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Saliva leukocytes rather than saliva epithelial cells represent the main source of DNA

Leucocitele salivare şi nu celulele epiteliale din salivă reprezintă principala sursă de ADN

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Abstract

Introduction. *Several alternative methods to peripheral blood DNA extraction have been implemented so far. Saliva seems to represent a very advantageous type of sample, easy to harvest and able to generate DNA yields comparable to those extracted from blood mononuclear cells.*

Material and methods. *8 patients suspected of ankylosing spondylitis, 9 patients with various hematological malignancies, displaying post-chemotherapy leucopenia and 30 healthy volunteers were included in our study. DNA was extracted with various commercially available kits and used for HLA typing either by PCR amplification, or by PCR followed by hybridization.*

Results. *Our data regarding HLA typing support already published results regarding the good DNA quality that allows its use in various molecular biology techniques. However, when attempting to use saliva from immunosuppressed patients for DNA extraction we have generated very low yields, comparable again with the ones obtained from peripheral blood. Flow cytometry and immunocytochemistry investigations confirmed the low number of leukocytes present in the saliva of these patients, while the number of epithelial cells was virtually unchanged.*

Conclusions. *The main source of saliva DNA seems to be represented by leukocytes present in this fluid and not by the epithelial cells. Under these circumstances, for immunosuppressed patients saliva cannot represent an alternative to blood when attempting DNA extraction.*

Keywords*: Saliva, DNA, leukocytes, epithelial cells*

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Rezumat

Introducere. *Există mai multe metode de extracţie a ADN-ului implementate ca alternativă la sângele periferic. Saliva pare să fie un tip de probă avantajos, uşor de recoltat şi cu posibilitatea de a genera ADN comparabil cu acela extras din celulele mononucleare ale sângele periferic.*

Material şi metodă. *Am inclus în studiul nostru 8 pacienţi suspecţi de spondilită anchilopoetică, 9 pacienţi cu diverse afecţiuni hematologice maligne, prezentând leucopenie post-chimioterapie şi 30 voluntari sănătoşi. ADN-ul a fost extras cu variate kit-uri comerciale disponibile şi utilizat pentru tiparea HLA prin amplificare PCR, urmată de hibridizare.*

Rezultate. *Rezultatele noastre referitoare la tiparea HLA sunt în concordanţă cu date publicate deja, referitoare la calitatea bună a ADN-ului extras din salivă, ceea ce permite utilizarea acestuia în diverse tehnici de biologie moleculară. Totuşi, extracţia ADN-ului din saliva de la pacienţi imunodeprimaţi a avut un randament scăzut, comparabil cu cel din sângele periferic. Investigarea prin citometrie în flux şi imunocitochimie a confirmat numărul mic de leucocite în saliva acestor pacienţi, în timp ce numărul celulelor epiteliale a fost similar cu cel al loturilor de comparaţie.*

Concluzii. *Principala sursă de ADN din salivă pare să fie reprezentată de leucocite şi nu de celulele epiteliale. În aceste circumstanţe, la pacienţii imunodeprimaţi, saliva nu este o alternativă superioară sângelui pentru extracţia de ADN.*

***Cuvinte cheie**: Saliva, ADN, leucocite, celule epiteliale*

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Introduction

For genetic analyses human DNA can be extracted from a variety of sources. Peripheral blood DNA extraction seems to be the method of choice for most laboratories, a procedure for which there are also clear guidelines regarding the blood drawing and storage [1]. However, alternative DNA sources have been considered and correspondent extraction methods have been implemented and standardized. While for cadaveric donors the spleen represents an important option and tumor biopsies might be critical in particular circumstances, for living patients or healthy individuals, collection methods like mouthwash, cytobrush, mucosal swabs and even saliva collection tend to become increasingly used. Each such method presents advantages and disadvantages.

While whole blood generates, in most cases, a sufficient amount of good quality DNA, harvesting peripheral blood is an invasive method which requires a phlebotomist, presents an important discomfort, a risk of infection or hemorrhage. Furthermore, the stability of blood samples is limited at room temperature (RT) and blood tubes require 4^0C storage conditions and special packaging conditions if they are to be shipped [2].

Brushes or swabs generate good DNA yields [3], but they require careful harvesting, by well trained medical personnel, and the extraction can pose technical difficulties, as supplementary steps to transfer the sample from the brush or swab into solution are mandatory.

The mouthwash collection method, even if very simple, requires a solution with a relatively high content of alcohol, which can be ill-tolerated by many persons, especially children [4]. Hence, it is considered that for children, swabs and cytobrushes are more advantageous for DNA extraction [5-7].

On the other hand, saliva sampling is a very comfortable method, easily accepted by patients, however still regarded with reluctance by many physicians for a number of potential problems that might be considered. As the number of cells present in this type of sample is projected by many to be lower than in a regular blood sam-

ple, a major concern is regarding the amount of DNA that can be extracted. However, there are an increasing number of studies that have shown that high yields of DNA can be extracted from saliva [8-10]. Another important issue is about the purity of the saliva DNA, as biological contaminants, especially bacteria, but also viruses and fungi are clearly present, even in the fluids of healthy individuals. As a consequence, concerns were raised regarding the purity and overestimation of DNA concentrations [8], but again, many studies demonstrated the high quality of the extracted DNA [9-12] that can be used for RT-PCR, PCR-RFLP [13], and even Sanger genotyping [8] or for New Generation Sequencing (NGS) [11].

Both DNA quantity and quality are critical for any type of gene analysis and this is why whole blood DNA extraction is still preferred and imposed by accrediting procedures for most current molecular biology investigations, HLA typing, and DNA sequencing. On the other hand, should large studies, based on an important number of volunteers, are to be considered, the compliance of participants represents an important issue for the success of the study and, when faced with a non-invasive method, the participants are much easier to convince [14].

Commercial kits are made available for saliva harvesting. They consist of a vial where the donors are required to spit, the optimal saliva volume being indicated by the manufacturer. A solution that contains chemicals able to prevent DNA degradation and bacterial growth is then added and the two liquids are mixed. Manufacturers claim that such samples can be stored at RT for at least one year and still be able to generate a reasonable amount of undegraded DNA [15,16]. By consequence, these features make saliva sample collection an important option for DNA extraction due to the simplicity of the sampling, which allows self harvesting and stability, which further reflects in storing conditions and shipping.

Several studies attempting DNA extraction from saliva using various commercial harvesting kits, some performed on very large cohorts, have already been published [11,12,17,18], confirming that this biological sample is a reliable one for extracting human DNA.

However, as not the fluid but the mixture of cells to be found in saliva represents the proper source of DNA, the question which further arose was regarding the type of cells that constitute themselves as the main source. Due to the rather large number of epithelial cells, around 4.3 x 10^5/ml [19] and due to the continuous turn over that enables the replacement of the surface layer at approximately every 3 hours [19] one would expect that these particular cells will be able to provide a sufficient amount of DNA [13].

This was our assumption as well when we have decided to approach saliva for DNA extraction as an alternative to blood, having in mind primarily the immunosuppressed patients, for which the regular blood DNA yields are often insufficient with respect mostly to PCR amplifications.

To the best of our knowledge, this study brings evidence for the first time that the saliva epithelial cells do not represent the main DNA source but rather the cells originating from the blood stream and crossing into the oral cavity are the ones targeted by the various commercially available DNA extraction kits.

Materials and methods

Patients and healthy individuals

Blood and saliva were harvested from 8 patients suspected of ankylosing spondylitis, for which HLA-B27 genotyping was recommended, and from 9 patients with various hematological malignancies, displaying post-chemotherapy leucopenia.

Also, a group of 30 healthy volunteers were included in this study, for which only saliva was harvested.

Saliva was harvested and used after an informed consent was obtained.

Blood harvesting

As we intended to use the extracted DNA in several types of PCR, the blood was harvested using vacutainers with either EDTA or citrate based anti-clotting agents, but not with heparin.

Saliva harvesting

The saliva was harvested using the commercial Oragene DNA device from DNA Genotek (Canada). The harvesting kit contains a stabilizing liquid which is mixed with a certain volume of saliva, indicated by the manufacturer. Once the liquids are mixed, the samples can be stored at room temperature (RT) before DNA extraction.

DNA extraction

The purification of the genomic DNA from saliva was initially attempted using a demo prepIT^{TM} CD2 Genomic DNA MiniPrep kit, recommended by the same manufacturer (DNA Genotek, Canada), according to the provided protocol. The kit is based on columns endowed with a matrix able to retain the precipitated DNA. After gently shaking the saliva harvested as described above, the tubes were incubated for 1.5 hours at 50^{0}C in a dry bath. Then, 500 µl of saliva were mixed with 350 µl of PT buffer and vortexed at top speed for 15 sec. The entire mix was transferred into the MiniPrep Column and centrifuged at 8,000 rpm for 1 min. After discarding the flow through, the column was washed with 500 µl of Wash Buffer 1 (60% ethanol) for 1 min at 8,000 rpm and then twice with 700 µl Wash buffer 2 (70% ethanol) at 12,000 rpm for 1 min. The DNA was eluted by centrifuging the column for 1 min at 12,000 rpm with 100 µl Elution Buffer pre-warmed at 65^{0}C.

The DNA was then extracted using an adsorbtion columns based "mini" Life Technology commercial kit (Invitrogen, Life Technologies, Carlsbad, CA, USA), dedicated to small blood volumes, usually between 200 and 300 µl. The protocol we have used, similar to the one described above, was the same as for peripheral blood, according to the manufacturer's indications. Thus 200 µl of sample (saliva and stabilizer mix, or blood) were incubated with 20µl of RNase and 20µl of proteinase K for 2 min. at RT. Then, 200 µl of proteinase K buffer were added and the mix was thoroughly vortexed. After 10 minutes incubation at 55^{0}C, the DNA was precipitated with 200 µl of 100% ethanol and introduced in a Life Technology column that was centrifuged at 10,000 rpm for 1 min. Two washes were performed at 10,000 rpm for 1 min, and 13,000 rpm for 3 min, using two different ethanol based buffers. The elution was performed by centrifugation at 13,000 rpm for 1 min, with 200 µl elution buffer.

DNA concentration measurement

DNA concentration was quantified with a Beckman Coulter DU800 spectrophotometer, in a 100 µl cuvette, at a 1/10 dilution in water. The absorbtion was measured simultaneously at 260 nm and at 280 nm as well, in order to determine the potential protein contamination.

PCR

An HLA-SSP (Sequence Specific Primers) approach was considered for this test. We have used a commercial kit dedicated to HLA-B27 typing, based on a single pair of primers (BAG Health Care Gmbh, Lich, Germany). The alleles amplified by these primers are, according to the manufacturer, HLA-B2701-2717, 2719-2721, 2724-2728, 2730-2732, and 2734-2745. The size of the control band is of 1070 base pairs (bp), while the amplicon should have a size of 420/85 bp. The reaction is set for a 10 µl volume, and the following components were mixed: 1 µl of DNA (20-40 ng/µl), 1 µl of PCR buffer (that includes MgCl), 0.1 µl of Histo-Taq polymerase

(BAG Health Care Gmbh, Lich, Germany) and 8 µl of molecular grade H_2O (Sigma Aldrich, Germany). The mix was then added to the tube containing the dehydrated primers and pipetted up and down until the primers were completely solubilized. The amplification set-up is as follows:

1	1 cycle	94^{0}C	5 min	Denaturing
2	5 cycles	96^{0}C 68^{0}C	20 sec 60 sec	Denaturing Annealing and extension
3	10 cycles	96^{0}C 64^{0}C 72^{0}C	20 sec 50 sec 45 sec	Denaturing Annealing Extension
4	15 cycles	96^{0}C 61^{0}C 72^{0}C	20 sec 50 sec 45 sec	Denaturing Annealing Extension
5	1 cycle	72^{0}C	5 min	Final extension

PCR and hybridization

The simultaneous genotyping of HLA-B27 and of the cytochrome P450 2D6 genes was performed by PCR multiplex amplification followed by a reverse hybridization assay, using a HLA-B*27 and CYP2D6*4 commercial kit (GenID Gmbh, Strassberg, Germany). The PCR amplification was set in a 25 µl reaction volume using the following amplification conditions:

1	1 cycle	95^{0}C	5 min	Denaturing
2	10 cycles	95^{0}C 68^{0}C	30 sec 120 sec	Denaturing Annealing and extension
3	22 cycles	95^{0}C 55^{0}C 72^{0}C	10 sec 30 sec 30 sec	Denaturing Annealing Extension
5	1 cycle	72^{0}C	8 min	Final extension

The amplification was followed by hybridization of oligonucleotides fixed on nitrocellulose strips. 20 µl of the PCR mix were denatured using an equal volume of denaturing solution provided by the kit, for 5 min at room temperature. Then, each strip was covered by 1 ml of hybridization solution, previously heated at 47^{0}C. The hybridization was performed at 47^{0}C, for 30 min, in a water bath with a shaker tray. After the removal of the hybridization buffer, the strips were washed twice for 1 min and once for 15 min at 47^{0}C with the stringent wash buffer provided by the kit. Once the stringent wash buffer was removed, the strips were then twice washed with a 1:5 diluted rinse buffer, for 1 min at RT. After the rinse buffer removal, the strips were incubated for 30 min at RT with 1 ml of 1:100 dilution of streptavidin-enzyme conjugate. After 3 rinses performed at RT for 1 min, the strips were covered with 1 ml of substrate and the color reaction was monitored. As the bands started to become visible, the reaction was stopped by removing the substrate buffer as soon as possible and by rinsing the strips with distilled water.

May-Grünwald Giemsa staining

Saliva smears were stained for 5 min at RT with a May Grünwald (Sigma Aldrich, Germany) solution (previously diluted with an equal volume of water), then immersed for 30 min in a 1/10 dilution Giemsa solution, after which they were washed with distilled water and dried.

Immunocytochemistry

Identification of the various cells present in saliva was initially performed by immunocytochemistry. The epithelial cells were labeled with monoclonal anti-cytokeratin antibody (MNF16, Dako, Denmark) [20] while the leukocytes were identified with the monoclonal anti-CD45 antibody (Dako, Denmark)]. Neutrophils were labeled with monoclonal anti-CXCR1 (IL-8 RA) and anti-CXCR2 (IL-8 RB) antibodies (BD Biosciences Pharmingen, USA) [21,22].

The cells from saliva samples were attached by centrifugation at 300g on glass slides and fixed for 15 minutes in 100% ethanol, at room temperature (RT).

The slides were incubated first with 3% H_2O_2, 5 min, RT, to quench the endogenous peroxidases, washed in distilled water, 5 min and phosphate-buffered saline (PBS), 5 min. Then, the cells were incubated with 2% fetal bovine serum in RPMI 1640 (Sigma Aldrich, Germany), for 30 min, RT, to block non-specific binding sites, followed by washing in PBS, for 5 min, RT. The incubation with the primary antibodies was performed at the concentrations recommended by the manufacturers, at 4°C, overnight, followed by two washings in PBS, 5 min, RT. The binding of all primary mouse anti-human antibodies was detected with the EnVision Dual Link System-HRP kit (Dako, Denmark). The slides were initially incubated with the secondary antibodies conjugated with an HRP labeled polymer for 30 min, washed in PBS, 5 min, RT, then incubated with DAB/hydrogen peroxide, 15-20 min, RT. The slides were counterstained with Mayer's hematoxylin (Dako, Denmark). Secondary antibodies and Streptavidin-HRP were used as negative controls.

Flow cytometry

The saliva samples were transferred into 5 ml tubes and washed with at least 4.5 ml PBS by centrifugation at RT, 500g, 5 min. After discarding the supernatant, the pellet was vortexed and resuspended in 100 µl isotone fluid. The cells were labeled with 2 µl anti-CD45 antibody PeCy5, clone MEM-28, Exbio, CZ) for 30 min, at RT, and washed with 2 ml isotone fluid. After removing the supernatant, the pellet was resuspended and subjected to analysis with a BD FACSAria III flow cytometer (FACSDiva software).

Results

For 4 patients with ankylosing spondylitis the saliva was collected with the Oragene harvesting system and the DNA yields obtained using a demo prepIT™ CD2 Genomic DNA MiniPrep kit from the same manufacturer (Genotek, Canada) are presented in Table 1.

Table 1. DNA concentrations obtained with the Oragene DNA saliva harvesting system and the prepIT™ CD2 Genomic DNA MiniPrep kit, as compared with DNA yields obtained from peripheral blood with the Life Technology DNA extraction kit.

Patient initials		
B G	260nm/280nm ratio	Concentration µg/ml
blood	1.9669	23.05
saliva	1.9239	51.53
C M		
blood	2.0027	25.37
saliva	1.6984	13.77
L V		
blood	1.8372	19.13
saliva	1.8865	30.24
S D		
blood	1.9264	42.49
saliva	1.8068	51.20

DNA average concentration extracted from 200 µl of blood: 27.51 µg/ml
DNA average concentration extracted from 500 µl of saliva: 36.68 µg/ml

In a similar manner, for the other 4 patients with ankylosing spondylitis saliva was collected with a demo Genotek saliva harvesting system and extracted with the Life Technology DNA extraction kit. The yields are presented in Table 2. The patient CM was not compliant and despite our indications, the saliva volume that he supplied was three times more than required, hence, due to the sample dilution, a low concentration of DNA was obtained.

For this group of patients, when comparing the blood and the saliva DNA extraction from a volume of sample of 200 µl, despite the differences evidenced by the measurements presented above, we have noticed that the average

Table 2. DNA concentrations obtained with Genotek saliva harvesting kit as compared with DNA yields obtained from peripheral blood with the Life Technology DNA extraction kit.

Patient initials		
B G	Ratio 260/280	Concentration µg/ml
blood	1.9669	23.057
S5	1.8672	25.96
C M	Ratio 260/280	Concentration µg/ml
blood	2.0027	25.3724
S5	1.6649	8.1686
L V	Ratio 260/280	Concentration µg/ml
blood	1.8372	19.1307
S5	1.9295	35.8471
S D	Ratio 260/280	Concentration µg/ml
blood	1.9264	42.4929
S5	1.9514	40.5

DNA average concentration extracted from 200 µl of blood: 27.51 µg/ml
DNA average concentration extracted from 200 µl of saliva: 27.61 µg/ml

yields are remarkably similar: 27.51 µg/ml for blood, versus 27.61 µg/ml for saliva, even considering an outlier, as is the case for patient CM.

Under these circumstances, we were encouraged to pursue DNA extraction from saliva samples and we have opted for the second set-up, given the fact that the Life Technology extraction kit is currently used in our laboratory. The yields obtained from 200 µl of saliva (diluted in equal volumes with the stabilizing agent) harvested from 30 healthy donors are presented in Table 3.

The mean DNA value obtained in this set-up was 30.96 µg/ml, which is slightly higher than the amount of DNA that we currently manage to extract from peripheral blood samples (roughly around 28 µg/ml). However, it should be noted that this extraction method, unlike DNA extraction from peripheral blood, may generate in some cases important yield differences between samples, and that can be a cause of major concern.

Table 3. DNA concentrations obtained with the Genotek saliva harvesting system and the Life Technology "mini" DNA extraction kit.

Patient initials	260nm/280nm ratio	Concentration µg/ml
M S	1.94	89.23
S G	1.98	80.41
M L	1.89	64.73
N M	1.96	62.61
N A M	1.94	46.82
A C	1.72	42.19
C M	1.92	41.85
B G	1.89	40.32
G M	1.9	36.8
S I	1.94	36.62
C P	1.95	31.81
P D	1.88	31.15
M M	1.91	31.12
S S P	1.95	30.82
B I	1.88	30.21
T M	1.88	30.08
P C	1.87	28.18
C I P	1.9	25.49
P D	1.81	19.19
L E	1.9	16.4
N S A	1.85	16.1
C I	1.85	15.9
F C	1.95	14.63
R R	1.64	13.03
S B	1.82	12.02
S E C	1.67	9.28
G G	1.66	8.7
D A	1.71	8.37
G I	1.71	8.04
A M R	1.62	6.81

Saliva DNA average concentration: 30.96 µg/ml

We have also checked if saliva DNA is appropriate for PCR amplification and hybridization in the current set-up of our laboratory, and the results were absolutely comparable with the

ones obtained using PBMC DNA (Figure 1 and Figure 2).

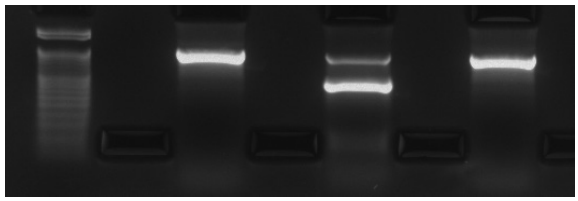

Figure 1. Amplicons generated in a PCR reaction using a BAG kit by three samples tested for HLA-B27. Sample 2 is positive, while 1 and 3 are negative.

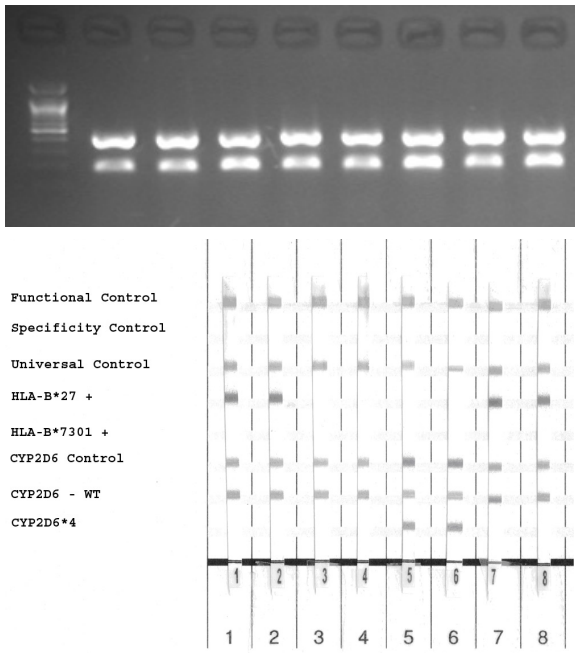

Figure 2. Saliva DNA checked in a multiplex PCR reaction followed by hybridization. A. The amplification is initially verified by loading 4 µl of the 25 µl total PCR volume in a 1.5% agarose gel. B. Different hybridization patterns generated by these samples in a test targeting simultaneously for HLA-B27 and the CYP2D6*4 allele. The strips are placed in a frame supplied by the manufacturer, indicating the position of the various oligonucleotides. For example, sample #1: HLA-B*27 positive, CYP2D6 Wild Type positive, CYP2D6*4 negative; sample #6: HLA-B*27 negative, CYP2D6 Wild Type positive, CYP2D6*4 positive.

We have further decided to approach saliva as an alternative for DNA extraction in the particular cases of cytopenic patients, as these patients do not present a sufficient number of white blood cells (WBC), hence they do not generate a sufficient amount of DNA, required for various molecular biology techniques currently employed by our laboratory. We have considered 9 patients with various hematological malignancies, subjected to various chemotherapy regimens.

The DNA yields obtained from blood versus saliva are presented in Table 4.

The average DNA concentration obtained from these blood samples was 10.4 µg/ml, slightly higher than the 8.81 µg/ml yield obtained from saliva.

Given the rather unexpected low DNA yields generated by the saliva of these particular patients, we have raised the question if indeed

Table 4. DNA yields obtained from saliva and blood of leucopenic patients

Patient initials	WBC count*	ANC		260/280 nm ratio	Concentration µg/ml
AG	0.24	0.01**	Blood	1.72	10.55
		4.2***	Saliva	2.1	4.36
CV	0.3	0.12	Blood	1.73	10.39
		40	Saliva	2.28	14.12
CP	1.48	0.01	Blood	1.75	11.42
		0.7	Saliva	1.67	6.53
FM	0.45	0.02	Blood	1.74	11.51
		4.5	Saliva	2.34	2.8
GGC	0.56	0.15	Blood	1.66	13.17
		26.8	Saliva	2.12	4.48
SM	0.3	0	Blood	1.57	13.58
		0.1	Saliva	2.05	10.5
VAC	0.14	0.02	Blood	1.84	10.65
		14.3	Saliva	1.99	11.36
GC	0.71	0	Blood	1.64	11.56
		0	Saliva	1.71	9.27
OO	1.6	0.19	Blood	1.63	11.22
		11.8	Saliva	1.85	24.72

*Normal = 4-10 x 10^9/L
**Normal = 2-8 x 10^9/L
***Normal = 45-80%
ANC= absolute neutrophil count

the epithelial cells represent the main source of DNA, as it is currently accepted [13].

In order to verify the content of such cells in the saliva, we have initially checked saliva smears stained using May-Grünwald-Giemsa method (Figure 3).

While the May-Grünwald-Giemsa staining was able to clearly show the epithelial cells as large cells with clear cytoplasm and small nucleus, the identification of the smaller cells present in the saliva requires specific labeling. Hence, saliva from both healthy donors and immunosuppressed patients was further analyzed by flow cytometry. We have targeted the panleukocyte CD45 molecule (Figure 4).

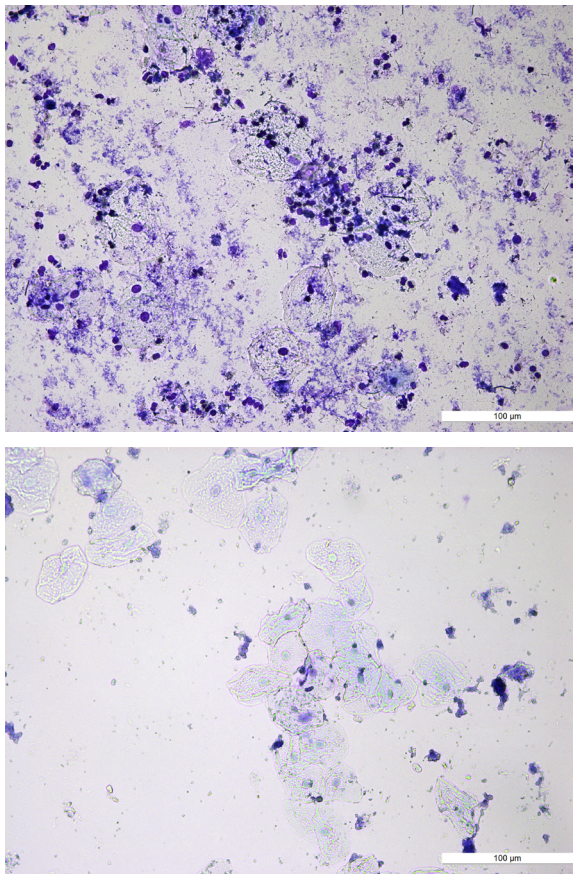

Figure 3. May-Grünwald-Giemsa staining of saliva smears: A. Saliva from a normal individual reveals epithelial cells with a large diameter and small nucleus, small cells, with a large nucleus, probably lymphocytes and cells with multilobed nuclei, probably neutrophils. B. Saliva from a patient subjected to chemotherapy shows a comparable number of epithelial cells, while the leukocytes are extremely rare (x20).

This analysis confirmed, as somehow expected, the low number of WBC in the saliva of the cytopenic patients, while the number of epithelial cells and epithelial debris was virtually the same.

Alternatively, the presence of leukocytes, epithelial cells and neutrophils was verified by immunocytochemistry. The labeling targeted CXCR1 and CXCR2 present on the neutrophils surface (Figure 5), epithelial cells (cytokeratin and EMA) and leukocytes (CD45) – data not shown. The epithelial cells are easily distinguishable, due to their large size. Many of the cells are broken; hence saliva smears are typically displaying much cellular debris.

Discussions

Molecular biology diagnosis is becoming increasingly used for a large array of conditions. Genomic studies have revealed gene abnormalities like mutations, deletions, rearrangements or alterations of the gene copy numbers that can pin point a certain condition. Furthermore, such genetic modifications are of maximum interest as many of them are transmitted and this leads to inherited genetic susceptibility, with a major impact in oncology if the targeted gene is a protective one [26,27]. Last but not least, therapy tends to become personalized as many new drugs are targeting particular alterations of key molecules or even entire cell signaling pathways [28]. Hence an accurate gene and molecular characterization is of great importance for both diagnosis and treatment. A key issue for such techniques is the ability to obtain a sufficient amount of good quality DNA.

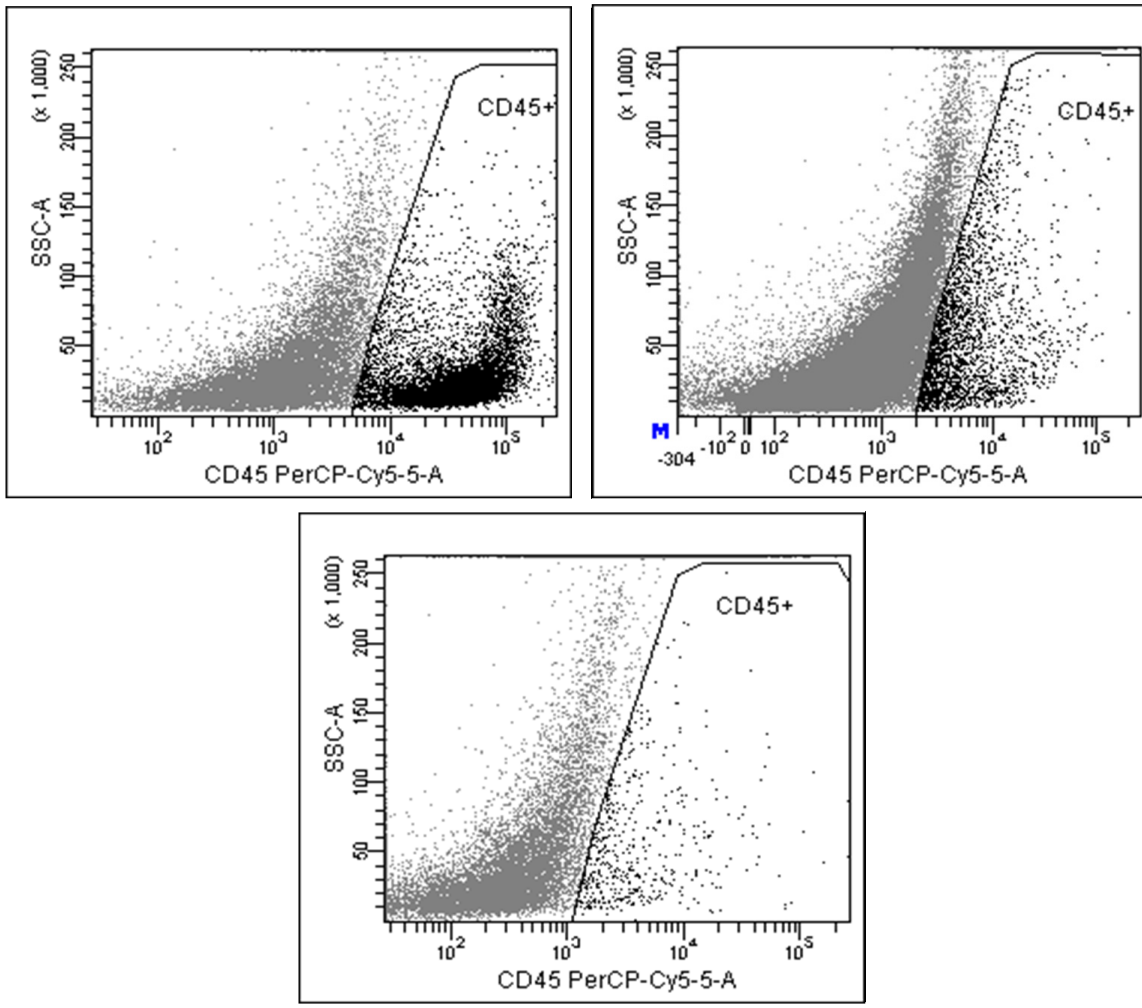

Figure 4. Flow cytometry analysis of saliva labeled for the CD45 pan-leukocyte marker. A. saliva from a normal individual, displaying 38% CD45+ cells; B. saliva from patient AG with a concentration of 4% CD45 + cells; C. saliva from patient FM, with a concentration of 2.7% CD45+ cells

The preferred source of DNA are the PBMCs (peripheral blood mononuclear cells) or, alternatively, in the case of cadaveric donor transplantation, spleen cells. As peripheral blood harvesting represents an invasive method, this raises several issues: availability of proper harvesting conditions, trained personnel, tubes with a proper anti-clotting agent, or the risk of infection. Furthermore, blood samples require refrigeration, proper tube transportation conditions and the DNA should be ideally extracted in a matter of days [29]. Largely used in forensic medicine, non-invasive alternatives are the DNA extraction from buccal swabs or brushes [30] and plucked hairs [31]. However, DNA extraction from saliva seems to be a much more advantageous method. There are several arguments in favor of this procedure. It is a very easy non-invasive harvesting method that can be performed even by the patient, and able to generate, in most cases, yields comparable to peripheral blood, as our results, obtained in different set-ups, are showing. We

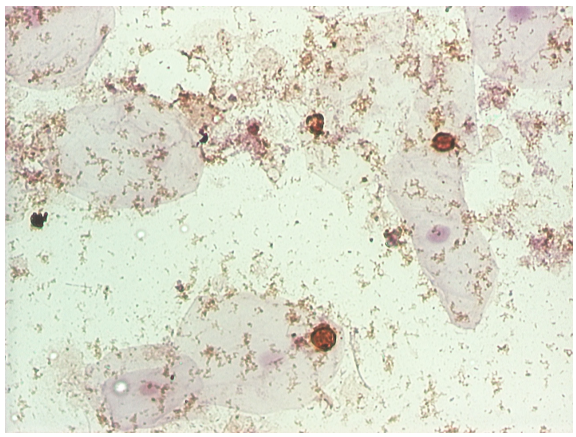

Figure 5. The cells form the saliva of a normal individual, positive for CXCR1, are, most probably, neutrophils (x20).

have used not only different saliva harvesting systems but also different DNA extraction kits, some dedicated to saliva, some dedicated to blood, and the results, as shown, were similar.

This can become thus the method of choice when the investigation targets healthy persons for various studies, patients for which veins are difficult to access, or children. The manufacturers of various saliva collecting devices put a special emphasis on the fact that saliva mixed with stabilizers proves to be a stable product [15,16], which is not only easier to transport but also gains acceptability from major carriers, unlike blood or tissue samples.

It is important to note that, due to the presence of various microorganisms within the oral cavity a substantial proportion of the DNA might be non-human [8], so one would expect this approach might require specific quantitation methods [23]. On the other hand, several studies have demonstrated that not only the proportion of human DNA extracted from saliva is higher if compared to other non-invasive methods [24] but also the non-human DNA will not interfere in analyses targeting the human DNA [25]. The good quality saliva DNA, extracted using mostly commercial kits, proved to be of good quality hence it could be used including for DNA sequencing, both with Sanger method [8] and NGS [11].

In order to check ourselves if the presence of non-human DNA extracted from saliva might have an impact on the outcome of various PCR amplifications and hybridization procedures we are currently performing in our laboratory, we have used this DNA for HLA genotyping. The results were fully satisfactory, and no difference could be noticed between the DNA extracted from blood or saliva.

As many molecular biology investigations are destined to oncological patients, given the impact of low cell count in the peripheral blood upon the DNA yield, we were interested to investigate if saliva can represent a viable alternative in this situation and if the exfoliated epithelial cells can represent a source of DNA. Nine such patients, with various hematological malignancies for which they were undergoing chemotherapy, were considered. A rather surprising match of the very low DNA yields extracted from both blood and saliva emerged this time as well.

It is known that in the oral cavity there is a quite remarkable turnover of epithelial cells, the layer of superficial epithelial cells being replaced roughly every 3 hours [19]. Our results demonstrated that while the number of epithelial cells remains virtually unchanged in saliva, the leukocyte number dramatically decreases in this fluid as well in immunosuppressed patients. Leaving aside the fact that in both saliva and blood extracellular (cell free) DNA is present [32], the match between blood and saliva DNA yields indicate that the most important source of DNA in the saliva samples is represented by cells travelling from the peripheral blood to the oral cavity, cells which are meant to play an important role in the protection against pathogens [33]. Hence, saliva DNA concentrations might even offer indirect information about the number of such cells reaching into the oral cavity and the

impact upon a number of conditions that might affect the mouth.

Unfortunately, the DNA extraction from saliva proves not to be a feasible method for this category of patients, which could have benefited otherwise tremendously of an alternative method. Should saliva still be considered as a valuable sample, perhaps a different approach would prove useful, one that would rather utilize DNA extraction methods dedicated to solid tissues, as the currently dedicated available kits seem to target leukocytes.

We conclude that DNA can be easily extracted from saliva and many commercially available kits might be adapted for this purpose. As saliva harvesting is a non-invasive method, this could represent an appealing alternative for many patients and healthy persons, especially when it is necessary to recruit participants for large scale studies or family investigations and samples often need to be shipped. However, as our results are showing, it is imperative to assess the DNA concentration, as sometimes significant yield differences compared to the expected average concentration can be noticed, that will thus call for appropriate adjustments.

In the case of non-compliant persons (mostly children and old patients) or of patients with xerostomia, saliva is obviously not a good choice. Furthermore, saliva cannot represent an alternative to peripheral blood when dealing with hematologically depleted patients since saliva DNA yields will practically match the yields generated by total blood, and these patients should not be approached with this method, at least not using the currently available dedicated DNA extraction kits.

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