statistically significant difference, increasing the risk of a type II error (false negative). Hence, the lack of a statistical difference in the IGF-1 levels between “peak” and “post-peak” groups (P=0.5009) cannot be regarded as a conclusive evidence. Furthermore, the small sample size also compromised the ability to

make a comprehensive comparison between all growth groups, particularly the "Pre-peak" group which, with only 3 subjects, was numerically insufficient for a valid statistical analysis compared to the other groups. However, the trend in IGF1 levels observed in the different groups strongly encourage in proceeding the study to complete the sample collection and the relative analysis before drawing the final conclusion.

Another challenge of this study is the difficulty encountered in the correct saliva sample processing for the assessment of LFIA prototype. In particular, matrix effect of saliva due to the protein-enriched composition of this biological fluid also detected during ELISA testing, may reduce the performance of this device. In general, LFIA development is a complex procedure due to multiple technical difficulties. Therefore, further efforts will be required to improve our prototype described in this study.

Nevertheless, we found a considerable disparity in experimental protocols of similar studies in the literature[50]. Heterogeneity related to ethnicity, sample size, sexual dimorphism, and early or late maturity combined with difference in assay sensitivity, laboratory practice, and populations may interfere with consistency of results and conclusions.

Therefore, to identify and develop a robust LFIA skeletal maturity biomarker as a chairside, sensitive, reliable tool to aid clinical decision-making and choice of orthodontic treatment alternatives a standardized protocol for saliva sampling and processing is highly recommended as first step. Then, further experiments and technical adjustments will be required to optimize our LFIA prototype.

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## Tables

**Table 1:** Patients Demographics

| Patients | Age (year/month) | Sex | BMI | BMI category | MPS Class |
|---|---|---|---|---|---|
| 1 | 10 | F | 18 | HW | 2 |
| 2 | 15.1 | F | 20.9 | HW | 5 |
| 3 | 10.8 | F | 24.8 | OB | 3 |
| 4 | 14.5 | M | 22.1 | HW | 3 |
| 5 | 13.8 | F | 17.2 | HW | 3 |
| 6 | 12.8 | F | 20.7 | HW | 5 |
| 7 | 11.8 | F | 17.5 | HW | 2 |
| 8 | 14.9 | M | 20.3 | HW | 5 |
| 9 | 9.4 | M | 17 | HW | 1 |
| 10 | 13.1 | F | 18 | HW | 4 |
| 11 | 11.4 | F | 15.9 | HW | 3 |
| 12 | 10.5 | F | 17.6 | HW | 3 |
| 13 | 13.8 | M | 24.5 | OW | 4 |
| 14 | 8.8 | F | 16.3 | HW | 1 |
| 15 | 12 | F | 17.9 | HW | 5 |
| 16 | 13.7 | M | 17.6 | HW | 4 |
| 17 | 12.7 | F | 22.2 | OW | 5 |
| 18 | 12.11 | F | 16.4 | HW | 5 |
| 19 | 9.4 | M | 17 | HW | 2 |
| 20 | 6.6 | F | 20.6 | HW | 2 |
| 21 | 12.2 | F | 16.8 | HW | 4 |
| 22 | 12.11 | M | 22.6 | OW | 2 |
| 23 | 14.5 | F | 21.9 | HW | 5 |
| 24 | 13.7 | F | 20 | HW | 5 |
| 25 | 11.6 | F | 19.4 | HW | 3 |
| 26 | 12.7 | M | 17.9 | HW | 3 |
| 27 | 12.10 | F | 18.4 | HW | 4 |
| 28 | 11.11 | M | 16.1 | HW | 3 |
| 29 | 15.1 | M | 15.6 | HW | 5 |
| 30 | 15.6 | F | 23.2 | HW | 5 |
| 31 | 10.11 | M | 15.6 | HW | 1 |
| 32 | 13.5 | F | 21.4 | HW | 5 |
| 33 | 15.1 | M | 18.3 | HW | 5 |
| 34 | 11 | F | 15.8 | HW | 3 |
| 35 | 12.2 | M | 16.6 | HW | 2 |

**Table 2:** Third finger middle phalanx maturational stages description and related information and drawings. MPS = middle phalanx stage.

(Modified from: [25] Perinetti et al., 2017)

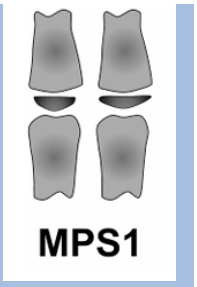
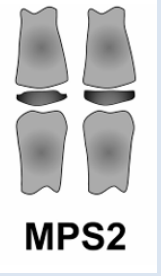
| Stage | Description | |
|---|---|---|
| MPS 1 | When the epiphysis is narrower than the metaphysis, or when epiphysis is as wide as metaphysis but with both tapered and rounded lateral borders. Epiphysis and metaphysis are not fused. |   |
| MPS 2 | When the epiphysis is at least as wide as the metaphysis with sides increasing thickness and showing a clear line of demarcation at right angle, either with or without lateral steps on the upper contour. In case of asymmetry between the two sides, the more mature side is used to assign the stage. |   |
| MPS 3 | When the epiphysis is either as wide as or wider than the metaphysis with lateral sides showing an initial capping towards the metaphysis. In case of asymmetry between the two sides, the more mature side is used to assign the stage. Epiphysis and metaphysis are not fused. |   |
| MPS 4 | When the epiphysis begins to fuse with the metaphysis although contour of the former is still clearly recognizable. Both sides of the epiphysis form obtuse angle to distal border, and the capping may still be detectable. |   |
| MPS 5 | When the epiphysis are totally fused with the metaphysis. |   |

**Table 3.** The four parameters resulting from logistic equation (SigmaPlt v 14.0, Systat Software, Inc.)

| min | max | EC50 | Hillslope |
|---|---|---|---|
| **~ 0** | 650 ± 30 | 1,3 ± 0,1 | 1.2±0.1 |

**Table 4:** Comparison between the LFIA values of the MSP3 samples analyzed with and without dilution

| Patient | Variables | 1:2 diluted (a.u.) | Not diluted (a.u.) |
|---|---|---|---|
| 3 | C | 417 | 308 |
| " | T | 26 | 14 |
| 4 | C | 398 | 340 |
| " | T | 46 | 47 |
| 5 | C | 43 | n.d. |
| " | T | n.d. | n.d. |
| 12 | C | 249 | 169 |
| " | T | 19 | 11 |

**Table 5:** Comparison between the "Peak" group and the "Post-peak" group. P-value 0.05

| Variables | Peak (N=15) | Post-peak (N=17) | P-value |
|---|---|---|---|
| Gender (F) | 9 (60%) | 12 (71%) | 0.7120 |
| Age (year) | 11.3±1.9 | 13.8±1.3 | <0.0001 |
| BMI | 18.6±2.7 | 19.7±2.5 | 0.2694 |
| IGF-1 (ng/ml) | 4.08±1.89 | 3.60±2.11 | 0.5009 |

**Table 6:** Comparison of patients classified with MPS class, BMI and IGF1 levels (ng/ml ± SD)

| MPS Class | N° and % | Female | Age | BMI | IGF1 |
|---|---|---|---|---|---|
| 1 | 3 (8%) | 1 | 9.6±1.2 | 16.3±0.7 | 3.44±1.39 |
| 2 | 6 (17%) | 3 | 10.4±2.3 | 18.7±2.4 | 3.62±1.76 |
| 3 | 9 (25%) | 6 | 11.9±1.4 | 18.5±3.1 | 4.39±2.01 |
| 4 | 5 (14%) | 3 | 13.2±0.7 | 19.1±3.1 | 4.43±3.06 |
| 5 | 12 (34%) | 9 | 14.1±1.4 | 19.9±2.4 | 3.25±1.62 |

**Table 7:** Comparison of patients classified with MPS class, BMI and IGF1 levels (ng/ml ± SD) and grouped with peak class.

| Peak class | MPS | N | Female | Age | BMI | IGF-1 |
|---|---|---|---|---|---|---|
| Pre-peak | 1 | 3 | 1 | 9.6±1.2 | 16.3±0.7 | 3.44±1.39 |
| Peak | 2.3 | 15 | 9 | 11.3±1.9 | 18.6±2.7 | 4.08±1.89 |
| Post-peak | 4.5 | 17 | 12 | 13.8±1.3 | 19.7±2.5 | 3.60±2.11 |

**Figures**

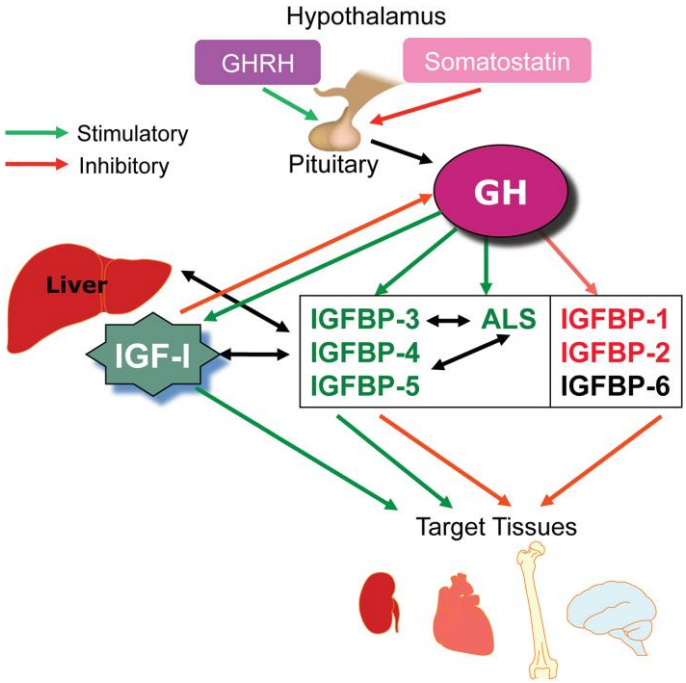



**Fig.1.** The IGF-1/GH axis involves a positive and negative feedback control mechanism. The production of the IGFBP- transport proteins are also involved in this control mechanism. From**:** Blum WF, Alherbish A, Alsagheir A, El Awwa A, Kaplan W, Koledova E, Savage MO. The growth hormone-insulin-like growth factor-I axis in the diagnosis and treatment of growth disorders. Endocr Connect. 2018 Jun;7(6):R212-R222. doi: 10.1530/EC-18-0099.

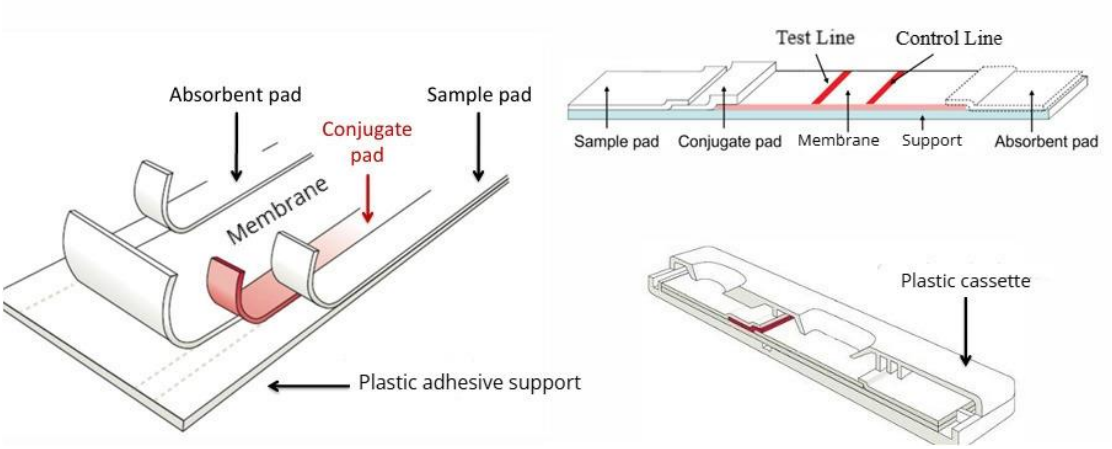



**Fig. 2.** The LFIA is composed of several components. The saliva sample is placed on the Sample Pad and flows through the nitrocellulose pad by capillary action toward the Control line, binding the target molecule to the various antibodies. Modified from; Di Nardo F. (8 march 2023): LFIA: dai fondamenti ai recenti sviluppi, https://elearning.unite.it/pluginfile.php/267332/mod_resource/content/1/Lateral%20flow%20immunoassays%202023.pdf

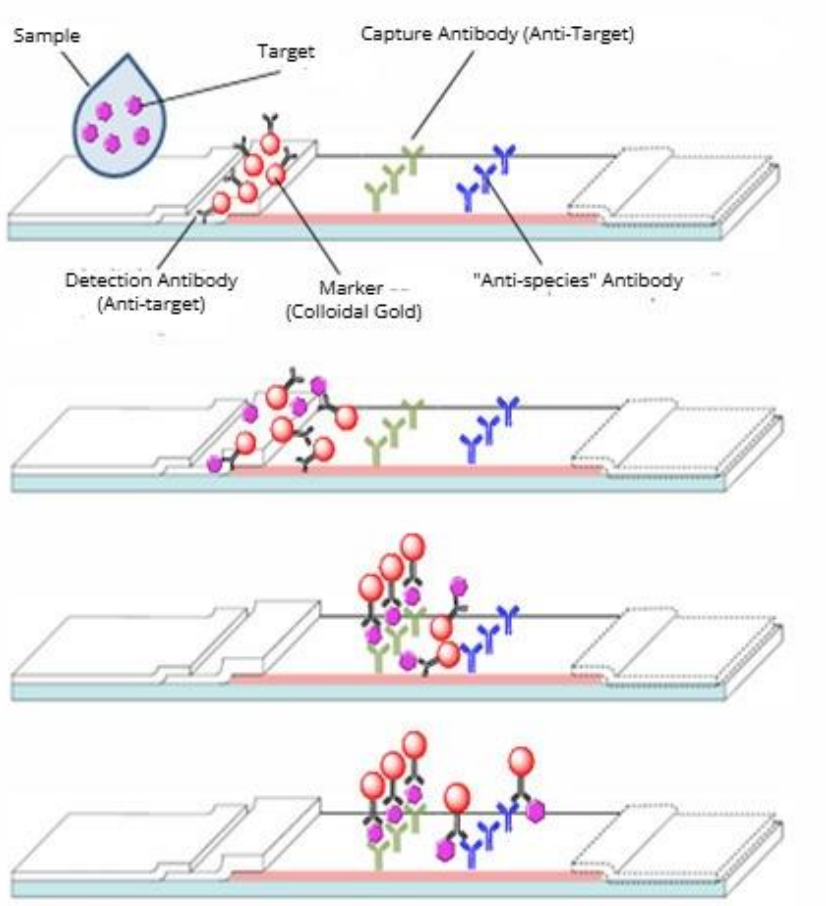



**Fig.3.** The target molecule flows along the adsorbent paper, first binding to an antibody pre-labeled with a color marker. Near the Test line, it binds to an antibody specific for the target, with the remaining unbound portion binded to another antibody on the Control line. Modified from; Di Nardo F. (8 march 2023): LFIA: dai fondamenti ai recenti sviluppi,

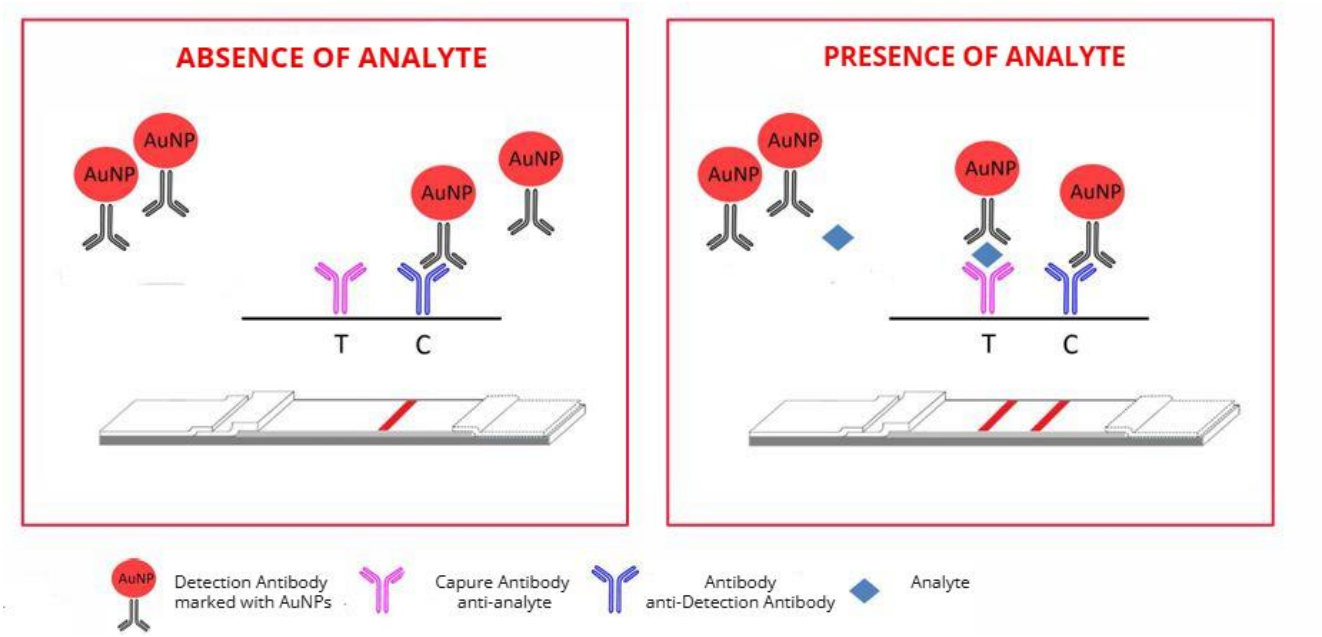



**Fig.4.** In non-competitive LFIAs, the appearance of only the control line indicates a negative result, while the presence of both the test and control lines indicates a positive result. The absence of both lines indicates an invalid test. Modified from; Di Nardo F. (8 march 2023): LFIA: dai fondamenti ai recenti sviluppi

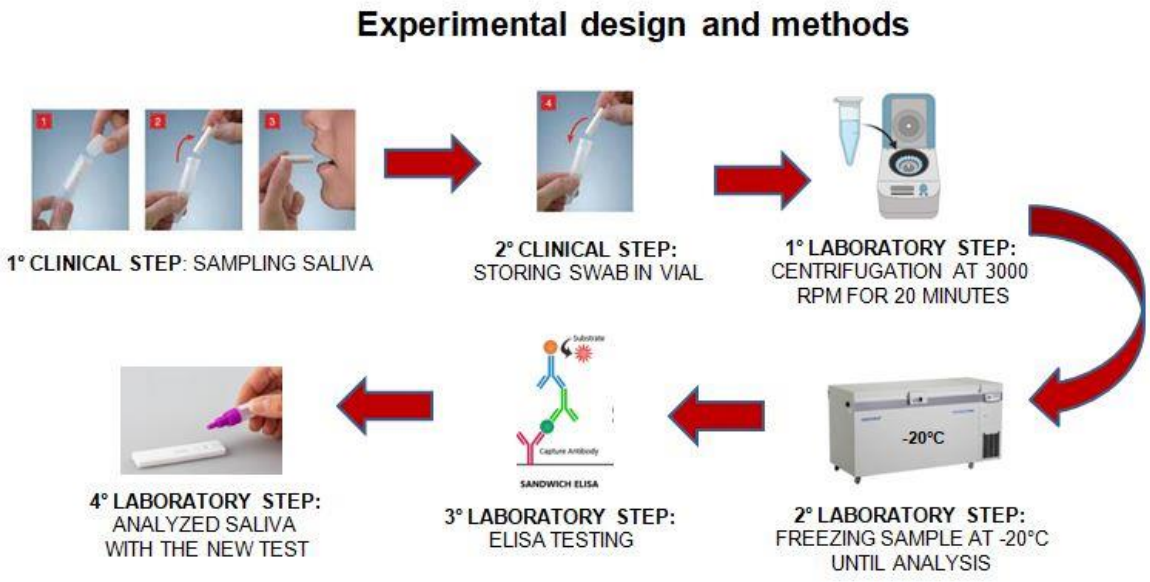



**Fig.5.** Salivary sampling and analysis includes some clinical and some laboratory steps where the sample is analyzed both with ELISA and with the LFIA prototype.

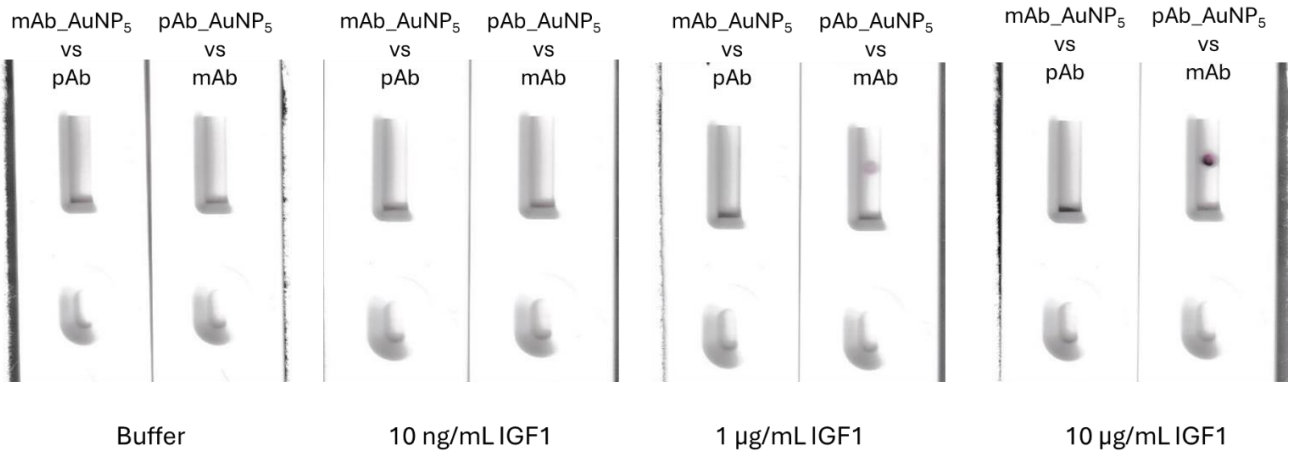



**Fig. 6.** With the ppo test, using the pAb on the Test line and the mAb labelled to the AuNPs, the presence of IGF1 did not reveal any results. On the contrary, using the pAb as detection Ab and the mAb as the capture Ab reveal a specific signal for IGF1 at 1 and 10 µg/mL.

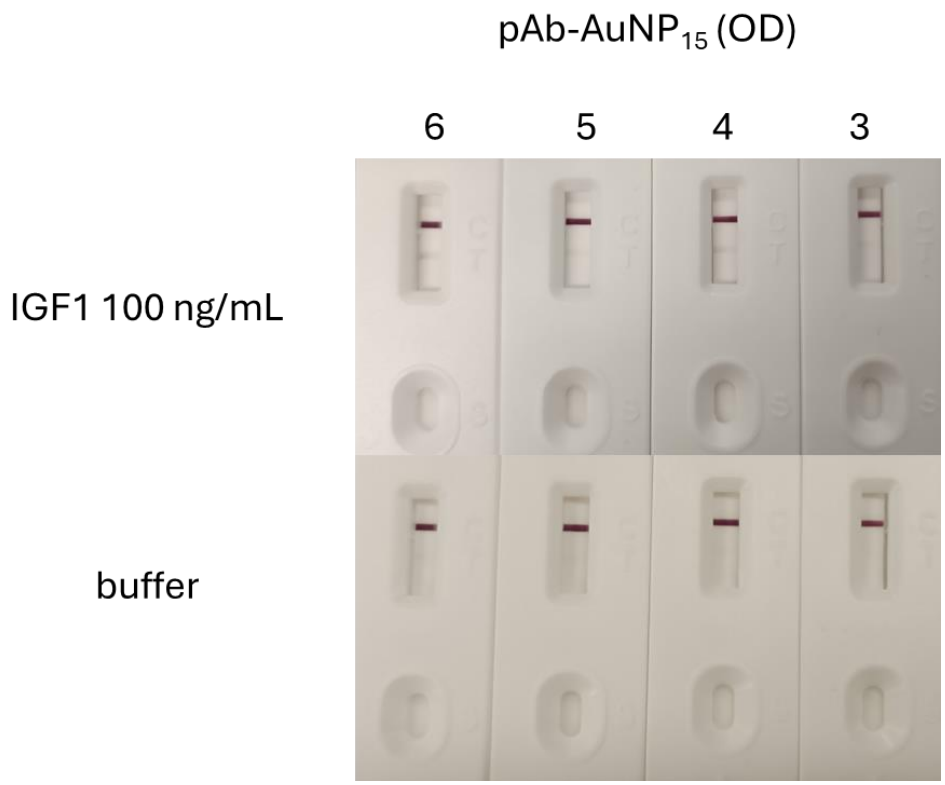



**Fig. 7.** To assess the sensitivity of the LFIA, the Ab-AuNPs concentration (expressed as the optical density) must be evaluated. A not-specific signal was clearly visible from OD4 onwards, whereas specific signals in the presence of IGF1 100 ng/mL were clearly distinguishable from the buffer. Therefore, Ab-AuNPs OD 3 was chosen for the vLOD evaluation.

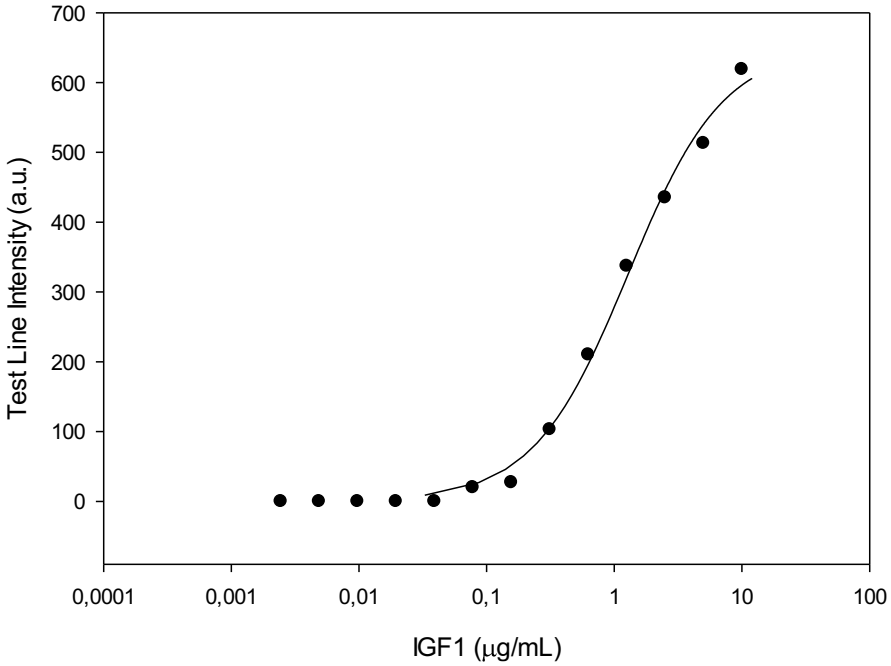



**Fig. 8**. Calibration curve referred to Fig.9

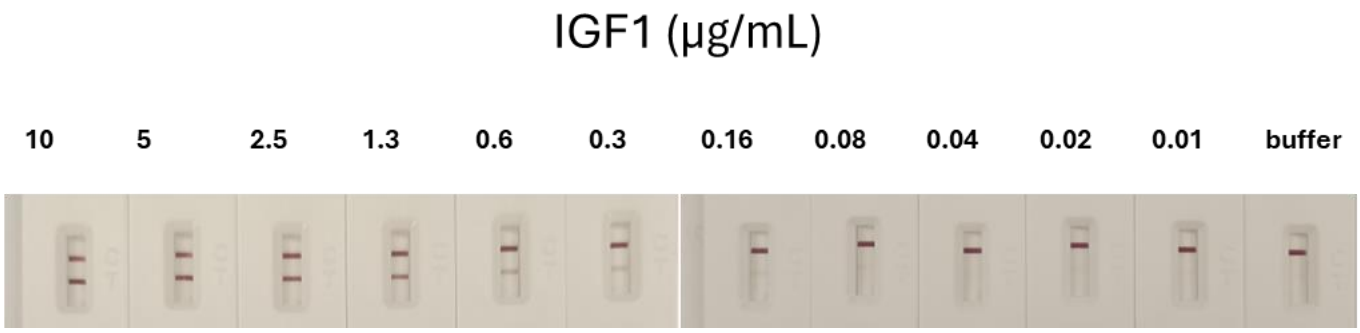



**Fig. 9.** The strips showed a visual LOD between 0.08 and 0.04 µg/mL.

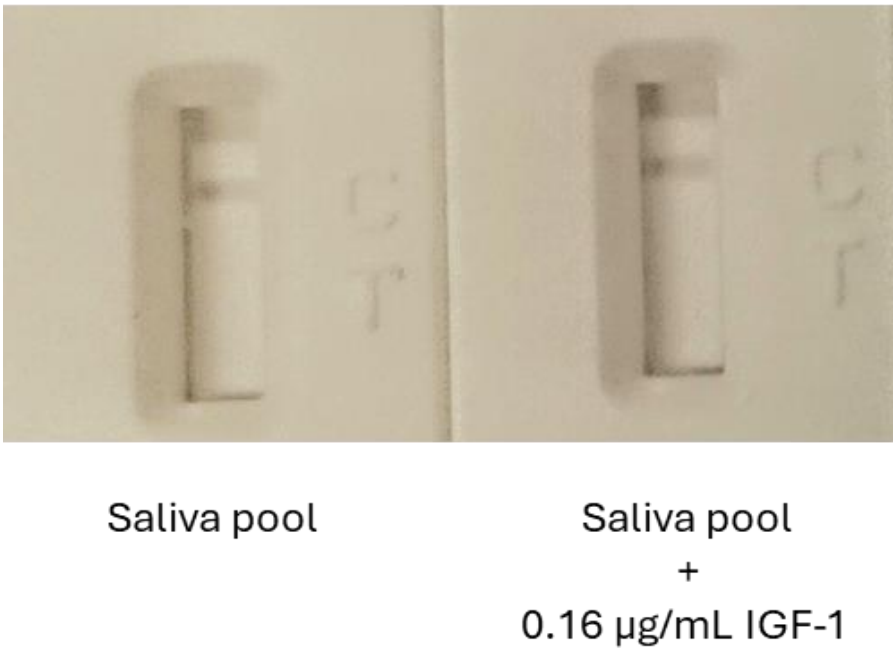



**Fig.10.** IGF1 was not visible in saliva of adult subjects, even in presence of 0.16 µg/mL IGF1

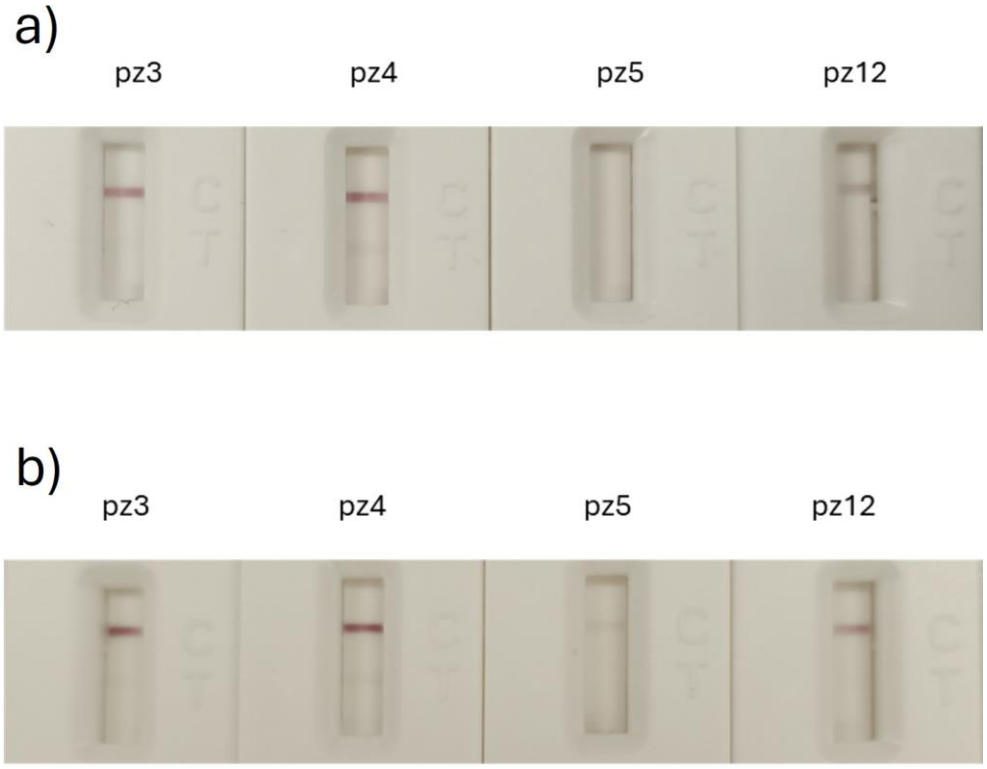



**Fig. 11**. Images of the LFIA strips by analysing the salivary samples of patients pz3, pz4, pz5 and pz12 without dilution (a) and diluted 1:2 using the running buffer (b)

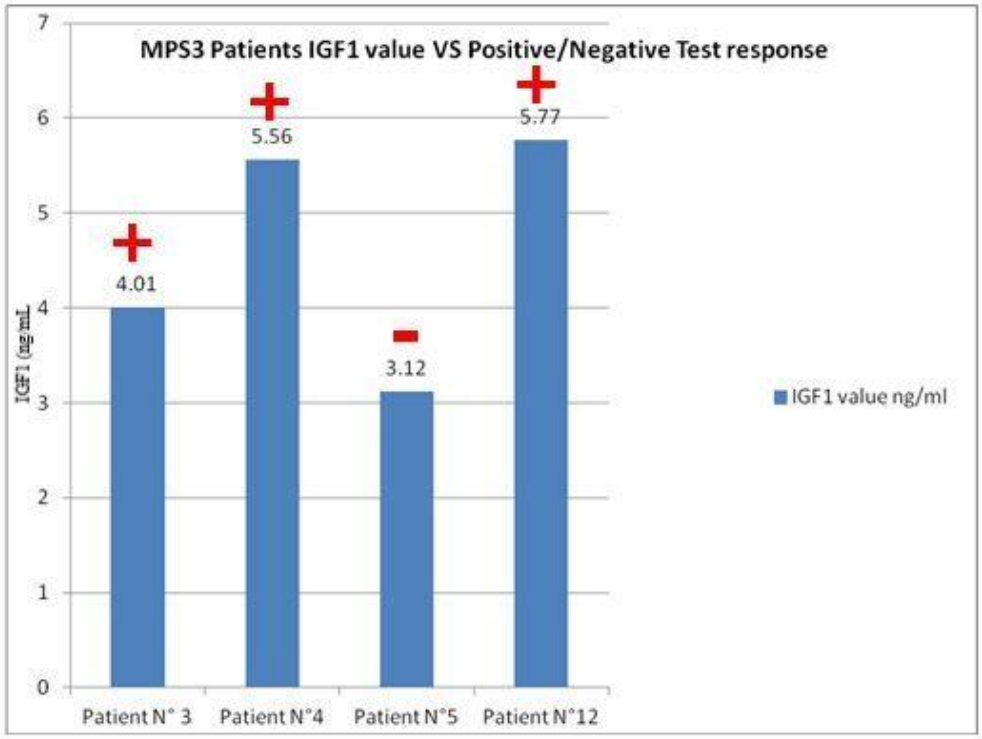



**Fig. 12.** Test response in MPS3 patients. MPS3 patients tested with the LFIA prototype showed a positive (+) result only for IGF1 value over 3.12 ng/mL. Patient N°5 showed a negative (-) response.

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