Insights into cerumen and application in diagnostics: past, present and future prospective

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Abstract

Cerumen or earwax is an emerging bio-fluid in clinical diagnosis that has been very little exploited during the past decades in spite of its high diagnostic potential. It is highly abundant in diagnostic biomarkers such as genetic material, lipids, proteins, chemical elements, internal and external metabolites (e.g. hormones, volatile organic compounds, amino acids, xenobiotics etc.) reaching earwax from the blood circulation. Thus, it is able to reflect not only physiology, pathophysiology of the human body but can also detect recent and long term exposure to environmental pollutants, without the need of invasive blood tests and in the same time overcoming many disadvantages faced by using other diagnostic biological fluids. This review discusses the biology, functions, chemistry of earwax, past and current approaches for the study of its chemical composition, emphasizing how a detected variation in its composition can offer information of high clinical value, which can be useful in diagnosis of many diseases such as metabolic disorders and tumours as well as in forensic applications. It also presents details about techniques of sample collection, storage, and analysis. Moreover, it highlights concerns about the use of earwax for diagnostic purposes, which should be addressed to make earwax diagnostics a reality in the future.

Key words: cerumen; laboratory diagnosis; metabolomics; proteomics; genomics

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Introduction

Cerumen, commonly referred to as earwax, is a rich biological fluid that has distinct advantages as a biomonitoring medium of a high diagnostic potential. Although many studies have been concerned with the elucidation of the chemical composition of cerumen, the literature shows that until today very little attention has been dedicated to the analysis of cerumen with the aim of laboratory diagnosis. Cerumen, being composed of a large diversity of biomarker compound classes including lipids, proteins, amino acids (AA), carbohydrates, volatile organic compounds (VOC), chemical elements in addition to hormones, antibodies, enzymes and their products, makes it a reflection of the physiological functions of the body and a potential alternative biological matrix. This review provides an overview of the biology, functions of cerumen, past and current investigations performed on its chemical composition and its applications in laboratory diagnosis with emphasis on pre-analytical, analytical, and post analytical aspects. It also highlights its advantages and limitations in comparison to classical fluids.

Composition and functions of cerumen

Cerumen is a waxy substance secreted by ceruminous “apocrine sweat” glands located subcutaneously in the external ear canal (1,2). Ceruminous glands in combination with the sebaceous glands produce earwax which is therefore, considered a mixture of sweat secretions and fatty material from the sebaceous glands (1,2). Regarding the chemical composition, it is composed of fatty acids, alcohols, ceramides, wax esters, triacylglycerols, long chain hydrocarbons, and cholesterol pre-
cursors as lanosterol, squalene, and cholesterol which are the final products in the hydroxymethylglutaryl-CoA (HMG-CoA) reductase pathway (3) with physical consistency ranging from wet, sticky and yellow or brown to dry, crumbly and white or greyish. Earwax production is affected by some factors such as working conditions, climate, and even increased cholesterol concentrations can block the HMG-CoA reductase pathway by negative feedback (2,4-5).

The earwax phenotype is determined by two alleles at a single gene termed as ABCC11 gene (6). A single-nucleotide polymorphism (SNP) in this gene encodes an ATP-driven efflux pump protein responsible for the variation in the apocrine gland secretion which affects the earwax type being wet or dry as well as the axillary odour commonly called “underarm odour” (6,7).

The earwax phenotype was also linked to ethnicity/race, where the dry type is commonly prevalent in East Asians (95%) but rare in Europeans and Africans (3%). A mixed rate of dry and wet types with dry wax incidence of 30-50% is seen in populations of Native North Americans, the Pacific Islands, Central Asia, Turkey and those of Asian ancestry (8,9).

Among its main functions is to moisten, clean, lubricate, and protect the skin of the human ear canal, in addition to acting as an antibacterial maintaining the environment in the ear canal acidic and a barrier against foreign substances as water, insects and dust (10). Moreover, it can provide important information about an individual including race, ethnicity, gender, diseases, food eaten and exposure to surrounding environmental pollutants (11).

Cerumen and implications to health, diagnostics and forensics

Cerumen sampling, transport and preservation

Cerumen, being secreted inside the ear canal, is protected against external contamination which is a serious problem limiting the diagnostic potential of many biological samples. Despite this fact, certain precautions must be considered during sample collection to maximize the credibility of data obtained upon its analysis as: performing hand hygiene, using disposable gloves and apron during sample collection, swabbing/removing cerumen from the inner portion of the ear as opposed from the lobes (where soap/shampoo flakes are more prevalent), transferring the sample to vials/containers that are air tight and at low risk of cross contamination, putting a label with patient information; transportation to the laboratory for analysis (12,13).

For cerumen sampling, different techniques were applied depending on the quantity required for analysis including for instance ear swabs commonly applied in forensic testing, DNA paternity testing, etc. (14). Ear swabs could be performed at home by oneself placing a sterile swab (cotton buds or Q-tips) into the inner portion of the ear and gently rotating to collect the sample 2-3 times if possible (the more the better), whereas a full swab tip may be enough (14). Other techniques for sample collection were applied using tools such as sterilized metal scoops, plastic curettes, wooden spatulas, and the Jobson-Horne probe (15-18). Ear scoops (picks) are more efficient in the removal of the dry type of earwax, while the Jobson-Horne probe (Figure 1) being fenestrated is effective in the removal of either the dry or moist-type (18,19). Generally, use of ear picks is better done by a health professional to avoid risks of damaging the ear and causing infections (20).

The sampling time required to obtain samples with detectable concentrations involving casual cerumen is variable. Normally, the subjects involved are instructed not to clean the ears one week to ten days to allow for sample build up before collection and to bathe/shower with fragrance-free liquid soap/shampoo, and avoid any kind of perfume or fragrance, in order not to affect the earwax volatile composition (21). In case of fresh cerumen sample, first casual cerumen is removed from the ears of the study participants; then the ears are irrigated with water and wiped with cotton swabs moistened with an alcohol-ether (3:1) mixture then the fresh cerumen is collected 24 or 48 hours later (21).
Samples are stored in sterile airtight containers (vials, bottles, Eppendorf tubes, etc.) kept away from sunlight, chemicals or conditions that may catalyse sample degradation (13,21,22). As for sample transport and preservation, the conditions are dependent on the nature of the compounds being analysed. For instance, in case of ear swabs for DNA testing, the sample can be shipped to the laboratory in a paper envelope and stored somewhere safe at room temperature for up to six months (14). In case of analysis of volatile organic composition; samples are usually stored in a deep freezer at -30 °C and analysed within one week to minimize the loss of the volatiles, while those used for lipidomics and proteomics are usually preserved at -18 °C and -80 °C, respectively (15,17,21). For samples that need immediate refrigeration/freezing, they can be kept in portable freezers with controllable temperatures available at the collection sites until transported to the laboratory (16).

Proposals were sought to enhance the use of dried biological samples, one of which is dried cerumen for clinical analyses with the aim of enhanced recovery of analytes and automated processing of dried specimen samples - particularly if elements of integrated sample preparation are necessary to preserve the recovery of integrity of a given biomarker class for later detection (23).

Analysis of cerumen

Cerumen has been subjected to different kinds of analyses of compound classes such as lipidomic, proteomic, genomic and metabolomic analyses in addition to analysis of chemical elements, and xenobiotics e.g. drugs, foreign pollutants, etc. Details on selected applications of cerumen analysis in laboratory diagnostics including compound classes involved, populations tested, pre-analytical, analytical and post analytical aspects are shown in Table 1.

Lipidomics

A great deal of effort was dedicated to the study of the lipid composition of cerumen, both casual and fresh as well as the variations detected with different earwax type, age, sex, season, menstruation etc. (2,4,24-30). The last approach was presented by Stransky et al. who performed a complete profiling of the cerumen lipid components in a sample (1.323 g) collected twice a week, throughout one year from both ears of a healthy 65 years old male (17). Then gradient column chromatography was used to separate cerumen into the aliphatic hydrocarbons, squalene, wax esters, cholesterol esters, triacylglycerols, free fatty acids, fatty alcohols, monoacylglycerols, cholesterol, sterols, and hydroxy acids which were then analysed separately, and identified by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) techniques.

Regarding the application of lipid analysis in cerumen, the majority of the studies of the lipid composition of cerumen were directed to the improvement of the developed ceruminolytic agents (25,31-33). This is due to the fact that the epidermal cells constituting approximately half of the mass of wax impactions are enveloped in a layer of bound lipids (w-hydroxyacids, free fatty acids, and ceramides) which contribute to the cellular cohe-
| Approach | Population tested | Sample collection | Sample pretreatment | Method of analysis | Disorder | Biomarkers detected | Detected ranges | Conventional bio-fluids | Reference |
|----------|-------------------|-------------------|---------------------|-------------------|----------|---------------------|-----------------|------------------------|-----------|
| **Proteomics** | 2 female, 3 male | Small metal scoop | 1D-PAGE pre-fractionation, online SC X-fractionation | LC-MS/MS | chronic otitis externa, otorrhinia, benign or malignant ear tumors, systemic diseases as (diabetic nephropathy, breast cancer) | zinc-alpha-2-glycoprotein | Qualitative | Plasma, saliva, urine | Feig et al. (2011) |
| **Genomics** | 40 patients (18 females, 23 males, 17-57 years) | Sterile swabs | Homogenization in saline, then DNA extraction | Real-time PCR | Diagnosis of Hepatitis B infection | Hepatitis B virus DNA | 4.2 x 10^2 - 4.7 x 10^6 copies per sample | Serum | Kalcioglu et al. (2004) |
| | 50 patients (21 females, 29 males, 20-40 years) | Sterile spoon /sterile swabs | Homogenization in saline, then DNA extraction | Real-time PCR | Diagnosis of Hepatitis B infection | Hepatitis B virus DNA | ND | Serum, saliva | Parizad et al. (2016) |
| | 35 patients (21 females, 29 males, 21-70 years) | Sterile swabs | Homogenization in saline, then DNA extraction | Real-time PCR | Transmission of Hepatitis C infection | Hepatitis C virus RNA | no copies per sample | Serum | Bayindir et al. (2005) |
| **Metabolomics** | 8 patients (24-48 years) | ND | Desiccation by electric plate at 110°C | Change in weight | Allergic rhinitis | Total volatile compounds | 28.4 ± 8.5 (43.8 ± 12.2 µg/g)* | Blood | Paiva et al. (1973) |
| | 17 patients (7-61 years) | ND | Desiccation by electric plate at 110°C | Change in weight | Otosclerosis | Total volatile compounds | 31.1±10.4 (43.8±12.2 µg/g)* | - | Paiva et al. (1973) |
| | 12 patients (46-72 years) | ND | Desiccation by electric plate at 110°C | Change in weight | Cancer | Total volatile compounds | 30.7±10.3 (43.8±12.2 µg/g)* | Blood, urine, saliva, sweat | Paiva et al. (1973) |
| **Volatile organic compounds** | Neonates (< 5 days) | Metal scoop | Solvent extraction and derivatization | GC, GC-MS | Maple syrup urine syndrome | Sotolone (4,5-di-methyl-3-hydroxy-2[5H]-furanone), a metabolite of isoleucine or allo-isoleucine | Qualitative (burnt sugar odour) | Urine | Kataoka et al. (2013), Liebich et al. (1983) |
| | 2 females patients (61 years) | ND | Solvent extraction | Paper chromatography, visualization with 5% ammonium silver nitrate | Alkaptonuria | Homogentisic acid | Qualitative | Urine | Frohlich et al. (1973) |
| | 26 patients (13 males, 13 females) | Plastic curette | No previous extraction | HS/GC-MS | Diabetes mellitus (Types 1 and 2) | Acetone, methoxyacetone, ethanol, isobutyraldehyde, hydroxyurea, acetic acid | Chemometric treatment of quantitative data | Blood, plasma, urine | Shokry et al. (2017) |
| Approach | Population tested | Sample collection | Sample pretreatment | Method of analysis | Disorder | Biomarkers detected | Detected ranges | Conventional bio-fluids | Reference |
|----------|-----------------|------------------|--------------------|-------------------|----------|-------------------|-----------------|------------------------|-----------|
| ND       | ND              | Solvent extraction | GC, GC-MS          | Exposure to toxic chemicals |          |  | Detected (0.0 µg/g)* |  | Breath, blood (short term exposure), adipose tissue, breast milk (long-term exposure) | Lauwersys et al. (1991), Wang et al. (1988) |
| 3800 (35-54 years) | ND | Solvent extraction | GC, GC-MS | DDT, HCH induced cancer mortality | DDT, HCH | Detected (0.0 µg/g)* |  | Breath, blood, adipose tissue | Wang et al. (1988) |
| 10 males and 7 females (≥ 18 years) | Plastic curette | Direct extraction with methanol | UPLC-MS/MS | Administration of drugs of abuse or drug facilitated crimes |  | lacosamide 13.2 ng/mg (0.0 ng/mg)*, lamotrigine 115.0 ng/mg (0.0 ng/mg)*, carbamazepine 13.2 - 295.5 ng/mg (0.0 ng/mg)*, phenytoin 8.7 - 243.3 ng/mg (0.0 ng/mg)*, levetiracetam 52.0 ng/mg (0.0 ng/mg)*, oxcarbazepine 50 - 326.5 ng/mg (0.0 ng/mg)*, valproic acid 186.5 - 4850.0 pg/mg (0.0 pg/mg)*, topiramate 9.8 - 175.5 ng/mg (0.0 ng/mg)*, clozadiazepam 186.5 - 4850 pg/mg (8.0 - 175.5 ng/mg) (0.0 pg/mg)*, anabasine 5.6 - 8.4 mg/mg (0.0 ng/mg)*, phenobarbital 5.6 - 6.3 mg/mg (0.0 ng/mg)*, clonazepam 3.7 ± 4.4 ng/mg (0.0 ng/mg)*, active smoker | Blood, plasma, urine, saliva (short term), hair (long term) | Shokry et al. (2017) |
| 38 postmortem samples | Cotton swab | Drying at room temperature for 24 h, solvent extraction | LC-TOF MS, LC-MS/MS | Drug abuse | Opiates, cannabinoids | 2-100 ng/0.42-8.2 mg cerumen | Blood, urine, hair, bile | Meier et al. (2017) |
| 24 females, 37 males (18-35 years) | Plastic curette | No sample pre-treatment | HS-GC/MS | Tobacco use/exposure | Nicotine 1.2 ± 0.5 ng/mg, passive smoker; 16.8 ± 32.9 ng/mg, active smoker | Blood, urine, saliva, sweat, hair, nails | Shokry et al. (2017) |
| Approach          | Population tested | Sample collection | Sample pretreatment | Method of analysis | Disorder                        | Biomarkers detected | Detected ranges                        | Conventional bio-fluids | Authors                      |
|-------------------|-------------------|-------------------|---------------------|--------------------|----------------------------------|---------------------|----------------------------------------|------------------------|-----------------------------|
|                   | 1 male, 1 female  | clean Q tip       | Desiccation, digestion in 10% nitric acid | Inductively coupled plasma atomic emission spectroscopy | Exposure to toxic elements | Lead                     | 13.5 ± 0.71 μg/g (0.0 μg/g)* | Plasma, sweat, skin          | Krishnan et al. (1992)     |
|                   | 10 males, 10 females | ND                | Incineration (for 6 hours) at 600°C | Flame Photometry   | Fungal infection in the ear       | Copper                | 0 ng/100gm (0.942- 3.314 ng/100gm)* | -                      | Yassin et al. (1966)        |
|                   | 4 male, 2 female (11-17 years), 1 male adult | Curette           | Desiccation under vacuum, digestion in nitric/perchloric acid mixture (5:1 V/V) | Atomic absorption spectroscopy | Cystic fibrosis | Zinc                     | 118 ± 103 μg/g (1857 ± 1341 μg/g)* | Blood, urine, saliva, sweat | Brand-Auran et al. (1972) |
|                   | 17 patients (7- 61 years) | ND                | Incineration in a muffle furnace at 550°C for 12 hours, dissolving in HCl | Clark-Collip method | Cancer (prostate, mandible, tongue, tonsils, larynx) | Calcium               | 6.87 ± 1.69 mEq/100 g (8.0 9 ± 1.86 mEq/100 g)* | Blood                  | Paiva et al. (1973)         |
|                   | 12 patients (46-72 years) | ND                | Incineration in a muffle furnace at 550°C for 12 hours, dissolving in HCl | Fiske-Subbarow method | Yellow titan                     | Sodium                | 29.15 ± 8.11 mEq/100 g (39.5 ± 14.42 mEq/100 g)* | -                      | Paiva et al. (1973)         |
|                   | 1 female (16 years), 2 males (22 and 28 years) | ND                | Histochemical examination | Wilson's disease (hepatotenticular degeneration) | Copper             | 7.17 (2.43 mg/100 g)* | -                      | Serum, urine, faeces, cerebrospinal fluid, bile, saliva | Canelas et al. (1963) |
|                   | 8 patients (24- 48 years) | ND                | Incineration in a muffle furnace at 550°C for 12 hours, dissolving in HCl | Clark-Collip method | Otosclerosis                      | Calcium               | 9.43 ± 3.03 mEq/100 g (8.09 ± 1.86 mEq/100 g)* | -                      | Paiva et al. (1973)         |
|                   |                  |                   |                     | flame spectrophoto-metry | Potassium                | 32.24 ± 4.17 mEq/100 g (37.66 ± 17.99 mEq/100 g)* | -                      |                          |                          |

1D-PAGE – one-dimensional polyacrylamide gel electrophoresis. LC-MS/MS – liquid chromatography/tandem mass spectrometry. DNA – deoxyribonucleic acid. PCR – polymerase chain reaction. RNA – ribonucleic acid. GC – gas chromatography. HS/GC-MS – headspace gas chromatography/mass spectrometry. DDT – dichlorodiphenyltrichloroethane. DDE – dichlorodiphenyl-dichlorodichloromethane. HCB – hexachlorobenzene. HCH – hexachlorocyclohexane. UPLC-MS/MS - ultra performance liquid chromatography - tandem mass spectrometry. *Reference ranges for the biomarkers studied. ND - Not defined.
siveness which justifies why studying the lipid composition in specific could be useful in the choice and development of new ceruminolytics (34,35).

On the other hand, very little work was concerned with the use of cerumen lipids as diagnostic biomarkers in laboratory diagnostics. However, in this review, we tried to highlight some reports potentially relating cerumen lipids with some pathological conditions, either local (inside the ear) or systemic.

In 1954, Akobjanoff et al. identified some of the fatty acids in earwax (capric, lauric, oleic, myristic, linoleic, palmitic, stearic acids) as an approach to determine the normal constituents of cerumen (36). This may allow the detection of pathological conditions of the ear through changes from the normal. For instance, external otitis caused by a malfunction of the epidermal glands of the skin of the ear canal would be expected to show changes in the cerumen composition, which if recognized could be used for prophylaxis and treatment (37).

Later, Inabi et al. investigated the lipid composition in earwax of patients with hircismus using thin layer chromatography (TLC) (38). Samples were collected from 20 patients with hircismus (wet earwax type) and 20 adult volunteers without hircismus, extracted with n-hexane, and resolved by TLC using different solvents (hexane, benzene, ether, acetic acid). Spots were visualized by spraying with 50% sulfuric acid and charring at 220 °C and the unidentified fat was further analysed by GC and high performance liquid chromatography (HPLC). Results show that wet earwax is due to the difference in quantity and quality of earwax lipids and hircismus is associated with higher incidence of wax lipids. For instance, in wet earwax, steryl-esters and wax esters were not found as compared to dry earwax type while two unidentified lipids were found only in wet earwax type.

In 1966, a preliminary report undergone by a Japanese research group suggested a correlation between the lipid composition of earwax represented in the earwax type with the incidence of a coronary heart disease (arteriosclerosis). Based on the investigations performed on 96 Caucasian and Japanese arteriosclerotic in- and out- patients (with no reported age range), results showed that the incidence of wet cerumen among the patients with arteriosclerosis, not accompanied by hypertension, was strikingly high (30.2%), whereas it was 13.8% among arteriosclerotic patients with hypertension (39).

Later, in 1976, further investigations showed that Caucasian and Japanese populations’ dry cerumen contains 18% lipid and 43% protein, while wet cerumen has about 50% lipid and 20% protein. Since the cholesterol fraction of the lipid material is similar, the absolute amount of cholesterol excreted by persons with wet cerumen is inferred to be greater. This supports the assumption that the cerumen cholesterol concentration can give an indication about cholesterol concentration in blood (40). However, unfortunately, no further reports were found correlating cholesterol in cerumen with blood cholesterol. Moreover, modern methods to characterize lipids and lipoproteins do not seem to have been applied to cerumen and since it is conventional, in studies of disease association, to treat the first claim with due suspicion, therefore the relevance of cerumen types to lipid metabolism and arteriosclerosis remained an unresolved issue that can be neither asserted nor rejected (40).

Wet cerumen was also related to the incidence of Tinea versicolor infection of the outer ear (41). Owing to its lipid composition, this type of earwax increases the susceptibility to lipophilic fungus “Malassezia furfur” responsible for Tinea versicolor. Two hundred and twenty three Japanese cases of Tinea versicolor were examined in Kumamoto, wet earwax was found in 90 cases (40.9%). These results indicate a significantly higher incidence of Tinea versicolor among individuals with wet earwax (41).

In another approach, 67 patients with Parkinson disease were subjected to the examination of their ear canals. By examination, the ear canals of one or both ears of 40 out of the 67 patients were found to be totally blocked with grossly excessive quantities of greasy hard wax (42). This is considered typical of the disease that causes increase in the activ-
ity of the sebaceous gland and thus the wax secre-
tion. Psoriasis can also cause an increase in waxy material in the ear (43).

**Proteomics**

The composition of the protein in fresh and casual cerumen samples was first investigated photo-
metrically by Chiang et al. (25). Later, an alpha-
2-globulin was detected in cerumen by double
diffusion and immune-electrophoresis but the
first description of the isolation and quantification
of total proteins of earwax was introduced by
Schwaab et al. (44,45). Samples at an average
weight of 77.75 mg were collected from ears of 16
healthy adults (wet earwax type) with a sterile
hook under otoscopic control. Then, they were
weighed, pulverized using a mortar and a pestle in
liquid nitrogen. Proteins were then isolated by the
Qproteome™ Mammalian Protein Prep Kit (Qiagen,
Hilden, Germany) in two different kinds of ways
(cell and lysate fraction). Afterwards, total protein
concentration was quantified using the BCA pro-
tein assay kit (Thermo Fisher Scientific, Rockford,
USA) method. This assay allows the colorimetric
detection and quantitation of total protein using a
unique reagent containing bicinchoninic acid.

The antimicrobial nature of cerumen has been in-
vestigated in relation to the levels of lysozyme and
immunoglobulins present. Cerumen samples were
collected by curette from 588 Caucasians, Black
people, and Chinese; suspended in a buffer solu-
tion and mixed by a vortex mixer. Then, the
lysozyme assay was performed as adapted from
Osserman et al. while the antibodies were tested by
immunodiffusion techniques set up with the ce-
rumen suspension and immunoglobulin (Ig) A/IgE
antibody (46,47). Lysozyme and immunoglobulins
were present in almost all samples of the dry type
but its frequency of occurrence varies significantly
among the wet type depending on the race (48).

Lower lysozyme content and less acid pH in ceru-
men were also related to the occurrence of malig-
nant otitis externa (MOE), an aggressive infection
involving the external auditory canal and tempo-
ral bone, characterized by high mortality rate, ag-
gressive disease progression and poor response to
treatment (49).

Further study of the antimicrobial role of cerumen
was carried out by quantitative estimation of 10
well known human antimicrobial peptides in ear-
wax using enzyme linked immunosorbent assay
(ELISA) (50).

Cerumen proteomic analysis was utilized for the
first time as a non-invasive tool for biomarker anal-
ysis and disease diagnosis by Feig et al. employing
liquid chromatographic-mass spectrometry (LC-
MS) (15). A number of 11,562 distinct peptides rep-
resenting 2013 proteins were identified in human
cerumen. Five hundred and ninety-nine proteins
(31%) were found unique to cerumen. Of these,
283 were successfully identified and by comparing
the proportions of proteins in cerumen to multiple
bio-fluids (saliva, urine and plasma), cerumen was
found equally efficient as a novel bio-fluid in clini-
cal diagnostics. In addition, the method allowed
the detection of high amounts of zinc-alpha-2-gly-
coprotein, cathepsin D, apolipoprotein D, serpins,
calpain, mucins and lysozyme C confirming the
antimicrobial role of earwax. Thus, proteomic
characterization of cerumen might provide expla-
nations for local pathologies of the ear such as ot-
omycosis, benign or malignant pathologies of the
outer ear and susceptibility to recurrent infections
such as chronic otitis externa, and can be applied
for disease stage stratification as well.

Apart from that, proteomic characterization of ce-
rumen could play a role in the diagnosis of system-
ic diseases where zinc-alpha-2-glycoprotein was
already described to serve as either a potential bi-
omarker for normo-albuminuric diabetic nephrop-
athy, apocrine activity in breast cancer or a cata-
bolic marker in cancer and noncancerous states
(51-54). Apolipoprotein D is a lipoprotein related to
increased total hydrophobicity and decreased sus-
ceptibility to infection by transport and binding to
hydrophobic molecules, e.g. cholesterol esters, in
the outer ear canal (3,55). An interaction of zinc-al-
pha-2-glycoprotein and apolipoprotein D also af-
fected prolactin-inducible protein (a molecule regu-
lating water transport in apocrine glands) (56). Prol-
actin-inducible protein is used as a potential mark-
er for grading of apocrine carcinoma of the breast
and interacts with IgG and CD4-T cell receptor (57).
Another glycoprotein was detected in cerumen, which is similar to salivary glycoprotein (EP-GP), a glycoprotein isolated from human saliva with homologues in several other body fluids (58). It was measured by quantitative ELISA in cerumen among other fluids and showed a wide variability while the EP-GP epitope bearing proteins were further characterized by electrophoresis and immunoblotting. The biological role of EP-GP is not exactly known but there were reports about its ability to bind different bacterial species both in vivo and in vitro (59).

Genomics
Cerumen was applied for the detection of different diseases using modern DNA testing techniques, such as for detection of chronic infection with hepatitis B (HBV) and ability to transmit hepatitis C viruses (HCV) (60-62). Detailed information on these applications is provided in Table 1.

An association between axillary odour and the wet-type earwax was first established only based on the phenotype more than 70 years ago (7). Later, this finding was confirmed using a SNP(rs17822931) of the ABCC11 gene, the determinant gene of the earwax types, and furthermore was successfully used as a diagnostic marker for axillary osmidrosis (AO), a clinical condition of individuals with a deep anxiety regarding axillary odour and had undergone the removal of bilateral axillary apocrine glands (7). Further genetic association was found between wet earwax type, breast cancer (63), and AO (64). For the purpose of fast genetic diagnosis of AO and potential risk of breast cancer, specific primers were developed that allow to clinically genotype the ABCC11 gene within 30 minutes (64). Further evidence was found on genetic association between wet earwax type, breast cancer, AO (63,64). The earwax VOC profile was indicative for some metabolic diseases as maple syrup urine disease (MSUD) and alkaptonuria, which were identified in earwax before being diagnosed using traditional techniques as blood and urine analysis (68-71).

Maple syrup urine disease was diagnosed by the characteristic burnt sugar odour that can be easily detected in patients’ earwax and in neonates < 5 days old. The smell is attributed to sotolone resulting from accumulation of branched chain AAs and 2-oxocarboxylic acids. On the other hand, alkaptonuria could be easily diagnosed at any age by the black earwax and the detection of homogentisic acid in samples by paper chromatography (71).

Most recently, cerumen was able to detect diabetes mellitus (DM) and to discriminate between its types 1 and 2 by monitoring of the changes in the volatile composition of samples collected from the mammary glands where the frequency of colostrum occurrence and its measurable volume are much higher among wet-type than the dry-type women. This could be important to provide anticipatory guidance for mothers about breast-feeding and the length of time that should be spent in feeding based simply on their earwax-type (65).

Metabolomics
Volatile organic compounds
Volatile organic compounds are a diverse group of stable carbon-based chemicals that are classified on the basis of their retention time and boiling point (ranging from 50°C to 260°C) (66).

Earlier, very little effort has been dedicated for analysis of VOC in cerumen, either alone (21) or along with other components as ash and electrolytes (sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), and phosphorus (P)) to monitor the change in its content in association with some disorders as allergic rhinitis, otosclerosis and cancer (21,67).

Recent studies have concentrated on the analysis of the volatile organic composition of earwax, as it is very lipophilic, and may act as an ideal substrate for retaining organic compounds indicative of physiological, dietary, environmental events and/or exposures and ethnic origins (11). The earwax VOC profile was indicative for some metabolic diseases as maple syrup urine disease (MSUD) and alkaptonuria, which were identified in earwax before being diagnosed using traditional techniques as blood and urine analysis (68-71).

Most recently, cerumen was able to detect diabetes mellitus (DM) and to discriminate between its types 1 and 2 by monitoring of the changes in the volatile composition of samples collected from
DM patients (types I and II) (16). Samples were analysed by headspace gas chromatography/mass spectrometry (HS/GC-MS) without previous extraction. Significant changes were obtained in the alcohols and ketones profiles, principally (ethanol, acetone, methoxyacetone, hydroxyurea, isobutyraldehyde, and acetic acid.

Amino acids

So far, AA analysis in earwax did not seem to have a role in medical diagnostics and the major part of it was dedicated to study the difference in AA composition between dry and wet earwax types as well as for development of new better ceruminolytic agents (72). For the later purpose, a study of AA composition of earwax as well as the carbohydrate content was done (31). The method employs an AA analyzer and LC with amperometric detection for AA and carbohydrate content, respectively. Glycine, glutamic acid, and serine were found to be the major AA components of earwax while galactosamine, galactose, glucose, glucosamine, mannose, and fructose were found in the carbohydrate part.

Carbohydrates

For medical diagnosis, studying the carbohydrate content in cerumen could be important as certain amounts of sugars in association with a nitrogen source and some AAs may prove advantageous to the growth of pathogens, and certain pattern of sugars in cerumen may be indicative of tumours and metabolic diseases (31). Only one abstract (in Russian) has been provided in this regard, about correlation of glucose concentration in cerumen with DM, both latent and manifest (73). In addition, the carbohydrate content in cerumen was investigated for development of new ceruminolytic agents, by anion exchange column chromatography (CC) with pulsed amperometric detection and a gold working electrode. The carbohydrate analysis was performed on earplugs obtained from 10 patients that needed ear plug removal. The results reveal in the order of their abundance: galactosamine, galactose, glucose, glucosamine, mannose, and fructose with ratios of 2.4, 1.0, 0.9, 0.7 and 0.3, respectively (31).

Xenobiotics

Some studies suggest that earwax is like nails, hair and teeth may indicate chronic exposure since the last three media facilitate cumulative deposition of xenobiotics (74). Cerumen has been used for detection of long-term exposure to bio-accumulat-ory xenobiotics like lindane, chlordane, dichlorodi-phenyltrichloroethane (DDT), dichlorodiphenyldichlororothyene (DDE), dieldrin, hexachloroben- zene (HCB), and hexachlorocyclohexane (HCH) which cause cancer mortality, using GC and GC-MS, as well as for detection of environmental exposure to metals (74-76). Despite that, the ceru- men sampling is much simpler and acceptable than the surgical sampling of other adipose tis-sues, the results can only reflect cumulative exposure over a period of months or years rather than recent exposure and information relating to the chronic health effects of concern is lacking (77).

On the other hand, earwax was most recently used as a medium for monitoring drugs specially to in-dicate administration of drugs of abuse or drug fa-cilitated crimes antiepileptics, anxiolytics, antipsy-chotics, etc. Cerumen could be even considered a more favourable surrogate to traditionally used biological fluids because of its non-invasiveness, ease of sample collection, minimum sample pre-treatment, and relatively less external contamina-tion in addition to being able to detect the ana-lytes recently administered as well as drugs ad-ministered some months ago (78). Further studies were extended to using post-mor-tem cerumen samples for detection of drugs of abuse, which may be correlated with the cause of death as opiates, amphetamine and derivatives, cocaine, methadone and/or derivatives (79). Sam-ples were collected using cotton swabs, dried at room temperature for 24 hours before extraction and analysis by (liquid chromatography/time of flight mass spectrometry (LC-TOF MS) and LC-MS/ MS (79).

It was also applied for detection of tobacco use/exposure by the monitoring nicotine and its relat-ed compounds (cotinine, anabasine and o-nico- tine). Moreover, it was able to distinguish non- or passive exposure to tobacco smoke from active
exposure. Samples of 20 mg were collected from 61 young adults (18-35 years) using a plastic cuvette and analysed directly by HS/GC-MS without previous extraction. Cotinine and anabasine were found to be the biomarkers capable of discriminating completely between the study groups due to the significant difference in their detected concentrations (80).

**Chemical elements**

In 1992, Krishnan *et al.* presented the first report of the use of earwax as a biological monitoring medium for metals (81). Thirty-eight elements were analysed in cerumen samples obtained from one Eurasian male in his middle forties and one female from the Indian subcontinent in her thirties, by inductively coupled plasma atomic emission spectroscopy (AES). Results demonstrated the non-detection of silver (Ag), boron (B), beryllium (Be), cobalt (Co), mercury (Hg), manganese (Mn), nickel (Ni), selenium (Se), and vanadium (V) which suggests potential usefulness of earwax as a biological monitoring medium for these toxic elements in people exposed to high concentrations in the environment or in the workplace, since no baseline correction is required unlike for the other elements. On the other hand, Pb and Cd were detectable in both samples which signifies possible use of earwax to assess their external exposure.

Later, several studies were carried out that related the metal content to the health status and pathological conditions as cystic fibrosis, allergic rhinitis, otosclerosis, cancer, and Wilson’s disease (82-84). For instance, cystic fibrosis patients show lower concentrations of all the detected electrolytes (Na, K, Ca, Mg, Cu, Zn), principally the later (82).

It was also indicative of ear infections where the fungal growth in the ear was investigated in relation to the content of iron and copper amounts detected in earwax samples obtained from 10 male and 10 female Egyptians (85). Samples were collected in Pyrex glass sterile tubes and microchemical analysis was carried out by flame photometry. High copper concentrations were found in samples of some subjects indicating the absence of fungal infections while iron, which is also toxic for fungal growth, was undetectable in all the experimental samples.

The metal content of cerumen was also used as a method to study the pathogen biodiversity of human cerumen by using an optical probe for metal content characterization (86).

**Future perspectives of cerumen analysis**

A great effort needs to be done to incorporate earwax diagnostics into daily use where collection methods and biomarkers need to be standardized and validated. Prospective specimen collection and retrospective blinded evaluation are typically used for this purpose to minimize bias and reinforce significance. To fulfil these protocols prior to diagnosis, large patient populations, procurement and categorization of their samples, and clinical information are required. Cerumen is assessed quantitatively to determine the specificity, sensitivity, and reproducibility of the biomarker(s) in question. Further evaluations are also required to explore the capability of detecting and accurately measuring the markers in relatively low concentrations. Then before cerumen is used in a clinical assay, it is subjected to five stages including: 1) pre-clinical testing where biomarkers are discovered in patient samples and confirmed either *in vitro* or *in vivo*; 2) feasibility analysis in which biomarkers are tested using small patient subpopulations to demonstrate their ability to detect disease; 3) validation process, which involves accurate testing for biomarkers; 4) statistical analysis to verify if statistically significant differences were obtained in a large patient population; 5) investigating the biochemical functions of the biomarker as understanding of the molecular mechanisms of biomarkers enables them to be more informative of the disease, progression and potential treatments.

In addition, new assays and devices need to be developed at a commercially feasible rate. This could make earwax-based diagnostic tests more accepted by health care professionals, consequently facilitating the generation of further studies to demonstrate and establish the accuracy, sensitivity, and specificity of earwax diagnostics in a much wider variety of diseases. This involves studying...
for instance, the impact of interindividual variations (e.g. age, gender, heath status, racial differences, etc.), the time course of the investigated biomarkers in earwax, and correlation of its levels with corresponding levels in blood and/or urine. Accomplishing this along with the establishment of defined guidelines for the procedures might make earwax diagnostics a reality in the future especially that there has been increasing applications of earwax analysis in forensics as evident in the recent literature. In this review, we highlighted some advantages and limitations of earwax as a diagnostic bio-fluid as shown in Table 2.

**Conclusion**

This review provides a summary of the biology, functions of cerumen, past and current investigations performed to establish biomarkers that could be potentially applied in disease detection combined with noninvasive sample collection. It also focuses on the potential role of earwax as medium for biological monitoring and a new frontier for medical diagnosis and forensic applications highlighting its advantages in comparison to traditional diagnostic tests. It also encourages further research on earwax as a promising alternative biological fluid. Some limitations were found in our review, where relatively less data was available on applications of cerumen in medical diagnosis and some of the references could be somehow outdated. That is probably due to the fact that earwax until recently have been looked upon as a neglected body secretion and thus many years were needed to reach a considerable amount of data supporting the diagnostic potential of earwax.

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**Potential conflict of interest**

None declared.

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**Table 2. Advantages and limitations of earwax testing for laboratory diagnostics**

| Advantages                                                                 | Limitations                                                                                           |
|---------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|
| Noninvasive, easy to collect, low cost                                    | Despite its accessibility, it has not been widely and sufficiently studied as a bio fluid              |
| No/minimum external contamination                                         | Earwax composition shows a high inter-individual variability depending on many factors (sex, age, season, menstruation, etc.) |
| No risk of disease transmission as in blood sampling                      | Timely reproduction, since time (few days) is required for the build-up of the sample thus it is not suitable for continuous monitoring |
| No need for trained medical staff                                          | Lack of standardized methods for earwax collection                                                   |
| Samples are easy to ship and store                                        | Till now, cerumen analysis involves complicated instrumentation as GC–MS, LC-MS that requires trained personnel |
| No/minimum embarrassment or discomfort associated with blood and urine tests |                                                                                                       |
| Requires less sample pretreatment or manipulation for diagnostic tests    |                                                                                                       |
| Can detect both recent and long-term exposure unlike blood, urine, saliva, etc. |                                                                                                       |
| Economical sampling, shipping and storage compared to blood               |                                                                                                       |
| Sampling can be done at home                                              |                                                                                                       |

GC-MS – gas chromatography - mass spectrometry. LC-MS - liquid chromatography-mass spectrometry.
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