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Biomarkers of disease in human nails – a comprehensive review.

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Abstract

Diagnostic, monitoring, response, predictive, risk and prognostic biomarkers of disease are all widely studied, for the most part in biological fluids or tissues, but with steadily growing interest in alternative matrices of which nails are an example. Here we comprehensively review studies dealing with molecular or elemental biomarkers of disease, as opposed to semiological, pharmacological, toxicological or biomonitoring studies.

Nails have a long history of use in medicine as indicators of pathological processes, and have also been used extensively as a matrix for monitoring exposure to environmental pollution. Nail clippings are simple to collect non-invasively, to transport and to store, and the matrix itself is relatively stable. Nails incorporate, and are influenced by, circulating molecules and elements over their several months' of growth, and it is widely held that markers of biological processes thus included will remain in the nail, even when their levels in blood have declined. They thus offer the possibility, not only of looking back into a subject's metabolic history, but also of studying biomarkers of processes that operate over a longer time scale such as the post-translational modification of proteins.

Reports on unguis biomarkers of metabolic and endocrine diseases, cancer, and psychological and neurological disorders will be presented, and an overview of the sampling and analytical techniques provided.

Keywords: Fingernail, toenail, ungual, biomarker, disease

Abbreviations: 3-DG – 3-deoxyglucosone; AAS – atomic absorption spectroscopy; AD – Alzheimer's disease; AFS – atomic fluorescence spectrometry; AGEs – advanced glycation endproducts; CKD – chronic kidney disease; DHEA – dehydroepiandrosterone; FTIR – Fourier-transform infrared spectroscopy; HbA_{1c} – glycated hemoglobin; HDL – high-density lipids; HPLC-UV – high performance liquid chromatography-ultraviolet detection; ICP-MS – inductively coupled plasma-mass spectrometry; INAA – instrumental neutron activation analysis; *KRAS* – the gene that codes for the K-Ras protein; LC-MS – liquid chromatography-mass spectrometry; LDL – low-density lipids; LIBS – laser-induced breakdown spectroscopy; NIR – near-infrared spectroscopy; SDS-PAGE – sulfate-polyacrylamide gel electrophoresis; T2DM – type 2 diabetes mellitus; VTGE – vertical tube gel electrophoresis.

Introduction

Biomarkers are defined as characteristics indicative of normal biological or pathogenic processes or responses to an exposure or intervention, and can be physiological (eg. blood pressure), physical (eg. tumor size), molecular (eg. glycated hemoglobin (HbA1c)) or elemental (eg. trace elements) [1]. Diagnostic, monitoring, response, predictive, prognostic and risk biomarkers are all widely studied, with some being regularly used in clinical practice [2], and recent efforts having been made to formalize biomarker terminology and its usage [1,2,3]. While the overwhelming majority are studied in biological fluids or biopsy samples, recent decades have seen growing interest in “alternative” matrices such as exhaled breath, saliva, tears, sweat, hair and nails (see [4] and papers that reference, for example, [5] and [6]).

Nails have a history of use in medical semiotics dating back to Hippocrates [7], and their use in Pharmacology, Toxicology and Forensic medicine is also long-established (eg. [8,9]). Since the 1960s–70s, nails have been used as a matrix for the “biomonitoring” of exposure to pollution, and as a means of monitoring the homeostasis and dietary intake of certain elements (eg. [10-12]). However their potential for the study of molecular or elemental biomarkers of disease is difficult to evaluate from the literature and, with the exception of He’s review of trace elements in nails as clinical biomarkers [13], the topic lacks the review publications available for other fields centered upon nail analysis.

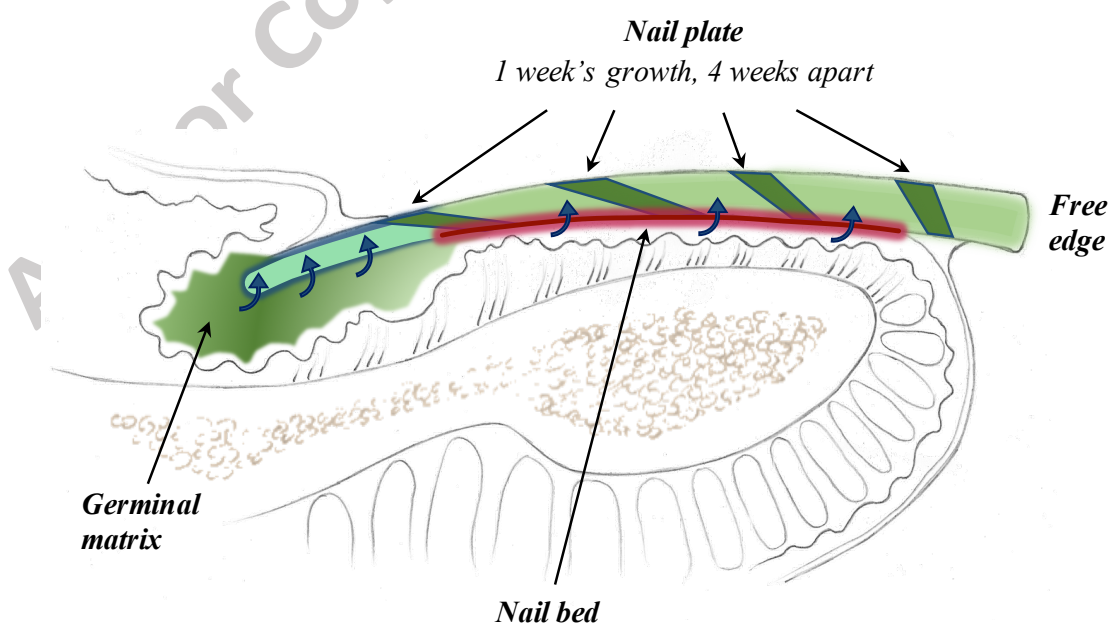
Nails present significant advantages over biological fluids in that they can be sampled non-invasively by untrained personnel and are relatively stable, making them cheaper to collect, transport and store. They thus offer not only the potential to study larger cohorts or maximize adherence to recommended clinical follow-up, as their non-invasive sampling is more widely

acceptable [14,15], but also the opportunity to study populations where refrigerated transport of samples is impractical or prohibitively expensive.

The nail plate is around 0.5mm thick and consists of dead, adherent cells lacking nuclei but with prominent cell borders [16]. Fingernails take about 3–5 months, and toenails around 6–12 months, to grow from the germinal matrix to the free edge (at around 3mm and 1mm per month, respectively) [17]. Keratinocyte proliferation in subungual tissues occurs at the anatomical boundary of the germinal matrix [17], which is visible from above as the white lunae. The flat layers of keratinized cells laid down in the germinal matrix assume a vertical orientation as the nail grows distally toward the free edge (Fig. 1).

Figure 1. Anatomy and growth of the nail (after [18,20]). Circulating biomarkers are incorporated at the germinal matrix during proliferation of keratinocytes, while the ventral layer of the nail plate is influenced by, as well as being in receipt of, molecules or elements in the circulation *via* the highly vascularized nail bed.

(↗ indicate biomarker incorporation / formation.)



The balance of current opinion holds that, while three distinct layers of the nail plate are discernable in compositional terms, all three originate in the germinal matrix [18]. The upper, dorsal layer of the nail is composed of a few cell layers of hard keratins; the intermediate layer contains softer keratins and constitutes the majority of the nail; the ventral (lower) layer is one or two cell layers thick and composed of soft, hyponychial keratins [16]. Both the matrix and nail bed are highly vascularized, and the ventral layer is therefore influenced by, and in receipt of, molecules and elements in the circulation. The nail bed and underside of the nail plate have an interlocking, longitudinally corrugated form, meaning the available surface area is quite large for any interaction between circulating molecules or elements and keratins in the nail plate, or indeed their incorporation by the nail [18]. Finally, incorporation of biomarkers from sweat and sebum has also been described [19].

The lifetime of the keratinized proteins that constitute >80% of the nail plate is longer than that of proteins in biological fluids, meaning post-translational modifications of proteins may accumulate more readily in nails than in these more commonly investigated matrices. Nails also integrate a longer period of metabolic history than blood or urine [18,20]: indeed, a key advantage of the nail as a matrix for the study of disease biomarkers is that, once incorporated into the nail plate, molecules or elements remain within it even after their levels in blood have changed, and fluctuations are thus evened out [20].

Associations between various nail pathologies and systemic disease are well-covered in the literature, while one motivation for this review was to disentangle biomarkers of disease from the many publications using nails in pharmacological or biomonitoring studies. We thus limit ourselves here to reports of molecular or elemental diagnostic, predictive, prognostic and risk biomarkers of disease in human nails. Reports on metabolic and endocrine diseases, cancer,

psychological and neurological disorders will be presented in dedicated sections, with occasional reference being made to other matrices when they are studied in parallel, and an overview of sampling and analytical techniques will be given.

Author Copy, Revised & Accepted

1. Endocrine and metabolic diseases

Two of the diseases presented in this section have an incidence among the global population that makes them of significant concern. Indeed, it is no exaggeration to say that, along with the increasing human lifespan with which they are intimately linked, they represent two of the biggest current and future challenges in the field of public health.

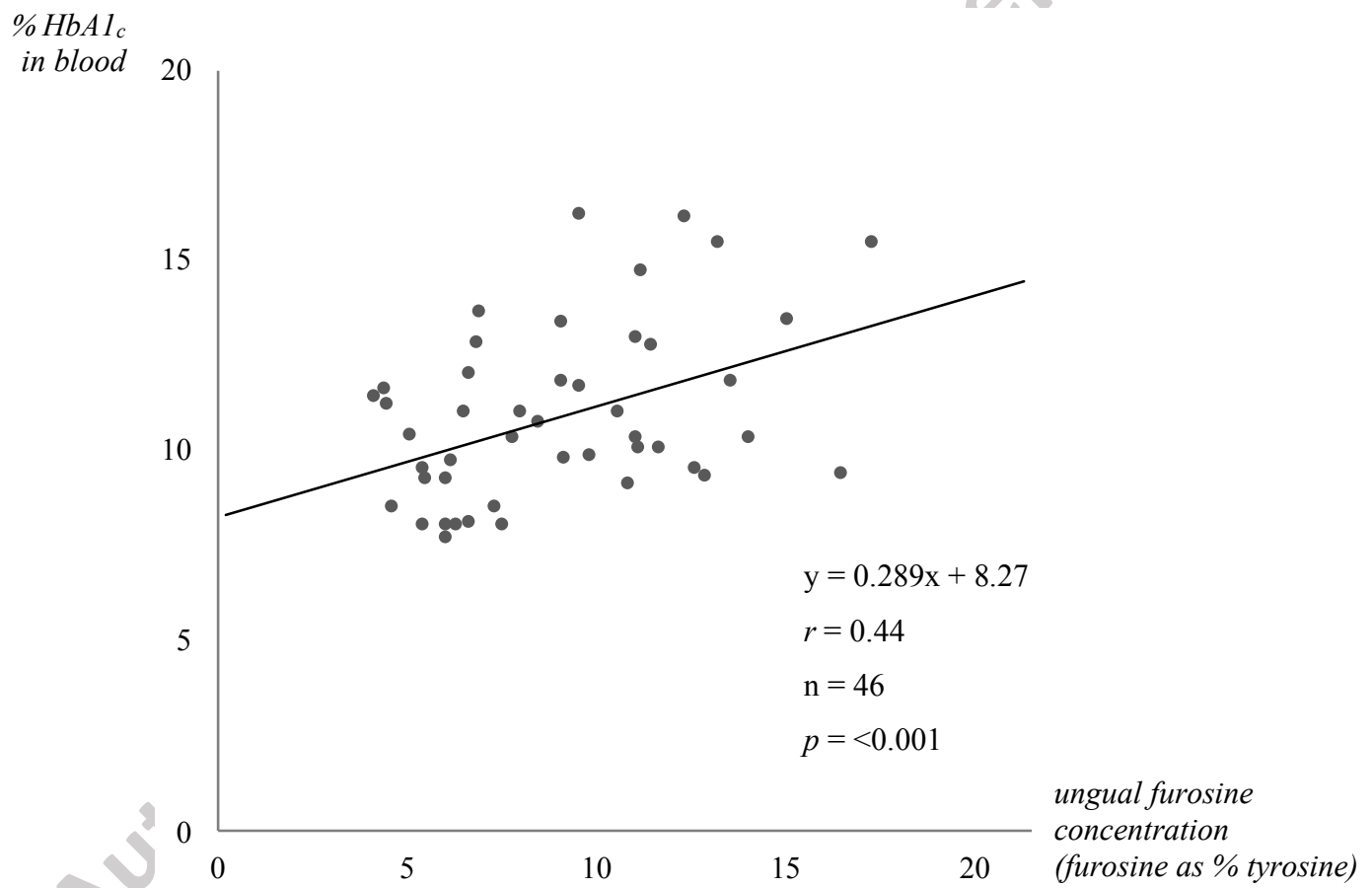
1.1 Type 2 diabetes mellitus

Diabetes mellitus is a metabolic disease with an incidence of *ca.* 463 million worldwide in 2019, though this is forecast to almost double over the next 25 years (*ca.* 700 million by 2045 [21]). The type-2 form (T2DM) represents 90% of the total incidence and has reached pandemic proportions, with attendant economic and societal costs, particularly in higher-income countries, though prevalence in low- and middle-income countries is rising rapidly. Early diagnosis of T2DM is key for successful clinical management but around half of sufferers are unaware they have the disease [21]: affordable, large-scale screening is thus a potentially key part of attempts to address this major health issue.

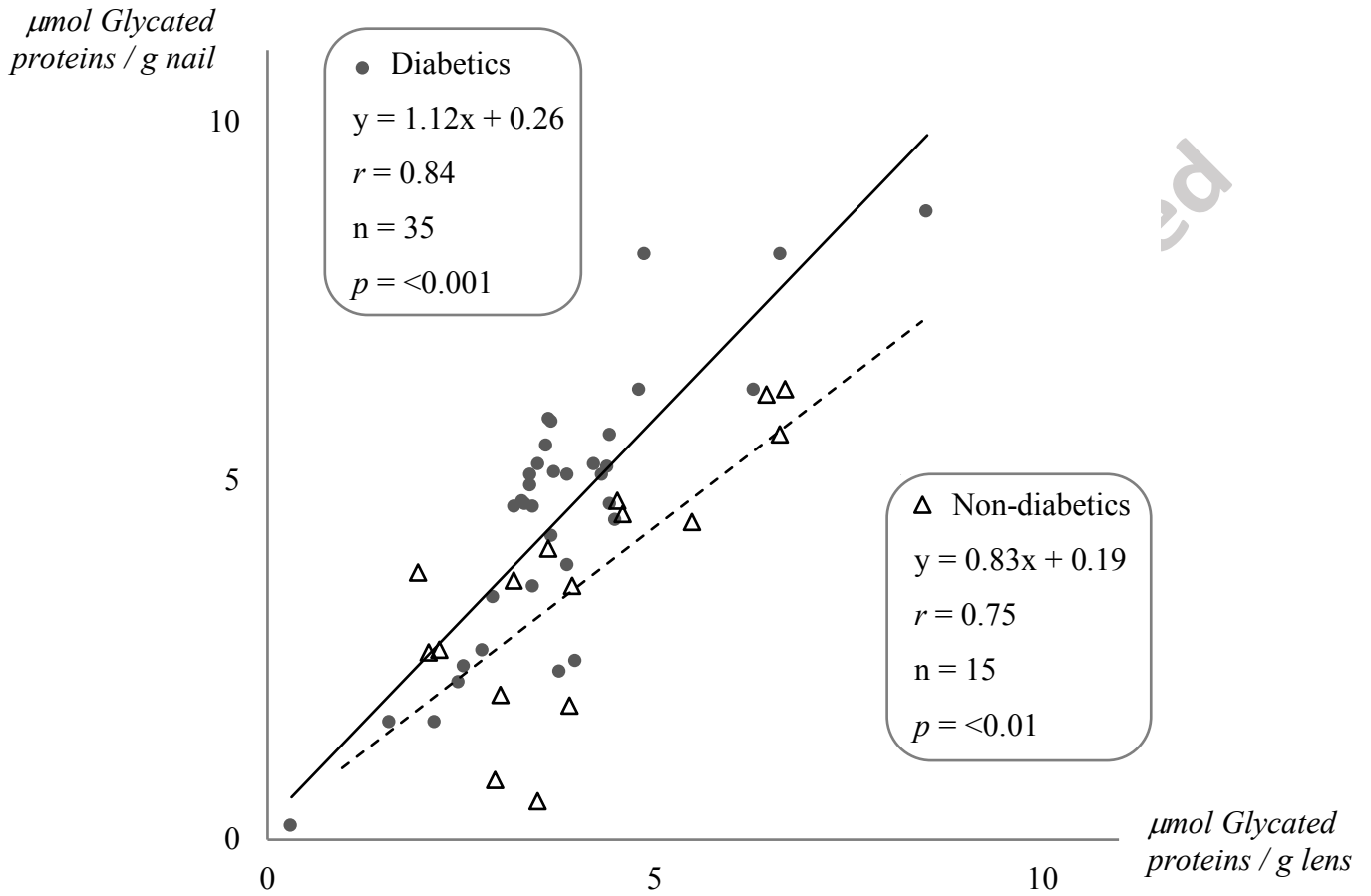
Non-enzymatic glycation of proteins is one of the key pathological mechanisms in diabetes-related complications and results from the chronic hyperglycemia characteristic of the disease [22]. The initial formation of the Amadori product fructose-lysine proceeds *via* rearrangement or oxidation to yield advanced glycation endproducts (AGEs) which are found to a greater or lesser extent in all tissues. Blood or plasma glucose or glycated hemoglobin (HbA_{1c}) are all widely used in diagnosis, with HbA_{1c} also considered to have some power as a prognostic and risk biomarker of complications, but none of these measures are entirely free of confounding factors and all require a blood sample. Nails have been the subject of significant research for diagnostic and prognostic biomarkers of T2DM.

Figure 2. Relationships between concentrations of glycation biomarkers in nails and in blood (A) or eye lenses (B).

A – Oimomi et al. [23] reported a significant association between diabetics' unguinal furosine and glycated hemoglobin (HbA_{1c}) in blood. (*With permission from Springer Nature.*)



B – Kishabongo et al. [27] reported a significant association between concentrations of glycated proteins in nails and lenses of diabetic cases and non-diabetic controls.



Author CC

Oimomi and colleagues [23] conducted pioneering work on the use of nails to assess glycation status among diabetics, and measured furosine (a derivative of fructose-lysine formed during acid hydrolysis in the laboratory), using a high-performance liquid chromatography (HPLC) method with ultraviolet (UV) detection. Expressing their data as a percentage of the tyrosine peak in their chromatograms, they reported mean furosine levels in the fingernails of 46 diabetic case to be greater than those in the 10 control subjects by a factor of *ca.*2 (9.13 vs. 4.65 %, respectively [23]). Ungual furosine was positively correlated with HbA_{1c} % in blood among the diabetic patients and the authors proposed it as a biomarker to assess glycemic control (Fig. 2A). Another Japanese group confirmed these findings in a case-control study of a similar size that included analyses of skin and hair: Sueki et al. [24] also reported higher furosine in the nails of diabetics than healthy controls and a significant correlation with HbA_{1c}.

More recently, Delanghe and co-workers built upon the work by Oimomi and colleagues in their study of another AGE intermediate. In particular, they sought to highlight the advantages of nail samples in settings where, for economic, practical and cultural reasons, blood samples are unfeasible in any large-scale, inclusive screening of the general population. In two case-control studies of several dozen participants in Belgium and the Democratic Republic of Congo, they showed that relatively simple photometric measurements of unguinal fructosamines are a feasible diagnostic biomarker for diabetes [25,26]. They went on to examine the relationship between glycation biomarkers in nails and eye lenses [27], reporting that unguinal fructosamine was associated with the amount of glycated proteins in diabetics' lenses (as measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE – Fig. 2B), as well as with HbA_{1c}. Further studies using Fourier transform infrared spectroscopy (FTIR) and near infrared (NIR) spectroscopy confirmed these relationships [28,29] and indicate that there is a robust association between glycation biomarkers in nails and distal tissues such as the lens,

thereby hinting that nails may be a valid surrogate tissue for the assessment of other diabetes-related complications.

Work from this research group also addressed methodological issues such as sample storage (ungual fructosamine levels were unchanged after 2 weeks at 37°C), the distribution of fructosamines in the nail plate (dorsal layer concentrations were about half those in the ventral layer), and the effect of an unspecified antidiabetic treatment (significant reduction seen in ungual fructosamines after 6 months' treatment).

Min et al. [30] studied three dicarbonyl AGE intermediates in a small (n = 20) case-control study of diabetic patients, reporting that ungual 3-deoxyglucosone (3-DG) was a potential diagnostic biomarker for diabetes but that there was no difference in methylglyoxal or glyoxal concentrations between cases and controls. Despite the small population sizes, their study covered a relatively broad age range (30–69 years old) and has the merit of including a rigorous characterization of their liquid chromatography-mass spectrometry (LC-MS) method. Earlier work by the same research group studied free amino acids in nails in a series of small case-control studies of diabetes and cancer (see next section). Derivatized, free L- and D-amino acids were analyzed in nails by HPLC with time-of-flight (TOF) mass spectrometric (MS) detection [31], and 20 diabetic cases were compared with 20 controls [32]. They reported a significant correlation among cases for the ungual D : L enantiomer ratios of alanine, valine, isoleucine and leucine that was absent in controls, but did not propose a mechanism for why this difference occurred.

There has been significant research interest in the role of minerals and trace elements in the etiology of T2DM (see [33] for a recent review), in particular selenium (Se – see [34-36]). The

involvement of selenoproteins in glucose metabolism underpins these investigations, but the mechanisms remain incompletely understood and apparently contradictory results have emerged from among the many *in vitro*, *in vivo*, epidemiological and clinical studies. Nails have a long history of use in the study of environmental or occupational exposure to elements, and Se concentrations in human nails are necessarily comprised of these exposures in addition to dietary intake or supplements: nevertheless, several workers have studied unguis Se as a predictive or risk biomarker for T2DM. Toenail Se has been used for more than 30 years as a means of assessing Se status, and the relationship between circulating and unguis Se is well characterized [37,38].

In a report of their prospective, 2008 study of over 7000 people in two separate United States cohorts (the Nurses' Health Study and the Health Professionals Follow-up Study), Park and co-workers [39] reported an inverse relationship between toenail Se and risk of T2DM, inferring that dietary Se reduces the risk of T2DM. They stressed that dietary supplements were not associated with toenail Se in these cohorts, and that "supradietary" doses of Se may in fact inhibit the normal, pro-oxidant responses of muscles to exercise and its consequent positive effect upon insulin sensitivity. On the other hand, Vinceti et al. [40] reported a lack of any significant association between toenail Se concentrations and T2DM in a cohort of several hundred people from Northern Italy.

Su and co-workers [41] studied an older population (>65 years old, n = 1856) in rural China and found a positive association between toenail Se and incidence of T2DM in their cross-sectional study. Highlighting the suitability of nails as a matrix in which to assess biomarkers of disease in this relatively remote population, their results are in apparent contradiction to the

findings of Park et al. [39] and Vinceti et al. [40], but accord with findings from an earlier study by Gao et al. [42].

A meta-analysis by Vinceti et al. [36] distinguished between cohort studies and case-control studies of unguinal Se and they report that the former lacked the positive association between unguinal Se concentrations and risk of T2DM reported in the latter. Taken together, these findings underline the complexity of the relationship between Se status and risk of T2DM and, indeed, Se status and risk of disease is characterized by a U-shaped relationship, with baseline Se concentrations linked to the Se content of regional soils (see Section 2).

Bahreini and colleagues have published several case-control studies of nails using laser-induced breakdown spectroscopy (LIBS). They combine this analytical technique with a multivariate analysis of variance, discriminant function analysis statistical approach, enabling them to discriminate between diabetes patients and controls on the basis of their LIBS spectra [43]. Though not a quantitative technique, the 82 emission lines in the spectra were attributable to 13 elements and one molecule, among which magnesium (Mg), potassium (K) and silicon (Si) were of lower intensity among diabetics compared with controls. While Si has no reported link with diabetes to our knowledge, low circulating levels of both Mg and K have been linked with T2DM [44,45].

1.2 Chronic kidney disease

Chronic kidney disease (CKD) affects more people than T2DM worldwide (697 million people worldwide in 2017), with greater prevalence in low- and middle-income countries, and is causally associated with risk of cardiovascular disease [46,47]. Biomarkers in urine and blood are commonly used in clinical management of the disease, and a recent review comprehensively

catalogues the origins for both classical and emerging biomarkers in these matrices [48]. Accumulation of urea and its breakdown products cyanate and isocyanate leads to increased carbamylation of proteins [49], a post-translational modification which accumulates with age in all tissues and has recently been associated with CKD progression [50].

De Bruyne and colleagues studied carbamylation biomarkers in the fingernails of hemodialysis patients using near-infrared (NIR) analysis to examine prognostic and risk biomarkers among patients with chronic kidney disease (CKD) [51]. In their cross-sectional cohort study, 53 healthy volunteers and 84 hemodialysis patients were examined and NIR spectra reflected the elevated levels of both a circulating uremic toxin and risk of mortality among the CKD patients compared with controls. The intensity of the peak at 1494 nm in the NIR spectra was significantly higher in dialysis patients compared with controls, and associated with plasma levels of the uremic toxins indoxyl sulfate, *p*-cresylglucuronide and hippuric acid. Even after adjusting for age, the intensity of this peak was positively associated with all-cause mortality. This study raises the possibility that a relatively simple, rapid and non-invasive analytical procedure could serve as a prognostic and risk biomarker to inform the clinical management of CKD, identifying in a timely manner those patients most at risk of death.

1.3 Gout

Colloquially known for centuries as the “King’s” or “Rich-man’s disease”, gout is characterized by elevated levels of circulating uric acid resulting from altered metabolism of purine nucleosides. While consumption of offal (a purine-rich foodstuff) and alcohol are known risk factors [52], diet alone causes only about 10% of cases [53]. Sufferers are at greater risk of cardiovascular disease, kidney disease, diabetes and metabolic syndrome [52], and early detection is important to initiate appropriate treatment.

Li and co-workers [54] compared uric acid concentrations in the nails of 26 healthy controls with 22 gout patients using HPLC with UV detection (see [55] for their methodology). They described significantly elevated mean levels among the gout patients (144 ng/mg fingernail vs. 45 ng/mg fingernail, respectively) raising the possibility that unguinal uric acid levels may be useful at least as a diagnostic biomarker of gout [54]. The same research group recently followed up this study with a case-control study of urate in fingernails from gout patients, reporting that the urate levels were also higher in cases than controls and that the concentrations were significantly correlated with the volume of monosodium urate crystal deposits [56]. They also described how urate-lowering therapy produced a significant reduction in both serum and unguinal urate at 3-month's follow-up, underlining the utility of nails as a matrix in which both diagnostic and prognostic biomarkers of gout may be quantified.

Thus, several molecules and elements in nails, as well as relatively non-specific spectroscopic measurements (*ie.* by Delanghe [25-29] and Bahreini [43] and their respective colleagues), have significant potential as diagnostic, prognostic and risk biomarkers of endocrine and metabolic diseases. Among them, T2DM and CKD rank highly among the major public health issues of our age and their clinical management stands to benefit from the potential for low-cost, large-scale screening that the analysis of nails enables. In this regard, nails' suitability as a sample matrix in areas where transport or healthcare infrastructure is limited is of particular interest.

2. Cancer

Cancer currently accounts for nearly 10 million annual deaths worldwide with over 19 million new cases per year (all types [57]). While metabolomics in particular has yielded several possible molecular biomarkers of cancer in recent years [58], and multi-omics studies within a systems biology approach have improved understanding of the disease, there remain relatively few published studies of molecular biomarkers of cancer in nails.

In a small case-control study (17 cases and 39 controls) of lung cancer, Min and colleagues [59] followed-up their work on diabetes by examining polyamines in nails using a rigorously validated derivatization technique followed by LC-MS/MS. Polyamines are essential for normal cell growth and elevated concentrations in cancer cells, resulting from altered polyamine metabolism, are linked with poorer outcomes and the recurrence of some cancers [60,61]. Min et al. [59] described higher levels in men's than in women's nails of putrescine and its acetyl form, and significantly higher spermine concentrations (by a factor of *ca.*2.5) in the nails of lung cancer patients compared with those from healthy controls.

In another small, case-control study on breast cancer (10 cases, 12 controls), Mitruka and colleagues [62] reported an absence in the finger nails of cases compared with controls of the free amino acids tryptophan, tyrosine, phenylalanine and histidine. They applied a novel, vertical tube gel electrophoresis (VTGE) separation method to nail lysates before analysis with high-resolution, quadrupole/time-of-flight mass spectrometry (Q-TOF-MS). Phenylalanine was the most abundant of the free amino acids in nails from controls, making its absence among cases the more remarkable. The authors draw upon a significant amount of work describing altered free amino acid profiles in the circulation of cancer patients (see for *eg.* [63]), and

proposed that this results from both local and generalized metabolic reprogramming by tumors which enhances catabolism of free amino acids. Although still in the pre-print stage at the time of our writing this review, these researchers also reported that their VTGE/Q-TOF-MS approach revealed an absence of the lipid metabolites choline, phosphorylcholine and lyso-phosphatidylcholine in nails from the same population of breast cancer cases [64]. Again, their small (and as-yet-unpublished) study is bolstered by the relatively well-described alterations of lipid metabolism in cancer (see for *eg.* [65]). Both the VTGE/Q-TOF-MS approach and the tantalizing findings from these studies require further confirmation, but they merit inclusion here for their innovation and the glimpse they offer of the potential for analyzing molecular biomarkers of cancer in nails.

In contrast to molecular biomarkers of cancer, the study of elements in nails as biomarkers of cancer has produced dozens of publications. Exposure to certain elements, or an imbalance in the levels of some essential trace elements, has long been thought to play a role in the etiology of several cancers. Selenium has received particular attention in cancer-related studies and a significant body of work spans four decades of research on the role of Se homeostasis in the etiology of cancer ([66,67] and references therein), while the interaction between dietary Se intake and genetics in determining cancer risk and outcome is also well described (*eg.* [68]).

Selenium status follows a U-shaped distribution in relation to both risk and prognosis of several cancers [69-71], and knowledge of individual Se status is also essential to determine the potential utility (or indeed risk) of any therapeutic Se supplementation. Baseline Se concentrations are generally higher in America than in Africa and Europe, but some Asian countries harbor extremes in close proximity (*eg.* China) [72]. Significant evidence has accumulated for a relationship with cancer risk that is dependent on Se homeostasis; in addition,

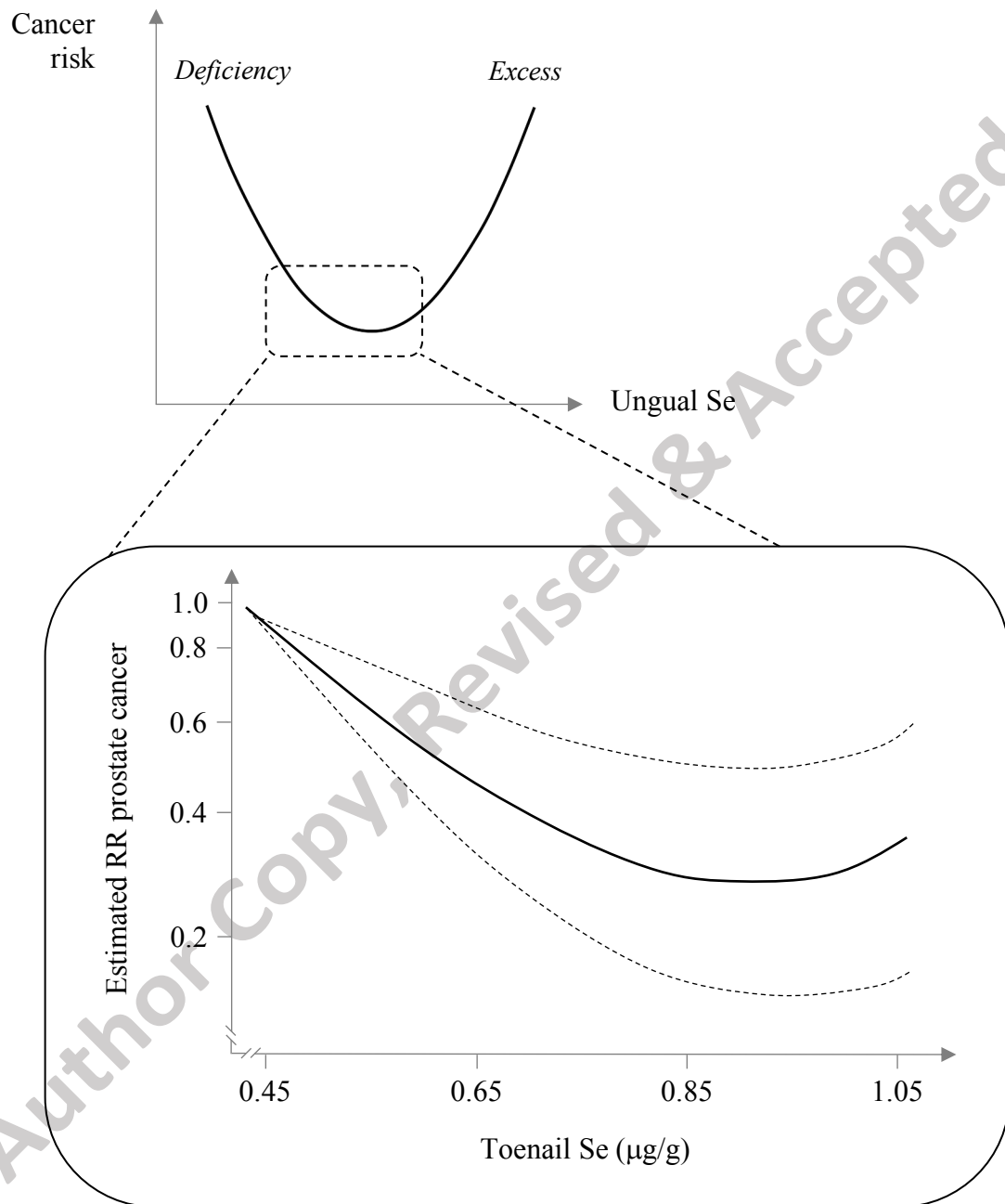
baseline Se status determines the potential risks and benefits of therapeutic Se supplementation.

Figure 3 graphically summarizes the relationship between cancer risk and Se homeostasis as measured by unguinal Se, and illustrates it with the specific example of relative risk of prostate cancer versus toenail Se concentrations.

In the last decade or so, several reviews and meta-analyses of the literature have examined the evidence for a link between the etiology of a specific type of cancer and Se status as measured by unguinal Se (these reviews also discuss Se levels in hair, blood or urine as biomarkers). Breast [73], lung [74], prostate [75-77] and bladder cancers [78] all enjoy this depth of research, while these and other cancers have also been the subject of significant cohort studies (*eg.* head-neck cancers [79]).

Given the accuracy with which Se in nails can be determined, unguinal Se is therefore a proven means of assessing Se homeostasis, an indicator of the potential risks or benefits of supradietary Se supplementation, and by extension may serve as a biomarker for assessing both the risk and prognosis of several types of cancer. Unguinal Se concentrations are generally within a small range, however, and significant differences in relative risk have often been reported only between the highest and lowest tertiles or quartiles of Se concentrations.

Figure 3. Generalized depiction of the relationship between cancer risk and Se homeostasis (after [71]) and a specific example of relative prostate cancer risk and toenail Se [75]. (With permission from Oxford University Press).



Best-fitting, fractional polynomial, dose-response plot (+/- 95% CI) of estimated relative risk (RR) of prostate cancer with toenail Se concentrations, from a meta-analysis of 3 case-control studies (total 500 cases, 1525 controls from United States of America, Canada and The Netherlands) [75].

Other elements have also been studied in nails, enabled in part by the more widespread use in recent decades of multi-element analytical techniques such as inductively-coupled plasma (ICP) with optical emission spectroscopy (OES) or MS detection. A study by Garland et al. [80] helped to establish the principle whereby unequal concentrations of elements as risk or prognostic biomarkers of cancer (and indeed other diseases) may be obtained from a single measurement. They studied 16 elements in paired toenail samples taken 6 years apart to determine the validity of a single measurement to assess exposure, reporting that a single measure of toenail Se and arsenic (As) was reflective of dietary intake. Other elements in toenails (mercury (Hg) and zinc (Zn)) were also reported as indicative of exposure, but copper (Cu), titanium (Ti) and chrome (Cr) were less reliable. Interestingly, they described the attenuating effect of inter-individual variation on the strength of associations (odds ratios), showing how even relatively minor within-person variability can reduce the strength of these associations [80]. Their work has recently been confirmed and updated by O'Brien and colleagues [81].

Amaral et al. [82] described a case-control study (118 cases and 399 controls) in which a significantly increased risk of exocrine pancreatic cancer was found among cases whose toenail concentrations of cadmium (Cd), As and lead (Pb) were in the highest quartile (mean odds ratios (OR) compared with controls of 3.58, 2.02 and 6.26 for Cd, As and Pb, respectively). They further reported that unequal Se and nickel (Ni) concentrations were inversely associated with risk of exocrine pancreatic cancer (mean odds ratio of 0.05 and 0.27 for Se and Ni, respectively) [82].

The same research group recently conducted a similar study on the most common form of pancreatic cancer tumors, pancreatic ductal adenocarcinoma (78 cases and 416 controls) [83].

They confirmed the positive association with risk of pancreatic cancer and unequal concentrations of Cd and Pb, as well as the negative association between risk and unequal Se. The authors also reported significantly different nail concentrations of several elements between *KRAS*-mutated and *KRAS* wild-type tumors among cases. Levels of Pb were higher, while Ni and Mn were lower in cases with tumors containing *KRAS* mutations at codon 12 compared with *KRAS* wild-type tumors. Specific mutations at codon 12 of *KRAS* are early events in pancreatic cancer, as well as in lung and colorectal cancers (eg. [84]), and are thought to influence tumor phenotype and outcomes. The association of unequal concentrations of Pb, Ni and Mn with these mutations therefore raises the possibility that nails could play a role in the much-vaunted development of personalized medicine.

The study of cancer biomarkers in nails could be on the verge of significant progress: early results for molecular biomarkers of the disease are tantalizing, while decades of work and the increased use of multi-element analytical techniques provide a solid foundation for recent, highly specific advances in the study of elements in nails as biomarkers of cancer risk or prognosis.

3. Psychological and neurological disorders

This section reviews studies that have examined nails for biomarkers of stress, depression, Alzheimer's disease, neurocognitive performance and epilepsy. Though perhaps not strictly within the scope of this review, stress biomarkers are included here because whether stress may increase susceptibility to disease has long been debated, and the study of stress biomarkers in nails is well developed with some excellent illustrations of the advantages of analyzing nails. Likewise, neurocognitive performance is not a disease *per se*, but its inclusion here is illuminating and of interest, particularly when viewed alongside studies on Alzheimer's disease, and within the current context of increasing human lifespan.

3.1 Stress and depression

While not defined as a disease as such, emotional or psychological stress may engender a predisposition to the development of disorders and pathologies including diabetes mellitus, acute coronary syndrome, bipolar I disorder and major depressive disorder [85,86]. Chief among the hormonal responses to stress is the production of cortisol, a steroid hormone secreted by the adrenal cortex, and the methodology underpinning the analysis of steroid hormones in nails is well described ([85-90], and references therein). The matrices generally used for the assessment of cortisol and dehydroepiandrosterone (DHEA, another abundant adrenal hormone) include blood, urine, saliva and hair, but the last 20-or-so years has seen significant interest in nails to evaluate responses to stress. Indeed, non-invasive sample collection is particularly useful in this field so as to avoid stressful sample collection (*ie.* venipuncture for blood sampling) which may alter circulating cortisol levels. In addition, because nail samples are relatively well accepted when compared with blood samples, stress biomarkers in nails have been examined in multicultural studies as well as in populations of newborns and infants [91,92].

A good example of this last is the study of newborns in intensive care units, in which plasma levels of stress biomarkers are susceptible to confounding by the pain experienced during blood collection. Maternal stress during pregnancy, and by extension stress *in utero*, has been linked to premature births and may even play a role in the child's risk of disease later in the life. In a cohort study of 80 mother-and-child nail pairs collected in the first 3 weeks after birth, LC-MS/MS analyses revealed higher concentrations of DHEA in nails from babies whose mothers had experienced stress during pregnancy [91]. In a later, prospective cohort study of 79 healthy mothers, the same team compared cortisol, cortisone and DHEA in maternal hair samples with infants' postnatal nail samples, and examined the endpoints of maternal depression and infant irritability at 3 months post-partum [93]. Stress in the 2nd- or 3rd-trimester of pregnancy was related to maternal hair cortisone levels, and these predicted depression symptoms at 3 months postpartum, while elevated unguinal DHEA in infants predicted maternal stress in the 1st trimester and infant irritability 3-months postpartum. In addition, infants' unguinal cortisol levels were positively associated with the cortisol : cortisone ratio in mothers' hair and negatively correlated with their birthweight. This work offers a non-invasive approach to study predictive and risk biomarkers in neonates of postnatal disorders resulting from pre- and perinatal stress. An excellent review recently focused on cortisol accumulation in the hair and nails of mothers and babies [94], while Liu & Doan [95] have examined the relationship between stress biomarkers in hair and nails.

Other work has described associations between the concentrations of cortisol in nails and saliva. A cross-sectional study by Izawa et al. [96] compared fingernail cortisol concentrations with levels in hair and saliva: they used an immunoassay technique to analyze methanolic nail extracts and found a moderate association between cortisol concentrations in fingernails and hair (n = 58 and 37 for the nail-hair and nail-saliva comparisons, respectively), while fingernails

reflected whole-day saliva concentrations 4 and 5 months previously [94]. Moreover, the raised cortisol levels in toe- and fingernails, arising from long-term periods of stress among 109 cancer survivors, were directly correlated with salivary cortisol levels [97]. Hence, unguinal cortisol offers not only a window on the preceding exposure to psychological stress at a specific time, but in conditions of chronic stress, also directly reflects elevated, circulating levels.

With respect to the greater acceptance of nails across cultures as a sample medium for epidemiological research, a recent cross-sectional, cohort study among 179 indigenous and 66 non-indigenous Australians showed similar median cortisol levels in nail samples between these populations. When data were adjusted for emotional distress and stressful events, indigenous participants had a higher median cortisol level which was negatively correlated with the number of stressful events [98]. This apparently unexpected negative association is nevertheless in accord with the proposition that while chronic stress may initially lead to hypercortisolism, advancing chronicity results in hypocortisolism [95].

Some researchers have explored unguinal cortisol concentrations in episodes of depression. In a case-control study of fingernail cortisol as a diagnostic and prognostic biomarker for depression, Herane-Vives et al. [99] built upon the work on stress and fingernail cortisol by Izawa et al. [96], again using an immunoassay technique. They showed that cortisol levels in fingernails were higher in depressed subjects than in healthy controls. Higher levels were also correlated with depression severity, a diagnosis of non-reactive depression and more prominent melancholic symptoms. They thus added weight to the proposition arising from studies on classical biological fluids and hair that elevated cortisol is a persistent phenomenon in depression, particularly in its more severe forms. With only one exception, the cortisol levels among depressed subjects were indicative of sub-clinical hypercortisolism leading the authors to suggest that depression be included within the remit of hypercortisolemia.

3.2 Alzheimer's disease and neurocognitive performance

Psychological and neurological disorders include dementia, characterized by an age-related deterioration of brain function, and of which Alzheimer's disease (AD) is among the most common condition. A recent review surveyed biomarkers of AD in both classical and non-invasive matrices: LDL- and HDL-cholesterol, thyroid stimulating hormone, ferritin, homocysteine and albumin, as well as the analysis of several elements in nails were all covered, but no correlations with incidence or severity of the disease were reported [100]. In this vein, Kuyumcu et al. [101] found very similar levels of Zn in their case-control study of fingernails of elderly Alzheimer's patients. Nevertheless, a small case-control study (n = 40) from 2021 [102] reported that median concentrations of both As and Se were higher in fingernails from Alzheimer's patients than controls. Using ICP-MS analyses, the difference for both elements was greater when "severe" patients were compared (factor of *ca.*5), but even "mild" AD patients had concentrations of As and Se 2-3 times those found in fingernails from healthy controls. In addition, a positive association between concentrations of these elements was evident only among the AD cases, though whether cause or consequence of AD remains unclear.

Nail samples have also been used by researchers seeking to define biomarkers of neurocognitive performance. A study by Rolle-McFarland and colleagues [101], in a population of Chinese workers occupationally-exposed to high levels of Mn, interestingly reported that higher unguis Mn levels were associated with poorer performance in auditory-verbal learning tests, a measure of cognitive function [103].

More directly pertinent to this review is a recent, prospective cohort study by Doherty et al. [104] who used ICP-MS to analyze maternal and infant toenails. They studied the neurodevelopmental effects of prenatal exposure to As, Cu, Mn, Pb, Se and Zn. Infant toenail

Mn was predictive of poorer performance on multiple composite scores of a behavior assessment system (the Behavior Assessment System for Children, 2nd ed. – BASC-2), while maternal toenail As was associated with worse scores on the BASC-2 Internalizing Problems and Behavioral Symptoms indices. They reported only weak or inconsistent associations for the other metals studied and BASC-2 scores, and concluded that exposure to Mn and As in mid-to late pregnancy may be harmful to neurodevelopment [104]. The same research group also interrogated these infant toenail data to examine the relationship between postnatal metal exposure and gut microbial diversity [103]. Among 179 infants studied, they found significant relationships between toenail As and Hg concentrations and decreases in the alpha diversity of the infants' microbiome, as measured by the Shannon and Simpson indices. Levels of toenail Pb were predictive of higher relative abundance of *Streptococcus* and Mn of lower relative abundance of neonatal *Clostridium*. While this last study may appear to be outside the scope of this section, it is interesting to consider that elements in toenails may provide an insight into gut microbial diversity, and we include it here since it addresses the growing research interest in the bidirectional gut–brain axis's importance in mental health (eg. [106,107]).

3.3 Epilepsy

Epilepsy affects around 50 million people worldwide and is a chronic neurological disorder, the causes of which remain poorly understood. The majority of epileptics live in low- and middle-income countries and sufferers have a higher risk of premature death than the general population, but timely diagnosis and treatment could mean most of them live seizure-free lives [108]. Ilhan and colleagues [109] published a case-control study of epileptic patients, comparing nail and serum levels of Cu, Mn and Zn analyzed by atomic absorption spectrophotometry (AAS). Though a relatively small study (n=50), and while they do not say whether finger- or toenails were analyzed, the authors report Mn levels in epileptic's nails to

be about twice those in controls (2 vs. 1 ppm), raising the possibility that unguinal Mn could be used to diagnose epilepsy [109].

While unguinal cortisol, in particular, benefits from a significant body of work describing its potential as a predictive or risk biomarker of psychological or neurological disorders arising from emotional or psychological stress, and may also be considered diagnostic and prognostic for depression, studies of sufficient scale and duration that confirm its utility are lacking. Some elements seem to have potential as predictive of neurocognitive performance, with Se once again being an element of potential interest in this regard. Similarly, unguinal Mn may have potential as a diagnostic biomarker for epilepsy, while others interestingly appear to be associated with gut microbial diversity, the relationship of which with psychological and neurological disorders is as yet in its infancy as a research topic.

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4. Sampling and Analytical Approaches

Table 1 presents a summary of the biomarkers studied, the analytical techniques employed and the key findings from the research reports reviewed here. While fingernails were preferred over toenails for the analysis of molecular biomarkers of disease, and toenails were generally used in studies of elements, some researchers did not indicate whether they analyzed fingernails or toenails.

There is often good agreement between biomarkers in nails and in fluids or tissues, in both direct (*ie.* the same biomarker) and indirect terms (*ie.* different biomarkers of the same process). For example glycated protein levels were highly correlated in nails and lenses among both non-diabetic and diabetic subjects [27], while unguinal furosine (a biomarker of early-stage glycation) correlated with HbA1c in blood (glycated hemoglobin, another early-stage glycation biomarker) [23]. Some authors have reported that fingernails integrate biomarkers more reliably than hair (*eg.* ethyl glucuronide as a biomarker of alcohol intake) [16].

While the minimum sample size is determined by a combination of the unguinal concentration of a biomarker and the sensitivity of the analytical method, nail analysis can be performed on samples as small as 1–5 mg in weight (*eg.* [98]). Most studies reviewed here analyzed about 10–25 mg : in our experience, this is roughly equivalent to a full-width, 2–4 mm-long clipping from the an adult's thumb, index- or middle finger.

Regarding storage of nail samples before analysis, most studies reviewed here stored samples at room temperature until analysis, and it is reasonable to assume that many of the publications lacking a description of a sample storage protocol also did so. Of note is the study by Park et al. [39] who solicited samples by mail for their cohort study of selenium in toenails and diabetes

incidence. Other workers reported that storage at 37°C for two weeks did not alter fructosamine levels [25], while cortisol concentrations were also unaffected by storage at room temperature for between 10–37 months [95]. Only a few studies refrigerated nail samples prior to analysis (at 4 °C [51], -18 °C [88] or even -30 °C [96]), and all were for studies of molecular biomarkers. De Bruyne and colleagues found no difference in the intensity of their spectra after more than 2 years of storage at 4°C [51], while He [13] recommended storage at room temperature prior to analyses for elements but apparently felt it unnecessary to stipulate a time limit.

Potential contamination of nail samples should be considered when deciding if a washing step is required before extraction and analysis [110]. Contamination prior to sampling from the environment (*ie.* exogenous molecule or element), from the scissors or clippers used to take samples (notably for analyses of nickel, iron or chrome), and during transport, storage or sample preparation in the laboratory, are all potential confounders. In addition, endogenous molecules or elements not incorporated by the nail, but present in sweat or sebum, may contaminate it – this was the case in cortisol analysis, for example, making it necessary to wash nail clippings as part of sample pretreatment [19,85].

It may not be necessary to wash the samples to analyse biomarkers which, due to their nature or origin, are unlikely to result from *ex-situ* or *in-situ* contamination. Although the potential for contamination was described by very few of the publications reviewed here, the majority nevertheless implemented a washing procedure before extraction. This usually involved a short (10 – 60 minutes) stirring or sonication in water (*eg.* [29,39]), different organic solvents (methanol, acetone, isopropanol, hexane or methylene chloride; *eg.* [96]) or surfactants (1% Triton X-100 or 0.1% SDS) [56,105], sometimes in combination. Other authors did not report any cleaning prior to the analysis of nail clippings. After washing, nail samples were often

dried at temperatures of between 37-95 °C in a desiccator or oven, or else left at room temperature overnight.

After the nail samples had been cleaned and dried, they were often cut into small pieces (1–9 mm) [26,40] or clippings were pulverized with a dental drill or a bead mill homogenizer, for example by adding 1–6 steel grinding balls to the tube at 30 Hz for 5–10 min [56,89]. Depending on the target analyte, then, it may be necessary to carry out specific studies to examine the effects of both washing and grinding or milling of nail samples before analysis.

With the exception of work by Delanghe et al. [25-29] and Bhareini et al. [43], who used spectroscopy to examine *in situ* nails or whole nail clippings, extraction is generally necessary to measure molecular biomarkers in nails. Furosine was analyzed after hydrolysis in 6M HCl at 95°C for 30h [23], while uric acid was determined after heating in a basic solution (0.1M NaOH at 90°C for 20mins) [54,56], and steroid hormones and ethyl glucuronide were extracted by sonicating nails in methanol [90]. Min et al. [59] employed a derivatization before their analysis of polyamines, while Mitruka and co-workers [62,64] developed a novel, vertical tube gel electrophoresis (VTGE) method to extract amino acids and lipid metabolites. Further purification was generally not performed, but some scientists have used solid-phase extraction plates or cartridges (*eg.* [111]) and others a liquid-liquid extraction to purify samples before analysis (*eg.* [90]).

A quick reading of Table 1 shows that, once extracted from the matrix, most of the molecular biomarkers reviewed here were analyzed by liquid chromatography with ultraviolet (LC-UV) or mass spectrometric detection (LC-MS), with only a few reports performing measurements by colorimetry [27] or immunoassay [97,99].

For the analysis of elemental biomarkers in nails by atomic fluorescence spectroscopy (AFS), atomic absorption spectroscopy (AAS), inductively-coupled plasma with mass spectrometric (ICP-MS) or atomic emission spectroscopy (ICP-AES), it is necessary to first digest the sample before analysis. Nails were generally mineralized in concentrated nitric acid, sometimes in combination with perchloric acid, and typically heated either in a microwave or on a heating plate [102,103]. Neither laser-induced breakdown spectroscopy (LIBS [43]) or instrumental neutron activation analysis (INAA, *eg.* [40]) require sample preparation.

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Table 1. Biomarkers of disease in nails, the analytical techniques used and key findings.

	Biomarker ¹	Type ²	Technique ³	Findings	Reference
T2DM ⁴	Furosine	D	HPLC-UV	Furosine reflects glycemc status	[23]
	Fructosamine	D, Pred	Colorimetry	Glycated nail proteins can diagnose & assess diabetics in remote areas	[25-27]
	IR spectra	D, Pred	FTIR & NIR	IR spectra of glycated proteins useful for diagnosis & clinical follow-up of diabetics	[28,29]
	3-DG	D	LC-MS	3-DG useable for diagnosis of diabetes	[30]
	Se	R	INAA or AFS	Mixed data on the power of toenail Se as a risk biomarker for diabetes	[39-41]
	LIBS spectra	D	LIBS	LIBS spectra for screening of diabetes	[43]
CKD ⁵	NIR spectra	D, Prog	NIR	Carbamoylated nail proteins associated with uremic toxins & mortality in dialysis patients	[51]
Gout	Uric acid & urate	D, Prog	HPLC-UV	Uric acid and urate useful for gout diagnosis & clinical follow-up	[54-56]
Lung cancer	Polyamines	D, Pred	LC-MS	Lower levels of spermine in cases	[59]
Breast cancer	Free amino acids & lipid metabolites	R	LC-MS	Reduced levels of free amino acids and lipid metabolites in nails of breast cancer cases	[62,64]
Prostate & Head-neck cancers	Se	R	INAA	Se associated with lower risk of prostate & head-neck cancers	[76,79]

Pancreatic cancer	As, Cd, Mn, Ni, Pb & Se	R	ICP-MS	Cd, As & Pb associated with higher risk of pancreatic cancer; Ni, Mn & Se inversely associated	[78,83]
Stress	Cortisol, cortisone & DHEA	D, Pred	LC-MS	Cortisol & DHEA associated with maternal, intrauterine & psychosocial stress, and predictive of postnatal depression & early-life behaviour	[91,93,98]
	Cortisol	D	Immunoassay	Ungual cortisol reflective of salivary cortisol measured 4-5 months previously	[96,97]
Depression	Cortisol	D, Prog	Immunoassay	Cortisol associated with severity & subtypes of depression	[99]
Alzheimer's Disease	As, Se	D	ICP-MS	As & Se higher among cases than controls	[102]
Neurocognitive Performance	As, Mn & Hg	D, Prog	ICP-MS	Prenatal exposure to As & Mn influences neurodevelopment & infants' microbiome	[104,105]
Epilepsy	Mn	D, R	INAA, AAS & ICP-MS	Higher Mn levels in epileptics also associated with poorer auditory-verbal learning	[109]

¹ see text for full names of molecules and elements;

² D – diagnostic; Pred – predictive; R – risk; Prog – prognostic;

³ HPLC-UV – high performance liquid chromatography-ultraviolet detection; FTIR – Fourier-transform infrared spectroscopy; NIR – near-infrared spectroscopy; LC-MS – liquid chromatography-mass spectrometry (in either LC-MS or LC-MS/MS configurations); INAA – instrumental neutron activation analysis; AFS – atomic fluorescence spectrometry; LIBS – laser-induced breakdown spectroscopy; ICP-MS – inductively coupled plasma-mass spectrometry; AAS – atomic absorption spectroscopy;

⁴ type 2 diabetes mellitus;

⁵ chronic kidney disease.

Concluding Remarks

While there have been reviews on the semiology of nails as indicators of systemic disease, a plethora of publications on unguis biomarkers of dietary or environmental exposure to molecules or elements, and significant work on the use of nails to assess trace elements' homeostasis, this is the first comprehensive review, to our knowledge, to focus solely upon biomarkers of disease in human nails.

It would have been possible to include other studies here, on the analysis of nails for biomarkers of alcohol abuse (*eg* [111] and references therein), acute myocardial infarction (*eg*. [112,113] and references therein) or brittle bone disease (*eg*. [114,115]). We have chosen to cite them only in passing, partly for reasons of space, but also because they were either indicative merely of a predisposition to disease or else were relatively few in number. The diversity of research on biomarkers in nails nevertheless bears witness to the breadth of interest in this topic.

There are very clear economic and organizational advantages to studying nails, but these cannot be fully exploited without more research on the mechanisms of biomarkers' incorporation, their mechanistic validity and sensitivity. Some excellent, innovative work seems to have languished unremarked since the 1980s and '90s, but there are signs that the topic is receiving renewed attention. It will be fascinating to see how investigations of this hitherto under-exploited biological matrix develop, and its fuller investigation within a systems approach to biology and medicine may benefit researchers, clinicians and, most importantly, patients themselves.

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