Potential application of body fluids autofluorescence in the non-invasive diagnosis of endometrial cancer

Potenciálne využitie autofluorescencie telových tekutín pri neinvazívnej diagnostike endometriálneho karcinómu

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Summary

Background: Endometrial carcinoma (EC) is the most common cancer of the female reproductive tract in developed countries. The prognosis and 5-year survival rates are closely tied to the stage diagnosis. Current routine diagnostic methods of EC are either lacking specificity or are uncomfortable, invasive and painful for the patient. As of now, the gold diagnostic standard is endometrial biopsy. Early and non-invasive diagnosis of EC requires the identification of new biomarkers of disease and a screening test applicable to routine laboratory diagnostics. The application of untargeted metabolomics combined with artificial intelligence and biostatistics tools has the potential to qualitatively and quantitatively represent the metabolome, but its introduction into routine diagnostics is currently unrealistic due to the financial, time and interpretation challenges. Fluorescence spectral analysis of body fluids utilizes autofluorescence of certain metabolites to define the composition of the metabolome under physiological conditions. ***Purpose:*** This review highlights the potential of fluorescence spectroscopy in the early detection of EC. Data obtained by three-dimensional fluorescence spectroscopy define the quantitative and qualitative composition of the complex fluorescent metabolome and are useful for identifying biochemical metabolic changes associated with endometrial carcinogenesis. Autofluorescence of biological fluids has the prospect of providing new molecular markers of EC. By integrating machine learning and artificial intelligence algorithms in the data analysis of the fluorescent metabolome, this technique has great potential to be implemented in routine laboratory diagnostics.

Key words

endometrial cancer – diagnosis – metabolomics – fluorescence

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Súhrn

Východiská: Endometriálny karcinóm (EC) je najčastejšou rakovinou ženského reprodukčného traktu vo vyspelých krajinách. Prognóza a päťročná miera prežitia úzko súvisia so štádiom pri diagnostikovaní. Súčasné rutinné diagnostické metódy EC sú buď málo špecifické alebo pre pacientku nepríjemné, invazívne a bolestivé. Aktuálne je zlatým diagnostickým štandardom endometriálna biopsia. Včasná a neinvazívnu diagnostika EC vyžaduje identifikáciu nových markerov ochorenia a skríningový test aplikovateľný do rutinnej laboratórnej diagnostiky. Aplikácia necielenej metabolomiky v kombinácii s nástrojmi umelej inteligencie a bioštatistiky má potenciál kvalitatívne a kvantitatívne prezentovať metabolóm, ale jej zavedenie do rutinnej diagnostiky je z dôvodu finančnej, časovej aj interpretačnej náročnosti v súčasnosti nereálne. Fluorescenčná spektrálna analýza telových tekutín využíva autofluorescenciu určitých metabolitov na definovanie zloženia metabolómu za fyziologických podmienok. ***Cieľ:*** Tento prehľadový článok poukazuje na potenciál fluorescenčnej spektroskopie pri včasnej detekcii EC. Dáta získané trojrozmernou fluorescenčnou spektroskopiou definujú kvantitatívne aj kvalitatívne zloženie komplexného fluorescenčného metabolómu a sú vhodné na identifikáciu biochemických metabolických zmien spojených s karcinogenézou endometria. Autofluorescencia biologických tekutín má perspektívu poskytnúť nové molekulové markery EC. Integráciou algoritmov strojového učenia a umelej inteligencie pri dátovej analýze fluorescenčného metabolómu má táto technika veľký potenciál byť implementovaná do rutinnej laboratórnej diagnostiky.

Kľúčové slová

endometriálny karcinóm – diagnostika– metabolomika – fluorescencia

Introduction

The most common malignancy of the female genital organs, endometrial cancer (EC), is a heterogeneous group of tumors whose biological behavior depends on a variety of factors (age, type, grade and stage of the disease), as well as several genetic and epigenetic alterations. Annually, more than 300,000 new cases are identified, accounting for around 8.2% of all female cancer cases worldwide [1]. The incidence of EC is increasing rapidly, the highest in North America and Western Europe, which is due to population aging, higher prevalence of obesity and metabolic syndromes [2]. More than 90% of EC cases occur in women older than 50 years, with a median age of 63 years. Only about 4% of patients are younger than 40 years.

When diagnosed at an early stage, EC is generally well treatable and has an excellent 5-year survival rate. However, a delayed diagnosis leads to progression of the tumor worsening the overall survival of the patient. Although relapse occurs in roughly 15% of cases, there is a lack of effective risk classification and limited progress in treating recurrent or metastatic disease [3]. This underscores the need to enhance the early-stage identification and stratification of patients with this form of cancer.

The dominant part of the cases is caught in the early stage of the disease according to The International Federation of Gynecology and Obstetrics (FIGO) classification. The prognosis for these patients is promising, e. g. with FIGO stages I or II, 75–90% of women survive 5 years. In advanced stages or recurrent tumors, the clinical course is very poor, and the overall survival is short – approximately 68% and 17 % in stages III and IV, respectively [4].

Obesity is a major risk factor for EC and is estimated to be responsible for 40% of all endometrial cancer cases [5]. Other risk factors include age, diabetes, hypertension, polycystic ovary syndrome, use of estrogen-only hormone replacement therapy, and tamoxifen [6]. Females may also have a familial predisposition to EC, especially those who carry a pathogenic variant in one of the genes required for DNA repair (Lynch syndrome) or in PTEN (Cowden syndrome) [7].

Early cancer diagnosis is crucial for successful and effective medical intervention. Nonetheless, many diagnostic procedures are often invasive, uncomfortable and unpleasant for the patient, which significantly limits the willingness of patients to undergo them as a preventive measure. Women with suspected EC undergo a variety of diagnostic tests, including transvaginal ultrasound scan (TVS), outpatient hysteroscopy (OPH), and endometrial biopsy (EB). The diagnostic value of these tests for EC is limited by their low specificity (TVS), invasiveness and high level of discomfort for patients (OPH, EB) [8].

The gold diagnostic standard is endometrial biopsy (EB) (Fig. 1). However, its disadvantages include severe pain in women who have not given birth yet and a high risk of failure during sample collection. Guided biopsy hysteroscopy, on the other hand, has better diagnostic sensitivity but is expensive and has a high outpatient failure rate. More than 30% of women experience severe pain or a vasovagal episode during the procedure. Additionally, there exists a theoretical risk of cancer cell dissemination into the peritoneum, with rare but life-threatening complications like uterine perforation [9].

An ideal diagnostic approach for EC detection should be simple, noninvasive, and could reliably detect all ECs at an early stage of disease with few false-positive or false-negative results. This approach should also be used in the screening of high-risk asymptomatic women with Lynch syndrome who have a high lifetime risk of developing EC [10]. Currently there are not any dependable and accurate methods that can be introduced as screening tests for this cancer among the general population.

Metabolomics in tumor detection

Metabolomics is a holistic approach in understanding biochemical processes in the biological system. It is a rapidly developing discipline that uses analytical techniques in conjunction with sophisticated statistical methods to comprehensively characterize the metabolome. The metabolome represents the total composition of metabolites present in an organism under physiological and pathophysiological conditions. Metabolites are not only substrates of metabolic reactions but also represent signaling

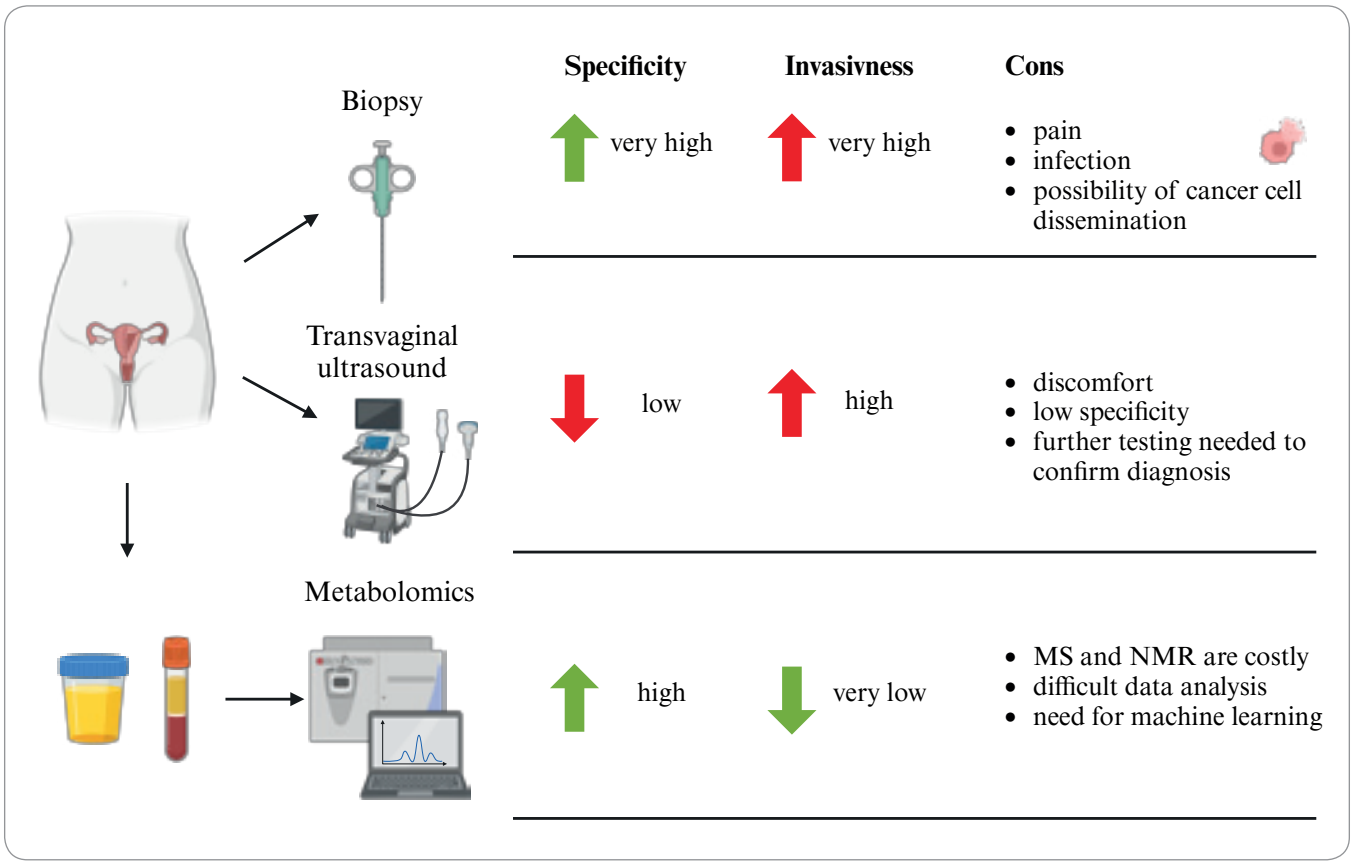

Fig. 1. Comparison of current diagnostic methods. (Created with Biorender).
MS – mass spectrometry, NMR – nuclear magnetic resonance

molecules controlling a wide range of cellular processes. All biological systems are defined by the genome, transcriptome, proteome and metabolome. However, only the metabolome characterizes the overall phenotype of an organism.

The metabolism of tumor cells differs from that of healthy cells. Metabolic reprogramming is a key feature of cancer. Oncometabolites provide cancer cells with energy and necessary substances used in biosynthesis, proliferation, invasion and metastasis, which can cause resistance to drug therapies. Cancer-related metabolites are by-products of cellular processes that result from neoplastic transformation and cell proliferation as well as the body's immunological (inflammatory) response to malignancy [11]. Such metabolites are quantitatively different from those resulting from non-malignant cellular processes and therefore have the potential to serve as biomarkers for cancer detection. The human metabolome is inherently dynamic and evolves in tandem with the progressive nature of malignancy. Therefore, studying the metabolome has the potential to identify biomarkers of a given carcinogenic process.

Most metabolomic studies conducted today are focused on tissues, organs, organoids or their extracts. This approach is valuable because it allows for a detailed exploration of the under normal conditions and detect any alterations or disruptions that may be linked to diseases or disorders. Unfortunately, organ and tissue extractions are highly invasive, so unless it involves a muscle or selective tissue biopsy, it is generally limited to studying animal models. On the other hand, biofluids can be obtained relatively noninvasively, which makes them ideal for studying living specimens, including humans. Biofluids serve as near-ideal proxies for organs or tissues as their constituents closely reflect the metabolic activity of the organ from which they are derived or the organs which they bathe. Because blood bathes all organs and tissues, it serves as a reasonably good metabolic proxy for the entire organism [12].

The human metabolome is a diverse group of relatively low molecular weight compounds that result from both endogenous and exogenous processes. The main goal in metabolomics is to generate a reliable clinical metabolomic profile for the maximum number of metabolites in a biological sample. However, target metabolites differ in several chemical and physical properties such as hydrophobicity, acidity, charge, pKa and particle size, moreover metabolites can be present in very different concentrations. Simultaneous identification of all metabolites in a single assay is extremely challenging.

Currently the dominant method in metabolomics analysis involves the combination of mass spectrometry (MS) with chromatographic separation to effectively isolate and identify metabolites

Tab. 1. Representative fluorophores identified in various cancers.

Identified fluorophore	Type of cancer	Biological material	Source
NAD(P)H	ovarian cancer	blood, urine	[18]
	prostate cancer	tissue	[19]
	breast cancer	tissue, blood	[20]
FAD	different etiology	urine	[21]
porphyrins	different etiology	urine	[21]
pteridines	ovarian cancer	urine	[22]
collagen	cervical cancer	tissue	[23]
tryptophan	malignant melanoma	urine	[24]
	kidney cancer	blood, urine	[25]
tyrosine	colorectal carcinoma	blood	[15]

FAD – flavin adenine dinucleotide, NAD(P)H – nicotinamide adenine phosphate

within biological samples [13].This approach provides high-resolution data for metabolite detection, allowing researchers to discern a wide array of molecules. MS is undoubtedly a powerful tool in metabolomics, offering exceptional sensitivity and the ability to detect a diverse range of metabolites. Yet, it is important to note that nuclear magnetic resonance (NMR) spectroscopy also plays a significant role in this field. NMR provides complementary information, offering advantages in terms of non-destructive analysis, reproducibility, and the capacity to identify and quantify metabolites without the need for extensive sample preparation. Hence, the choice between MS and NMR often depends on the specific goals of a metabolomics study and the nature of the samples being analyzed. However, the complexity of the data generated by both necessitates the use of specialized statistical and bioinformatics tools for effective data analysis, interpretation, and integration [14].

Fluorescence spectroscopy as a promising metabolomics tool

Significant diagnostic potential is provided by sophisticated fluorescence spectroscopy techniques, which are applicable to the analysis of both biological fluids and tissues. These techniques are particularly advantageous in the field of metabolomics, and their efficacy is enhanced by the innate fluorescence of certain biologically significant molecules, which are considered endogenous fluorophores.

To identify or characterize minimally altered biological material, three-dimensional recordings of various scanned fluorescence spectra (excitation-emission matrices – EEM, and synchronous fluorescence matrices – SFM), which describe the mixture as a whole, can be utilized (mixtures of fluorophores) [15]. The coordinates of the fluorescence centers, their intensity of fluorescence as well as the minor characteristics given by the contours create a unique image 'fingerprint'. This graphic depiction is exceedingly precise because it is the result of the distinct internal composition of metabolites and their mutual interactions. It represents fluorescent metabolome. Every change in the composition of the biological material disturbs the very fine balance of internal relationships, which results in a change in the fingerprint. The measured variation in the fingerprint automatically reflects the variation in the biological material's composition. If such fingerprints are related to specific physiological (pathological) changes, they provide enough information for diagnostic assessment [16].

Native fluorescent metabolites

Autofluorescence refers to the inherent property of certain molecules within biological materials to emit fluorescent light when excited by a light source. These molecules, known as endogenous fluorophores, include various compounds such as vitamins, coenzymes, structural proteins, and other metabolites that are naturally present in tissues, body fluids, or cells (Tab. 1). This intrinsic fluorescence property is characteristic of conjugated polycyclic and aromatic compounds, including vitamins like folic acid, essential coenzymes such as NAD(P)H and FAD, structural proteins like collagen and elastin, lipids, advanced glycation end products, porphyrins, aromatic amino acids, and their derivatives [17].

When exposed to an appropriate excitation wavelength, these endogenous fluorophores emit fluorescent signals that can be captured and analyzed. This intrinsic property of molecules allows for the development of diagnostic techniques that rely on analyzing the altered fluorescence signals in blood, urine, or tissues, which can provide valuable insights into cancer development and facilitate early detection and risk assessment (Tab. 1).

The key advantage of utilizing fluorescence lies in its sensitivity and ease of application, making it another choice for metabolic profiling. With remarkable sensitivity, fluorescence techniques can detect and quantify low concentrations of specific metabolites, a feature that is invaluable in discerning subtle changes within biological systems. Furthermore, the straightforward testing procedures associated with fluorescence assays and equipment enhance their accessibility to researchers and clinicians, facilitating a range of applications from basic scientific research to clinical diagnostics. Moreover, the natural fluorescence of biochemically significant molecules positions fluorescence techniques as a cost-effective option for rapid screening or diagnosis of various diseases. Changes in the concentration, ratios, or the presence of atypical fluorophores can be linked to disease-related processes within the body. For instance, carcinogenesis can profoundly influence metabolite concentrations, thereby affecting how tissues and body fluids scat-

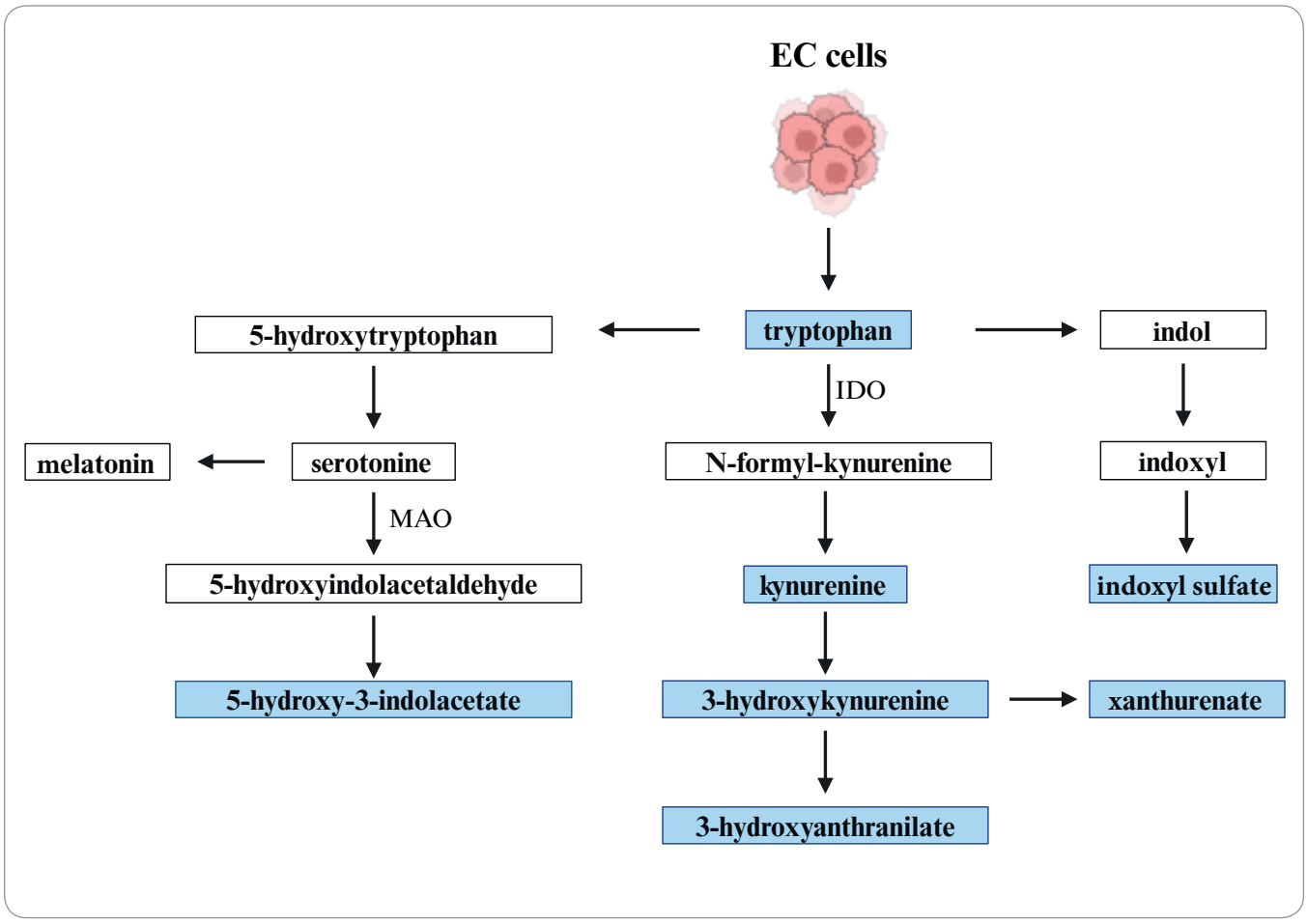

Fig. 2. Fluorescent metabolites of tryptophan. Tryptophan metabolic pathways produce fluorescent metabolites detectable in body fluids. Fluorescent intermediates are highlighted in blue. (Created with Biorender).
EC – endometrial cancer, IDO – indolamine 2,3-dioxygenase, MAO – monoamine oxidase

ter and absorb light. Therefore, fluorescence techniques are well-suited for metabolic profiling due to their exceptional sensitivity and the intrinsic fluorescence of biologically important molecules and non-destructive analysis. Fluorescence is a desirable option because of these characteristics for deciphering metabolic complexities and for diagnostic uses of selected diseases [26].

Tryptophan metabolism is a source of native fluorophores

Tryptophan, an essential amino acid, plays a significant role in the autofluorescence diagnostics of various cancers, including EC. In the realm of metabolomics, tryptophan and its metabolites have garnered attention for their potential as biomarkers due to their ability to influence the intrinsic fluorescence of biological materials. Alterations in tryptophan metabolism have been linked to numerous pathological conditions, and their detection through autofluorescence can offer valuable insights. Specifically, the concentration of degradation products of tryptophan in blood has been investigated in relation to conditions like breast cancer. The relationship between tryptophan metabolites and steroid hormones in breast cancer has shown promise [27]. While the diagnostic potential of tryptophan and its metabolites is still under exploration in EC, these compounds could provide valuable fluorescence signals for early detection, risk assessment, and diagnostic stratification, enhancing the non-invasive nature of EC diagnosis.

Tryptophan (Trp) is metabolized by three different metabolic pathways: kynurenine, serotonin and indole (Fig. 2). The intermediate products of these metabolic pathways are several fluorescent compounds that are excreted in the urine and blood. The primary pathway of tryptophan catabolism in the liver is its degradation via the kynurenine pathway (KP). It is estimated that up to 95% of dietary Trp is metabolized via KP, 90% of which is catabolized in the liver, with minor extrahepatic KP having a significant role in immune activation. Dysregulation of the kynurenine pathway is thought to be a mechanism of tumor immune escape via enzymatic activity of indolamine 2,3-dioxygenase (IDO) production. Immunometabolic dysregulation mediated by the IDO1 enzyme is thought to protect EC cells from T cell-induced cytotoxicity, thereby actively creating an immunosuppressive environment. It depletes the tissue microenvironment of the essential amino acid Trp by converting it into the immuno-

suppressive metabolite Kyn. Altered IDO enzyme activity is found in a wide range of human malignancies, e. g., including endometrial carcinoma [28].

Tryptophan is a precursor of serotonin (5-hydroxytryptamine – 5-HT), which is involved in the physiological regulation of several behavioral and neuroendocrine functions. The serotonin pathway is also localized in the lining of the gastrointestinal tract and is involved in 90% of serotonin production in the body. Serotonin is also accepted as a substrate of IDO1, mostly during prolonged IDO1 enzyme activity [28]. By mitochondrial monoamine oxidase, serotonin is metabolized to 5-hydroxyindole-3-acetic acid, which is excreted in the urine. According to recent findings, the serotonergic pathway is also involved in tumor angiogenesis. Several studies have demonstrated the role of serotonin and 5-HT receptor subtypes in cell proliferation, angiogenesis, invasion, migration and metastasis. Genetic models of several cancers, such as ovarian, breast, kidney, and pancreatic cancer cells, have shown that serotonin levels in tumors play a key role in tumor growth [29].

Microbial degradation of tryptophan produces a myriad of active indole derivatives via a pathway known as the indole pyruvate metabolic pathway. The microbiome is a key component of the tumor microenvironment and influences cancer initiation, promotion, and response to therapy; therefore, it is clear that tryptophan metabolism via microbial transformation to indole compounds is altered in carcinogenesis.

Autofluorescence of body fluids

One of the most common biological materials used for clinical examinations in human medicine is blood. However, it requires minimal invasive sampling. It contains many substances, such as proteins, whose composition is changed in various diseases. Masilamani et al. first demonstrated the relationship between porphyrins and cell proliferation in an animal model, and a year later, they extended the same study to human blood [18]. The work focused on the possibility of diagnostics of different cancer types by measuring porphyrins autofluorescence in serum, and it confirmed alterations in cancer patients, but different malignancies could not be distinguished from each other. The EEM application was used for screening and real-time diagnosis of cervical precancers with 75–90% specificity and high sensitivity [30]. In the field of gastroenterology, EEM can be used to diagnose colon cancer [31].

In the realm of oncological diagnostics, urine analysis has gained substantial attention due to its potential to uncover early-stage malignancies. Urine contains a plethora of biomolecules, including metabolites, proteins, and cellular components, whose fluorescence properties can be perturbed by neoplastic processes. Due to the close proximity of the urethra to the vagina, naturally released tumor metabolites have the potential to contaminate the urine. Urine is an ideal non-invasive specimen for biomarker detection due to the possibility of repeated collection and patient comfort during collection. A wide variety of substances can be found in urine that may serve as potential biomarkers of EC. These substances include many endogenous metabolites as well as tumor DNA, peptides/proteins, malignant cells, and secreted organelles such as extracellular vesicles. Investigating each of these targets to identify biomarkers requires the use of specialized techniques such as cytology, spectroscopy, genomics, transcriptomics, proteomics, and metabolomics [32]. To date, several studies have been published showing the potential of urinary biomarkers in the diagnosis of EC [33,34].

Proteins and peptides excreted in urine are less complex and more stable compared to plasma proteins, providing an advantage in identifying novel biomarkers. EC cells can be identified in urine, especially in women with symptoms of bleeding, for example by microscopic evaluation of urine (cytology) or using single-cell sequencing technology [35]. On the other hand, tumor DNA may be excreted by the kidneys or may result from the breakdown of malignant cells contaminating the urine. Characterization of tumor DNA, including assessment of DNA concentrations, presence of mutations, and methylation status in urine, has great potential to provide relevant biomarkers and requires further research [32].

Autofluorescence of corpus uteri

Researchers were able to identify altered levels of aromatic amino acids in tissue (eutopic endometrium) [36], serum [37], follicular fluid [38], urine [39] and endometrial fluid [40] of human subjects with endometriosis. Autofluorescence has been investigated for intraoperative guidance during gynecological cancer surgeries. Studies, such as the one by Ramanujam et al., have demonstrated the use of autofluorescence to differentiate tumor margins from healthy tissue in real-time, aiding in achieving complete tumor resection [41].

The metabolome is a dynamic system and is susceptible to environmental and genetic changes thus, hormonal variations throughout the menstrual cycle phases could have implications on the levels of amino acids. Dutta et al. state that the catabolic state induced in response to injury in endometriosis leads to increased breakdown of endogenous protein and release of free amino acids in circulation [36]. This statement agrees with their study, where they found an inverse relationship of amino acid levels between tissue and serum. The many similarities of endometrial cells to neoplastic cells, such as high proliferation, angiogenesis, anti-apoptosis, and cell invasion have been extensively recognized in the literature [42,43]. All these characteristics require a high catabolic state from which amino acids could be serving as an important supply since they can be interconverted to the TCA cycle intermediates and support energy requirements for fast-growing endometrial cells.

The potential application of autofluorescence analysis in diagnosing EC through blood or urine samples holds promise but requires further research and validation (Fig. 3). Although autofluorescence has proven its efficacy in diagnosing various cancer types using different body fluids, applying this method to EC diagnosis poses unique challenges.

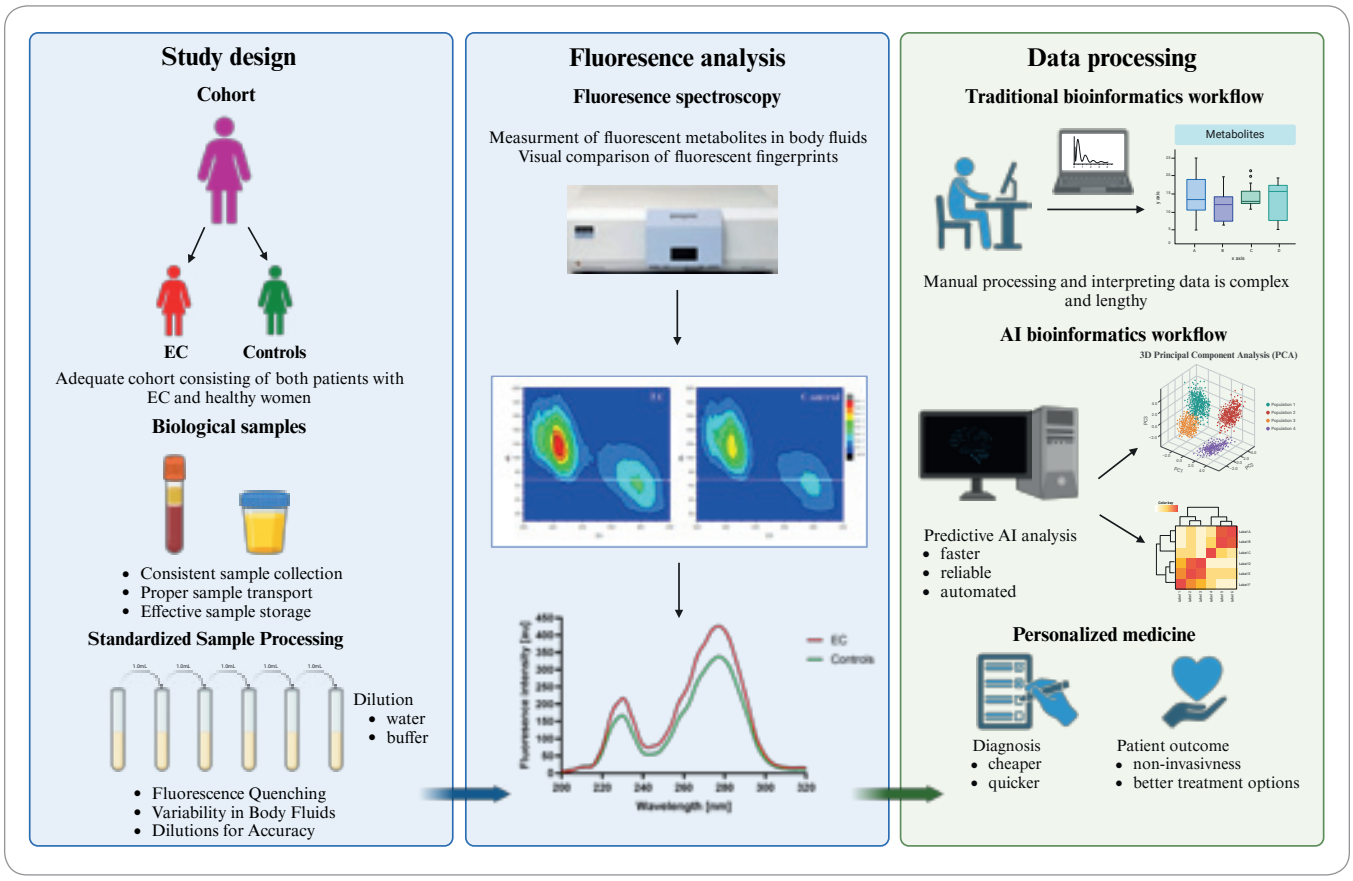

Fig. 3. Prospective workflow for fluorescence metabolite research in EC. The figure displays a visual comparison of the current fluorescent metabolomics (in blue frames) and outlines its perspective implementation in diagnostics through mathematical processing of fluorescence data, aided by artificial intelligence and machine learning (in the green frame). (Created with Biorender).
AI – artificial intelligence, EC – endometrial cancer

This malignancy, originating in the uterine lining, typically involves diagnostic techniques such as biopsies, imaging, and histopathology. Endometrial tissue consists of multiple cell types and undergoes dynamic changes throughout the menstrual cycle. This complexity could affect the reliability and specificity of autofluorescence signals.

Comparing traditional and fluorescent metabolomics

Traditional metabolomics methods, such as MS and NMR, have long dominated metabolic research due to their ability to provide data on thousands of metabolites. However, despite their prevalence in research settings, these methods come with significant drawbacks that limit their utility in routine diagnostics. Both MS and NMR are often expensive, analytically complex, and pose challenges in data evaluation, making their integration into routine diagnostic practices impractical. While these techniques excel in research and biomarker discovery, they remain less suited for widespread clinical applications.

When it comes to applying autofluorescence to EC diagnosis, the complexity of the disease and the use of blood or urine samples present significant challenges. The composition of body fluids, invaluable for non-invasive analysis, is influenced by various factors, including diet, hydration, and individual variations in metabolites. These variations can introduce hurdles in establishing standardized protocols for autofluorescence analysis, as the baseline levels of endogenous fluorophores may differ among individuals. Nevertheless, despite these challenges, the non-invasive nature of body fluid analysis through autofluorescence offers a promising avenue for improving EC diagnosis.

In contrast, 3D fluorescence spectroscopy, though historically underutilized in diagnostics, offers a promising alternative. It boasts advantages such as ease of use, simultaneous detection of multiple metabolites, non-destructive sample analysis, and monitoring of different metabolic changes. Overcoming the historical dominance of MS and NMR in metabolomics, recent advancements in artificial intelligence (AI) and machine learning present a solution to the challenges faced by 3D fluorescence spectroscopy. AI can assist in data analysis, metabolite identification, and standardization, making this technique a more attractive option for diagnostic applications. Its non-destructive nature and the ability to monitor metabolic changes have the potential to complement traditional metabolomic approaches, ultimately contributing to more comprehensive and effective diagnostic tools.

Conclusion

In conclusion, autofluorescence emerges as a promising diagnostic approach for endometrial cancer, offering insights through the examination of body fluids such as blood and urine, as well as direct analysis of tumor tissues. This non-invasive technique capitalizes on the intrinsic fluorescence properties of endogenous molecules, enabling the detection of subtle molecular changes associated with cancer development. By examining these altered autofluorescence patterns, we gain valuable insights into the metabolic alterations and biomarker profiles linked to this malignancy. In essence, autofluorescence diagnostics provide a promising avenue for improving the identification and understanding of endometrial cancer, underscoring its potential to transform the landscape of cancer diagnosis and patient care.

Contributors and supporting agencies

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