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Analysis of Bowel Diseases from Blood Serum by Autofluorescence and Atomic Force Microscopy Techniques

Autofluorescence & Atomic force microscopy in bowel disease diagnosis

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Abstract: Diagnosis of bowel diseases is often difficult and time consuming since it is not always possible to obtain adequate information by the conventional diagnostic methods to set up a diagnosis and exclude non-gastrointestinal causes of symptoms. The aim of this study was to investigate the structure of blood serum samples of patients with selected intestinal diseases. The blood serum samples of patients (N=35) with selected diagnoses (*mesenteric thrombosis, inflammatory bowel disease, duodenal ulcers, sepsis, enterorrhagia, sigmoid colon resection, small intestine cancer*) and of healthy subjects were evaluated by synchronous fluorescence fingerprint and atomic force microscopy. Autofluorescence of blood serum studied at λ_{ex} = 280 nm showed significant decrease of fluorescence intensity in patients with all types of diseases affecting bowels in comparison with the healthy control patients. The blood serum surface of ill patients showed significant differences in comparison with control group samples after atomic force microscopy evaluation as well. Irregularly placed small globular units of irregular shape in small amounts are possible to observe in patients with intestine ischemia. Fluorescence analysis and atomic force microscopy showed the ability to rapidly reflect qualitative and quantitative changes of proteins in blood serum samples of patients. These sensitive methods could be beneficial for monitoring the progression of both acute or chronic bowel diseases.

Keywords: bowel diseases; analysis; proteins; synchronous fluorescence fingerprint; atomic force microscopy.

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1 Introduction

Bowel diseases represent a group of different disorders affecting the digestive tract from the duodenum to the rectum. This group includes diseases such as chronic inflammatory bowel disease, several tumour types, bleeding, duodenal ulcers, but also sepsis characterized by impaired intestinal barrier function and translocation of bacteria from the lumen to the blood stream. These diseases are either chronic and their progress is slow, or acute, sometimes with fulminant course. A common feature of these diseases is the impairment of various layers of the intestinal wall based on the basic characteristics and pathomechanism of the certain

disease. The pathomechanism is based on the inflammatory changes, apoptosis and necrosis of certain cell populations, macroscopic changes such as bleeding, mucous edema which lead to the malabsorption of nutrients and ultimately to the changes in the composition of blood proteins in acute or chronic manner [1].

Crohn's disease (CD) and ulcerative colitis (UC) belong to the group of autoimmune-mediated inflammatory diseases of the intestines, which are known as Inflammatory Bowel Disease (IBD). Patients with the diagnosis of IBD must face a long-term medical therapy and even surgical interventions. 5-aminosalicylic acid (5-ASA) preparations (e.g., mesalazine, mesalamine) are well-established preparations used in the management of IBD, mainly the mild and moderate stages of these diseases [2,3].

Biological therapy represents a significant advance in the treatment of non-specific chronic intestinal inflammation. A key cytokine in the inflammation cascade of IBD is tumor necrosis factor alpha (TNF-α), which is also the target of biological treatment. Infliximab (IFX) as a monoclonal antibody against TNF-α, which may have revolutionized treatment for more than 50% of patients who have not responded to standard treatments. IFX has been introduced into therapeutic regimens for earlier stages, with the aim to avoid the side effects of steroids, surgical intervention, and the development of resistant disease [4].

The most representative disease of the intestine with involvement of the inflammatory, apoptotic, necrotic pathways with following regeneration is the acute mesenteric ischemia. Acute mesenteric ischemia is a set of conditions in which an interruption in the splanchnic circulation occurs. A severity of the disease is expressed by the mortality which typically ranges from 60% to 80% [5]. The main role in pathophysiology of acute mesenteric ischemia plays ischemic-reperfusion injury (IRI) and its diagnosis is difficult and in need of specific and sensitive markers.

Traditional and novel markers for the diagnosis of intestine damage show low diagnostic accuracy with problems occur on several levels. Firstly, the marker should be able to reflect complex tissue architecture which consists of mucosa, submucosa and external smooth muscles, in order to distinguish between the mucosal damage and intestinal infarction. Secondly, before reaching the systemic circulation, the marker must undergo the hepatic clearance and the hepatic metabolic events. It is also hard to distinguish between the organs (liver or intestine) of origin of expressed proteins [6]. The ideal marker should be noninvasive, specific for the intestinal tissue and easy to measure in peripheral blood. Finally, is its sensitivity in diagnosis of early intestinal ischemia.

This experimental work studied the blood serum of patients with various representative cases of intestinal diseases. The aim of this study is to investigate the blood serum structure, using several methods, e.g. fluorescence analysis, atomic force microscopy, of patients with selected intestinal diseases in comparison to a control group of healthy subjects.

2 Material and Methods

2.1 Experimental

Blood of selected patients with seven different intestinal diseases (n = 35) and blood of healthy volunteers (n =10) was collected by trained surgery personal in vacutainer tube with red cap at 2nd Surgical Clinic of Louis Pasteur University Hospital in Košice. The patients suffered from the following intestinal diseases; superior mesenteric artery (SMA) thrombosis (n=5), inflammatory bowel disease (IBD) (n=5), duodenal ulcers (n=5), sepsis (n=5), enterorrhagia (n=5), sigmoid colon resection (n=5) and cancer of the small intestine (*adenocarcinoma*) (n=5). After collection, the blood in the tube was not mixed and was allowed to stand at room temperature (20-25°C) and then centrifuged for five minutes at 4500 rpm. All collected samples of blood serum were frozen and stored at -20°C. All clinical investigations were conducted according to Declaration of Helsinki. Ethical consent for this study has been given by the institutional committee on human research and is compliant with ethical standards on human experimentation.

2.2 Material

Phosphate buffer with pH = 7.4, c = 0.3 mol/l was prepared from deionized water, potassium dihydrogen phosphate (KH_2PO_4) and dipotassium phosphate (K_2HPO_4) which were purchased from Fluka AG, Switzerland.

2.3 Synchronous fluorescence fingerprint of blood serum

In this research, we followed the methods [7]. All samples of blood serum were diluted (1: 10000) in phosphate

buffer (pH = 7,4) before each fluorescence analysis. The intensity of endogenous fluorescence of control and experimental samples was measured in a quartz cuvette (1cm width and volume V = 2.5 ml) by synchronous fluorescence fingerprint (SFF) analysis on a Perkin-Elmer Luminiscence Spectrophotometer LS 55 at 25°C. The setting of the instrument rate of scans was 800 nm/s, the setting of excitation slit was 5 and emission slit was 5 nm. The synchronous simple fluorescence spectra (10 scans) were measured in the wavelength range λ_{ex} = 200-390 nm with increment Δ10. The obtained fluorescence synchronous spectra were processed using the graphic WinLab software (version 4, 2001). Three-dimensional spectrum of the SFF was created from 10 scans of simple synchronous fluorescence spectra of blood serum which were measured at various Δλ located in an area with the increment Δ 10 [8].

2.4 Method of the atomic force microscopy

Blood serum (5 µl) of patients and healthy subjects was pipetted on specially cleaned slides (ultrasound, special nanopaper), which are used in classical microscopy. Subsequently, the blood serum was spread over the surface of the microscope slide using the blood smear method. Samples were analyzed using an atomic force microscope Dimension Icon (Bruker FastScan,) and each analysed sample was processed into graphic form by ScanAsyst ™ software.

2.5 Statistical analysis

The fluorescence intensities (for ten repetitions) of the patient's blood serum were statistically compared to the control group using both one-way analysis of variance (ANOVA) and Tukey – Kramer test at significance level, $p < 0.001$, which at the graph is marked with symbol (***).

3 Results and Discussion

Dealing with the complexity of blood matrix is a long-term challenge in which optical spectroscopy and especially fluorescence techniques could facilitate. However, for the early and fast diagnosis the optimization of analytical techniques is needed for examples optical spectroscopy and atomic force microscopy. Many pathological processes lead to detectable changes of endogenous fluorophores and we assume that the three-dimensional fluorescence spectra and excitation-emission matrix of human blood have the potential to reflect the pattern of signals that are characteristic for particular diseases. The excitation-emission spectrum of human blood serum consists of the peaks occurring at wavelength pairs of 260-630, 280-340, 340-460 and 450-520 nm which refer to porphyrins, tryptophan, NADH, NADPH and FAD, respectively [9]. These fluorescence measurements were examined in correlation with intestinal ischemia and reperfusion injury using samples of the jejunal part of the rat intestine [10]. Autofluorescence of blood serum has already been used for characterization of metabolic and pathological changes during thoracic aortic aneurysm [11] or monitoring the interactions between serum proteins (albumin, haemoglobin) and other substances.

3.1 Synchronous fluorescence fingerprint of blood serum

Blood serum is considered to be a dynamic, relatively stable biomaterial which represents the characteristic mixture of naturally occurring endogenous fluorophores that are able to fluoresce and present changes in an organism rapidly, which is an ideal case for study by fluorescence methods [12]. These endogenous fluorescence markers are the source of valuable information about both physiological and pathological processes and their research opens up new possibilities of early diagnosis of intestinal diseases using small samples.

Blood serum is the mixture of several endogenous fluorophores: aromatic amino acids, proteins, co-enzymes, vitamins and metabolites. These fluorophores influence each other, may enhance, attenuate or mutually quench their fluorescence [13]. Detection of the fluorophores depends on how fluorescence data is displayed, as on some occasions individual active substances cannot be seen because their fluorescence maxima overlap with the multi-fluorescent system of blood serum. For this reason, blood serum has been moved to the periphery of interest for utility of fluorescent spectral methods [14].

Fluorescence analysis showed ability to rapidly reflect the changes in the organism from blood serum as an appropriate research object. One center of fluorophore fluorescence is visible in the healthy subjects (Figure 1, Δλ = 60 / λ_{ex} = 280 nm, F = 611). Comparing different SFFs, the qualitative and quantitative changes are seen in comparison to a defined standard – the control sample. The changes in the fluorophores break the balance of the mutual interactions what will be manifested in the transformed graphical display, the intensity of the

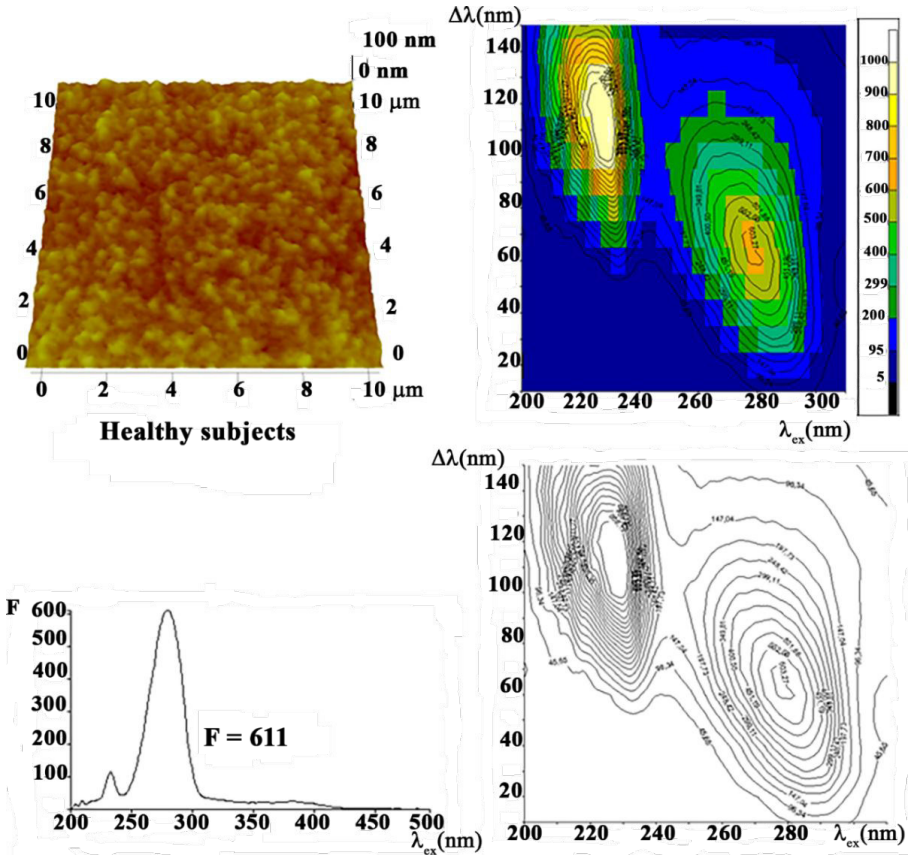

Figure 1: Blood serum of healthy subjects studied by synchronous fluorescence fingerprint and atomic force microscopy.

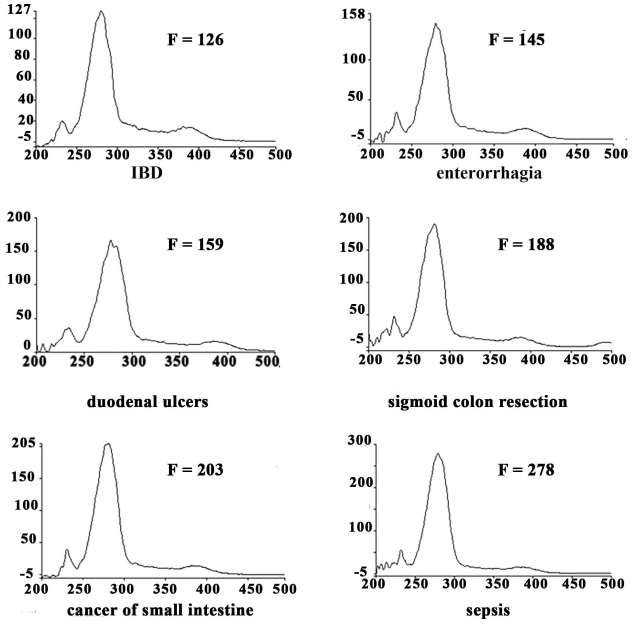

Figure 2: Simple synchronous fluorescence spectra show autofluorescence of blood serum of patients with inflammatory bowel disease (IBD), enterorrhagia, duodenal ulcers, sigmoid colon resection and cancer of small intestine (*adenocarcinoma*), sepsis.

fluorescent region of SFF or the new fluorescent region may appear or disappear. For a diagnostic aspect, the detailed analysis of the curve is not necessary. The qualitative or quantitative change of fluorescence of individual components already has the significant informative value.

If these fluorescence synchronous fingerprints are related to concrete physiological or pathological changes, they bring sufficient information for diagnostic assessment. It is possible to detect the characteristic markers by comparing the fluorescent profiles of healthy subjects and patients. This could be used for cheap, accurate and fast screening or diagnostics of the selected diseases [7].

Fluorescent contour maps are used for observing different biological fluids and tissues, e.g. they were used for monitoring of serum lipoproteins oxidation and their relation to atherosclerosis [7].

We implemented the fluorescent methods which are not set up in the practice for early diagnosis of intestinal ischemia by monitoring the fluorescence of blood serum endogenous fluorophores from experimental samples of patients with several selected intestinal diseases:

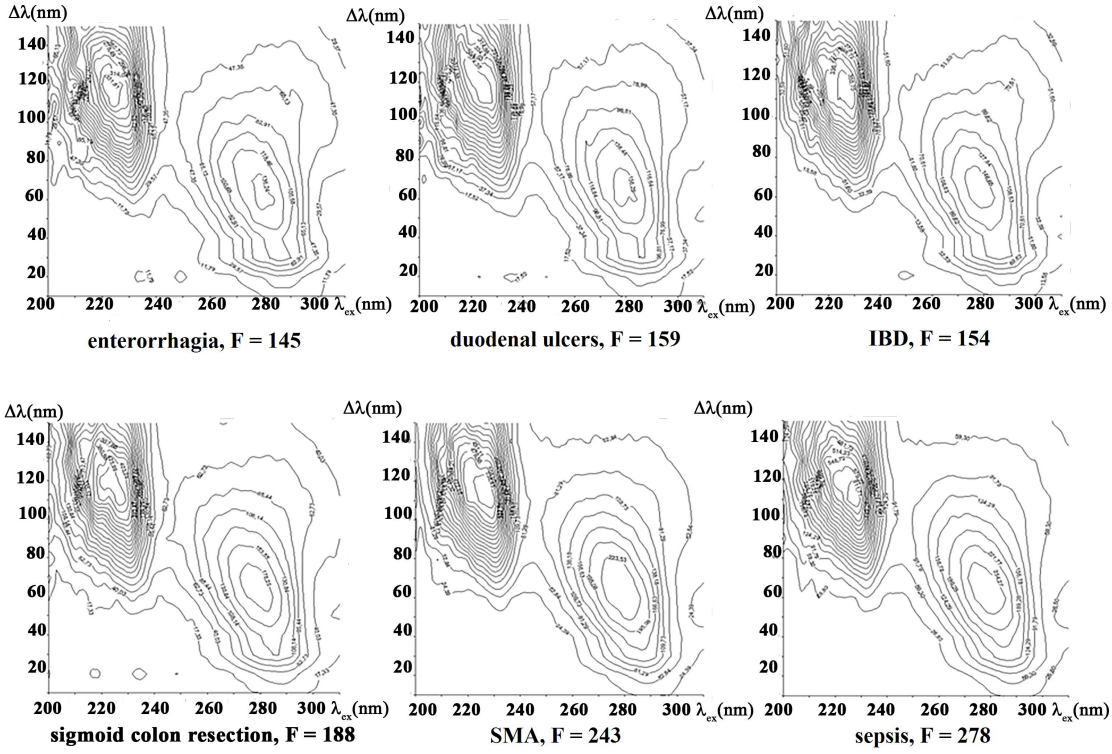

Figure 3: Synchronous fluorescence fingerprints in grey colours of contour lines show characteristic autofluorescence of blood serum of selected ischemic patients suffered enterorrhagia, inflammatory bowel disease (IBD), duodenal ulcers, sigmoid colon resection, superior mesenteric artery (SMA) thrombosis and sepsis.

mesenteric thrombosis (F = 243), inflammatory bowel disease (F = 126), duodenal ulcers (F = 159), sepsis (F = 278); enterorrhagia (F = 145), sigmoid colon resection (F =188), cancer of small intestine (F = 203).

Figures 1-4 show the results of patient blood serum samples compared to the control group samples (Figure 1) using SFF. Autofluorescence of blood serum studied at λ_{ex} = 280 nm showed a decrease in fluorescence intensity in long-term ischemic patients (Figure 2-4) in comparison to the blood serum of healthy subjects (Figure 1, F = 611). A horizontal cut off SFF at Δλ = 60 revealed simple synchronous spectrum with one main fluorescence center/peak at λ_{ex} = 280 nm as result of fluorescence intensity of all fluorophore (Figure 2). The most significant decrease of fluorescence intensity of blood serum at λ_{ex} = 280 nm in patients with intestinal ischemia (Figure 2) was observed during inflammatory bowel disease in comparison with (F = 611) blood serum of healthy subjects (Figure 1).

Statistical analysis of the average fluorescence intensity in blood serum of patients with several cases of intestine ischemia (Figure 5) revealed a significant decrease in fluorescence intensity compared to healthy subjects.

The blood serum of the experimental groups changed total intensity of fluorescence and graphical display as result of disruption of balance, mutual interactions of fluorophores, changed fluorophores in compared to blood serum of healthy subjects. A detailed analysis of the fluorescence curve is not essential for diagnosis because the qualitative and quantitative change of the individual components in the blood serum mixture in specific wavelength regions of the fluorescence spectrum provides a wealth of information.

3.2 Atomic force microscopy of the blood serum

In the recent years, the exploitation of atomic force microscopy (AFM) has been increasing due to significant interest and research in the field of biomedicine. AFM has the potential to be a helpful and effective tool in analysis of blood serum and might be useful in the early diagnosis of severe diseases such as heart or intestinal ischemia or thoracic aortic aneurysm [15]. AFM has the ability to display the components of blood serum with their mutual interactions and in their complexity, for example the

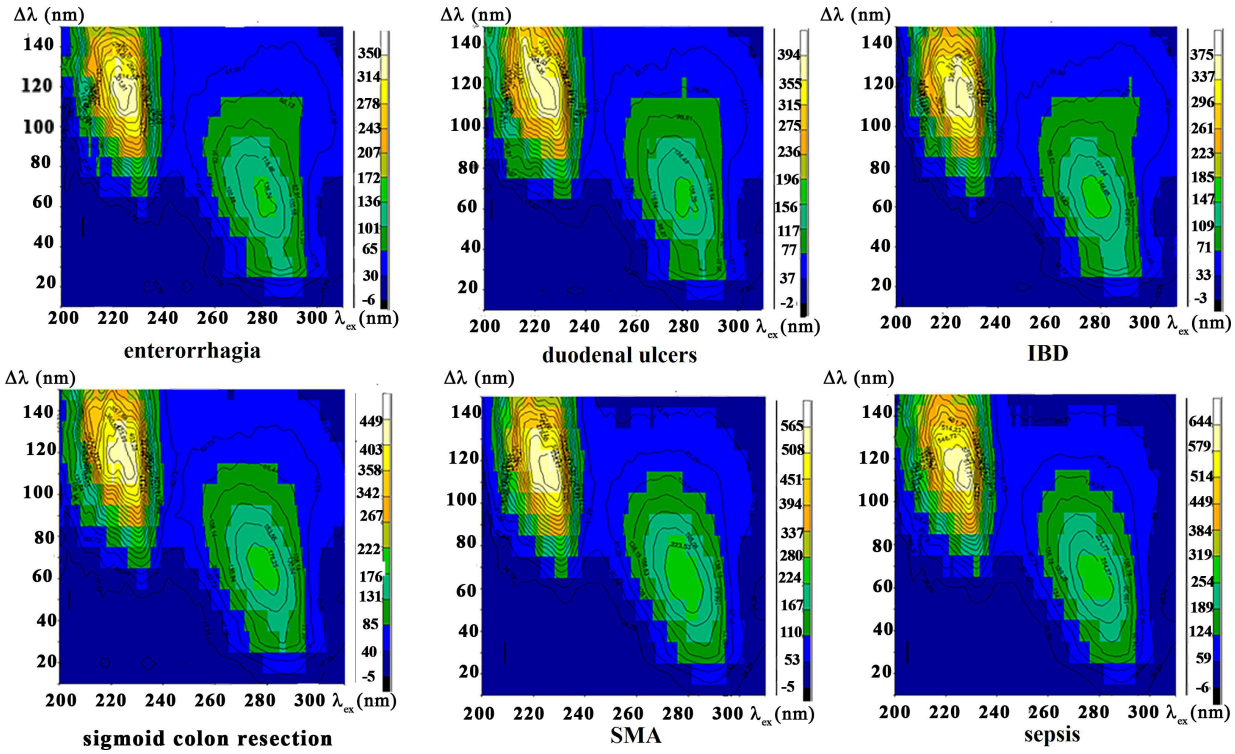

Figure 4: Synchronous fluorescence fingerprints in colours show more detailed intensity of autofluorescence of blood serum of selected ischemic patients suffering from enterorrhagia, inflammatory bowel disease (IBD), duodenal ulcers, sigmoid colon resection, superior mesenteric artery (SMA) thrombosis and sepsis.

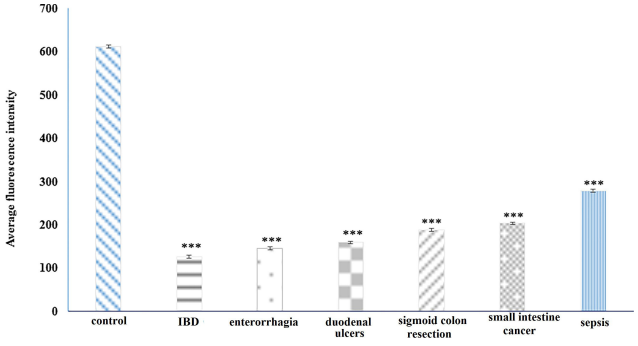

Figure 5: Statistical analysis of average fluorescence intensity in blood serum of healthy subjects and patients with selected diagnoses (*inflammatory bowel disease* (IBD)*, enterorrhagia, duodenal ulcers, sigmoid colon resection, small intestine cancer, sepsis*).

surface characteristics of blood serum during a thoracic aortic aneurysm have been indicated by a higher roughness of blood surface and notable well-defined globule particles [15]. Moreover, AFM also offers the detection of single proteins in multi-component films (Siedlecki, 2003), the drug interactions with the tissues and their delivery process of [16]. Additionally, by using AFM we are able to investigate changes to human red blood cells [17] and therefore it can be applied as an immediate assessment criteria for the applicability of stored red blood cells for transfusion [18], used as tool to identify patients with increased risk of cardiovascular diseases [19], improve our understanding of metastasis, or why average life span of the smoker's erythrocytes is significantly less than that of non-smoker's [20,21]. It is also possible to use AFM to detect microparticles (microvesicles) in blood plasma [22].

Using AFM we studied the surface of blood serum which displayed regular globules of proteins with unknown composition. The surface of the samples is measurable in small volumes, ~ 5 µl. The detailed differences of the graphically displayed analyzed blood serum surfaces of the healthy individuals samples (Figure 1) were examined and compared with graphically displayed blood serum surfaces of the patients with intestinal ischemia (Figure 6).

The results showed similar blood serum surface in the healthy individuals and characteristic pattern of globular units occurred for all with height 10 nm and width 200 – 250 nm formed by lipids, proteins, cholesterol and other organic compounds of blood serum. The blood serum surface of the patients with intestinal ischemia showed significant differences in comparison to the samples of the healthy individuals.

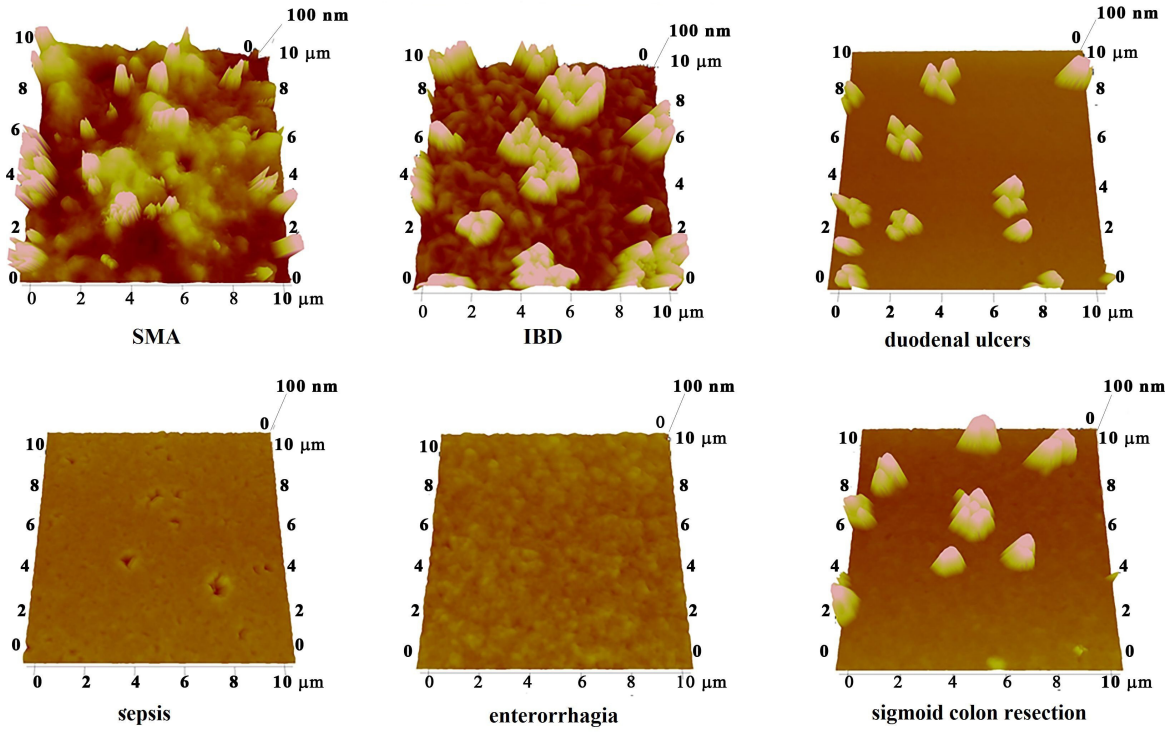

Figure 6: Surface of blood serum of selected ischemic patients suffering from superior mesenteric artery (SMA) thrombosis, inflammatory bowel disease (IBD), duodenal ulcers, sepsis, enterorrhagia, sigmoid colon resection.

The surfaces of particular samples of blood serum of ischemic patients in comparison with healthy individuals were identified using atomic force microscopy. There is not a significant collateral blood supply in the small intestine unlike in the heart which could be the reason why we observed poorer ischemic processes than in the ischemic extremities. The small intestine is the centre of nutrient absorption and diet influences its proper functions and therefore it is very sensitive to the presence of ischemia. A disruption of metabolism and nutrient absorption occurs due to the inhibition of enzymes which naturally metabolize disaccharides, triglycerides, proteins and cholesterol. The small intestine is also the place of chylomicrons formation which is also damaged during ischemia. In long-term ischemia, a significant amount of un-metabolized and undigested substances may get into the blood supply which is manifested as irregular big units – the globules with height 50 – 100 nm and width 1000 nm, presumably of lipoprotein composition, observed on the blood serum surface of severely ill patients. Irregularly placed small globular units of irregular shape 20 – 50 nm and in smaller amounts are also observed in patients with intestine ischemia (Figure 6).

These abundantly present units in blood serum could participate in atherosclerosis and ischemic disease. In the control, healthy subject, samples we observed the regular globular units with height of 10 nm and width of 250 – 500 nm. The regular structure of globules vanished and the sample became amorphous and smooth. In the control blood serum we observed the organized globule units with a height 10 nm and width 250 – 500 nm.

A detailed analysis of the components present in AFM images is not essential for the diagnosis because the information contains qualitative and quantitative change of the morphology of the blood serum with characteristic arrangement, shape, size, damage of individual particles in blood serum in comparison with blood serum of healthy subjects.

4 Conclusion

Synchronous fluorescence fingerprints of blood serum in a simplistic form define specific structure of blood serum mixture which is considered to be a characteristic “fingerprint”. Synchronous fluorescence fingerprints of healthy subject’s serum show significantly higher average autofluorescence intensity in comparison with patients with various intestinal diseases. Serum samples without changes in autofluorescence intensity might be a

good negative predictor of intestinal diseases. The future possible application of these novel methods (synchronous fluorescence fingerprint and atomic force microscopy) could offer a rapid screening for intestinal diseases and monitoring their progression which requires only a small sample of blood serum.

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