

NEUTROPHIL ACTIVATION AT HIGH-FAT HIGH-CHOLESTEROL AND HIGH-FRUCTOSE DIETS INDUCES LOW-GRADE INFLAMMATION IN MICE

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Nonalcoholic fatty liver disease (NAFLD), which can progress to nonalcoholic steatohepatitis (NASH), is a significant health concern affecting a substantial portion of the population. This study investigates the role of neutrophil extracellular traps (NETs) in liver inflammation induced by high-fat high-cholesterol diet (HFHCD) and high-fructose diet (HFD). The chronic nature of NAFLD involves low-grade inflammation with cytokine elevation. The research aims to visualize neutrophil elastase (NE) activity during HFHCD and HFD representing conditions of low-grade activation and assess neutrophil functional status. The study employs a mouse model subjecting animals to HFHCD, HFD or a standard diet (SD) for six weeks. Various analyses were used including histological evaluations, *in vivo* imaging of NE activity using a fluorescent probe, fluorescent microscopy, flow cytometry and assessment of neutrophil function through reactive oxygen species (ROS) levels. Mice on HFHCD and HFD display liver damage consistent with NASH, which was validated pathohistologically. NE activity in blood significantly increases after six weeks indicating systemic NETs involvement. *In vivo* imaging confirms NE activity in multiple organs. Cellular localization reveals NETs persistence even after neutrophil destruction in splenocytes indicating systemic involvement. Neutrophils under HFHCD exhibit a functional phenotype associated with low-grade inflammation, higher basal ROS levels and reduced activation potential. This study establishes the systemic impact of NETs in HFHCD- and HFD-induced liver inflammation, providing insights into the functional state of neutrophils. The findings contribute to understanding the mechanisms underlying chronic liver conditions and may inform future therapeutic strategies.

Key words: NASH, high fat diet, neutrophils, neutrophil extracellular traps, neutrophil elastase, *in vivo* imaging, low-grade inflammation.

Nonalcoholic fatty liver disease (NAFLD) affects an estimated 25% of the population and may progress to nonalcoholic steatohepatitis (NASH), now also referred to as metabolic dysfunction-associated steatohepatitis (MASH), a severe liver disease affecting 5% of the population [1, 2]. NAFLD is a chronic disease that features low-grade inflammation accompanied by increased cytokines and inflammatory mediators [2]. Accumulating recent data suggest neutrophil extracellular traps (NET) involvement in liver inflammation, reviewed in [3]. NETs were shown to initiate gallstone

formation [4], and upon excess amount of cholesterol, they serve as a source of pro-inflammatory cytokines, as neutrophils were shown to release NETs upon contact with microcrystals [5, 6] and specifically cholesterol microcrystals [7] at high-fat high-cholesterol diet (HFHCD). Besides, high-fructose diet (HFD) was shown [8] to induce abundant NETs formation in the hepatobiliary system. Its mode of action is probably related to the depletion of adenosine triphosphate levels in hepatocytes [9, 10] accompanied by increase in uric acid in the liver [11]. Cell death due to energy exhaustion and formation

of uric acid will stimulate formation of monosodium urate crystals, a known inducer of NETs formation [12]. Neutrophil elastase (NE), released from polymorphonuclear leukocytes (PMNs or neutrophils) on NETs, was shown to play a crucial role in degradation of extracellular matrix of organs (exemplified on lungs during COVID) [13] destroying elastin matrix and promoting replacement of elastin fibers with that of collagen. Here we hypothesize that the similar mechanism can take place in the liver during NET-stimulating diets, namely HFHCD and HFD. To check the current hypothesis, we aimed on direct visualization of NE activity during HFHCD and HFD as examples of conditions of low-grade activation, as well as on assessing the functional state of neutrophils during these conditions.

Materials and Methods

Animal and diet models. Studies involving animals – including housing and care, method of euthanasia and experimental protocols – were approved by the Ethical Committee of Danylo Halytsky Lviv National Medical University, protocol number 20201221/9, and all experiments were designed to comply with principles of the 3Rs (replacement, reduction and refinement). The study involved a NASH model in laboratory mice. C57BL6/N mice at eight weeks of age and weighing between 20–22 g were used for the experiment. Each group of animals, consisting of six individuals in R1 and five individuals in R2, received a specific diet. The investigated groups included a standard diet (SD), HFHCD (containing 1% cholesterol and 0.5% cholic acid, C1089, Altromin) and HFD (standard diet with a 10% fructose solution in water). The experiments were repeated twice, and the duration of each experiment was six weeks. Body weights of the mice were measured every three days, and blood samples were collected from the tail tip before the experiment and then every two weeks (Fig. 1, A). Serum was immediately frozen. To study neutrophils in air pouches for reactive oxygen species (ROS) levels, a separate experiment was performed where air pouches were induced on the back of the mice by sterile air injection as described [5], 1 mg of UV-sterilized cholesterol crystals was induced into air pouches at day five. To study NE localization in vivo, a separate experiment with six mice at HFHCD and six controls was performed. At the end of the study, animals were euthanized using tribromoethanol injection and cervical dislocation. Organ dissection,

including liver and gallbladder, was performed, and microphotography of the organs was conducted on calibrated stages allowing quantitative assessment of organ sizes. For the microphotography, a Nikon D7100 camera and a Micro-NIKKOR macro lens from Nikon, Japan, were utilized. Air pouch lavage was isolated by injecting 5 ml of sterile phosphate buffered saline (PBS) into air pouch and extracting suspension. Cell treatment was done in sterile conditions. For induction of ROS response, extracted air pouch lavage with predominating PMN cells were incubated with sterile suspension of 200 µg/ml of 10 nm nanodiamonds covered with peptide (S-protein RBD, aa. 1–194) and incubate for two hours at 37°C and 5% CO₂.

Histological analysis including tissue fixation, histological preparations and hematoxylin eosin staining (H&E) were made according to standard established procedures [14]. Morphometric evaluation of hepatocyte area and lipid content was performed using ImageJ software [15, 16]. For this reason, the high-resolution images of 5 µm-thick liver tissue sections (made with objective 40x, 0.95 NA air or 60x 0.9NA water) were subjected to “colour deconvolution” command using vector R:0.09283; G:0.95454; B:0.28324. In the resulting image, the contours of individual hepatocytes were manually identified by the operator, and the area of individual cells was measured, and intensity was evaluated. For each sample, at least three fields of view were captured and analyzed with a measurement of a minimum of 100 cells for each field. At least three references: slides representing completely normal cells without visible lipid inclusions, slides with lipid inclusions occupying maximal percentage of cytoplasm and the same where nuclei damage was additionally observed were analyzed to get reference lipid values for each condition, which are indicated on the images. Histological assessment of liver damage utilized criteria described in [17].

Fluorescent staining. Splenocytes were extracted from spleen by washing with 1 ml of PBS per organ, then immediately placed on glass, air dried and fixed with a drop of cold methanol. SYTOX Green (1:1000) solution (Helix NP Green, BioLegend, USA) and a-mouse-CD11b-PE labeled (BioLegend) (1:100) were added to splenocyte smears and incubated for 30 min, then washed with water and embedded in mounting medium.

Fluorescent microscopy. Fluorescent microscopy was performed with a Keyence BZ-X800 Fluorescence Microscope (Keyence, Japan) equipped with

custom Omega Filters filter set to image IVIS NE probe (Omega Filters, Brattleboro, USA) and typical filters for fluorescein isothiocyanate (used for SYTOX Green detection) and tetramethylrhodamine (used for propidium iodide detection). Both 40x 0.95NA and 100x 1.45NA oil immersion objectives were used for imaging. Native Keyence software was used for image processing and x, y, z stitching. Confocal imaging was achieved by utilizing optical sectioning module with grating set to 10, linear mode and z-pitch of 200 nm. ImageJ (National Institutes of Health, Bethesda, MD, USA) software was used for image analysis and quantifications. All image analysis was performed under a fixed setting of parameters including exposure and compensation.

Fluorometric assay for NE activity was done using frozen serum samples. The sample was incubated with the fluorogenic substrate MeOSUc-AAPV-AMC (Santa Cruz Biotechnology) in 96-well black microplates. Calibration was performed using human leukocyte elastase (Sigma), and analysis was conducted on a Perkin Elmer HTS 7000 Bio Assay Reader following the protocol detailed by [18]. In vivo imaging was performed at day 35 after diet induction under sevofluran-induced anesthesia using LI-COR Pearl Trilogy in vivo imager. Imaging was done at one hour and four hours postinjection of NE probe. Excitation was done with 685 nm laser, and fluorescence was analyzed at 720 nm. ROS detection was done by incubating cells population ($2 \cdot 10^6$ cell/ml) with 50 nM 2',7'-dichlorofluorescein diacetate (DCFDA, also known as H2DCFDA, DCFH-DA and DCFH from Sigma) for 20 min at 37°C and then analyzing mean fluorescent intensity (MFI) at FL1 channel using Beckman Coulter DxFlex flow cytometer. As positive control for ROS generation the viral-peptide covered nanodiamonds, shown by us to induce strong oxidative burst in PMNs population leading to systemic response, were added to the cells at 200 µg/ml [19]. The gating of PMN population was done using forward scatter and side scatter parameters. Data analysis was performed using CytExpert 3.0 software.

Statistical analysis. For comparisons of fluorescent signals of cells in the studied groups, the Mann–Whitney U test for numerical variables was employed. For comparisons of multiple groups vs NHD or the untreated group, Dunn's multiple comparisons test was used. Colocalization analysis was done with ImageJ (NIH) software with at least 20 high-resolution cell images analyzed, and Pearson's

correlation coefficient (r) was calculated using the embedded algorithm. All analyses were performed using Excel 2016 (Microsoft, Redmond, USA) and Prism 7.0 (GraphPad, San Diego, USA) software. A P -value of ≤ 0.05 was considered statistically significant.

Results

Assessment of liver damage at NET-related diets. The morphological assessment of the digestive system organs, analyzed after 42 days of either HFHCD or HFD, revealed visible discoloration of the livers (Fig. 1, A) and increased gallbladders' size (not shown) in animals under experimental diets compared to SD. HFHCD induced pronounced color changes in livers, while HFD led to a less pronounced but noticeable discoloration of liver tissues. Histological analysis of liver samples was conducted, involving cross-sections of the left lateral lobe for comparison (Fig. 1, B). In the SD group, high metabolic activity was observed, evidenced by numerous mitoses and the presence of binucleated (tetraploid) cells with eosinophilic cytoplasm. The bile duct epithelium was well-preserved, and liver vascularization was clearly defined. Conversely, HFHCD and HFD showed visible macrosteatosis and microsteatosis with most hepatocyte nuclei being pyknotic indicating irreversible necrotic processes. Mitoses or binucleated cells were absent, and hepatocyte cytoplasm was filled with non-stained lipid inclusions. Large fat droplets (macrosteatosis) were observed between cells, and the bile duct epithelium was occasionally disrupted. Visible hypertrophy of hepatocytes was observed in animals subjected to HFHCD, while lipid accumulation was most prominent at HFD. A quantitative assessment of hypertrophy and lipid accumulation was conducted by evaluating the hepatocyte area in digital microphotographs from different liver sections of each experimental group (Fig. 1, C). ImageJ software was utilized for quantitative analysis, revealing an almost twofold increase in hepatocyte area on histological sections. A statistically significant difference ($P < 0.0001$) was observed between the SD and HFHCD groups. Histological analysis of liver tissues on HFHCD and HFD confirmed morphological changes characteristic of NASH, including hepatocyte hypertrophy ($P < 0.01$), lipid accumulation ($P < 0.01$), pyknotic nuclei, reduced diploid cell count, hepatocyte necrosis and extracellular lipid accumulation in the form of lipid droplets. Leukocyte infiltration in the liver triad

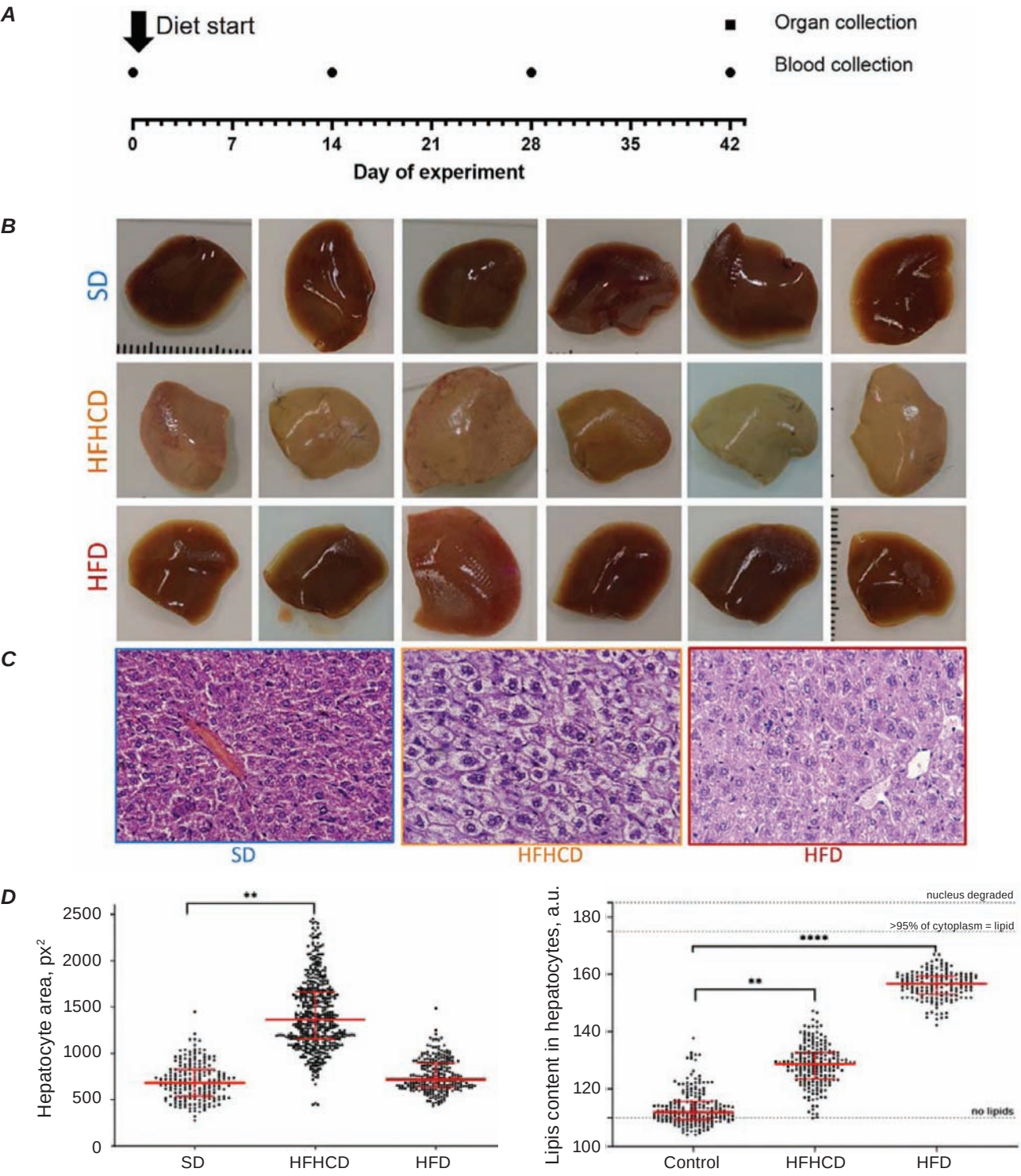


Fig. 1. Both HFHCD and HFD cause morphological symptoms of NASH after six weeks. **A** – time scheme of experiment, indicated time of blood and tissue collection. **B** – macrophotographs of livers after six weeks on the indicated diet. **C** – representative histological image of livers as in **B**, staining with H&E, objective 40x 0.95NA, z-stack. **D** – morphometric analysis of area and lipid content of cells in **C**

area was also noted. Thus, the used model diets were pathohistologically validated to cause NASH.

NETs formation at NASH. Since NETs are quickly cleared in normal conditions, their morphologically assessment is complicated, thus, we utilized NE activity in blood as a marker for NETs formation. To understand the extent of the impact of HFHCD and HFD on the formation of NETs, specifically whether NET formation is locally confined to the hepatobiliary system or affects the entire organism, we measured the activity of neutrophil elastase in mice blood serum. The measurements were conducted at four and six weeks (28 and 42 days). A specific fluorogenic substrate for NE enzyme allowed for the detection of small amounts with high specificity, and the analysis was performed using a fluorometric analyzer. A significant increase in NE activity was observed under both HFHCD ($P = 0.0148$, ANOVA) and HFD ($P = 0.0219$) conditions at six weeks into the experiment (Fig. 2). However, no such changes were detected at four weeks, and the difference between four and six weeks was statistically significant ($P < 0.001$). The blood of mice under SD and at day 0 was used as control for basal level of NE. Increase of NE activity at very late stage (six weeks) can be explained by high abundance of its natural inhibitors – serpins and α -1 antitrypsin – in the blood, thus, the activity is detected only when its levels cannot be suppressed

by natural inhibitors [20, 21]. Therefore, the NET-associated enzymes released at HFHCD and HFD are causing not a local, but systemic effect being detected in blood of peripheral circulation.

In vivo localization of NE activity. Recent progress in infrared (IR) imaging and specific enzymatic sensors allows a real-time monitoring of enzymatic activity in the body for specific enzymes. Here, we utilized a Perkin Elmer Neutrophil Elastase 680 FAST NIR fluorescent probe for *in vivo* visualization of neutrophil elastase activity in animals subjected to HFHCD and HFD. This probe utilizes two fluorophores with near-infrared fluorescence (NIR) connected by a short linking peptide. Neutrophil elastase cleaves this peptide, restoring the fluorescence of the fluorophores. Fluorophores are self-quenching while they are interconnected via peptide and start fluoresces upon peptide cleavage.

To measure fluorescence, an IR laser at 685 nm was used in the LI-COR Pearl Trilogy fluorescence analyzer enabling the detection of fluorescence after intravenous administration of the compound. The dynamic activation of the compound was monitored revealing a peak activity at four hours postinjection (Fig. 3) and also confirming the enzymatic activity as substrate accumulation was seen with time. Following intravenous injection of the compound, animals were withdrawn from the experiment, and fluorescence levels were analyzed in their bodies.

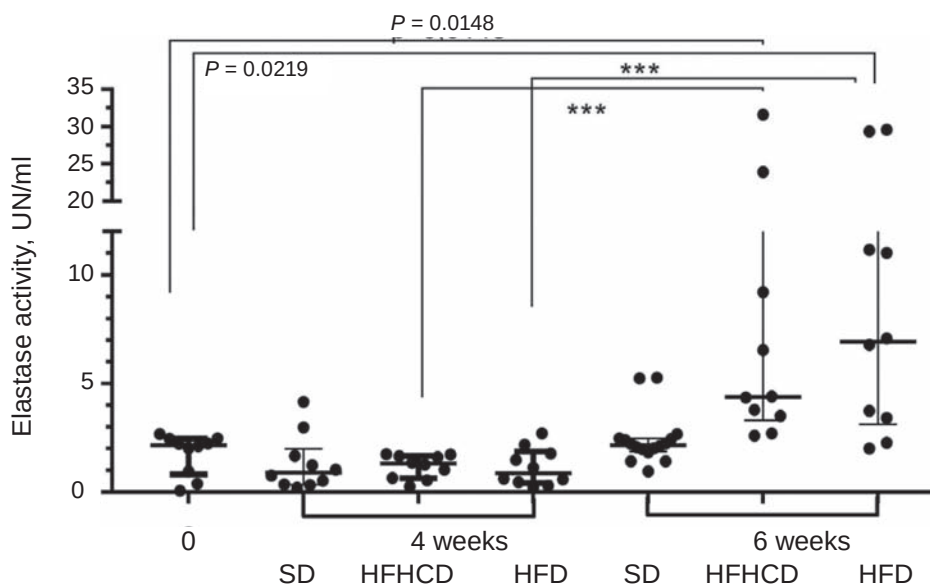


Fig. 2. Neutrophil elastase activity in serum of mice under studied diets. A significant increase in NE activity was observed at six weeks for HFHCD and HFD. Aggregated data of two independent experiments, each dot representing individual animals

Subsequent analysis of individual organs revealed increased fluorescence in the liver and spleen under HFD and liver, spleen and heart under HFHCD (Fig. 3, B). Thus, NE activity has a systemic increase under HFHCD and HFD affecting multiple organs.

Cellular localization of NE signal. For cellular localization on NE probe, we isolated splenocytes from spleen in the experiment shown at Fig. 3, B. We used Keyence BZ-X800 microscope equipped with custom NIR filters to track fluorescent probes.

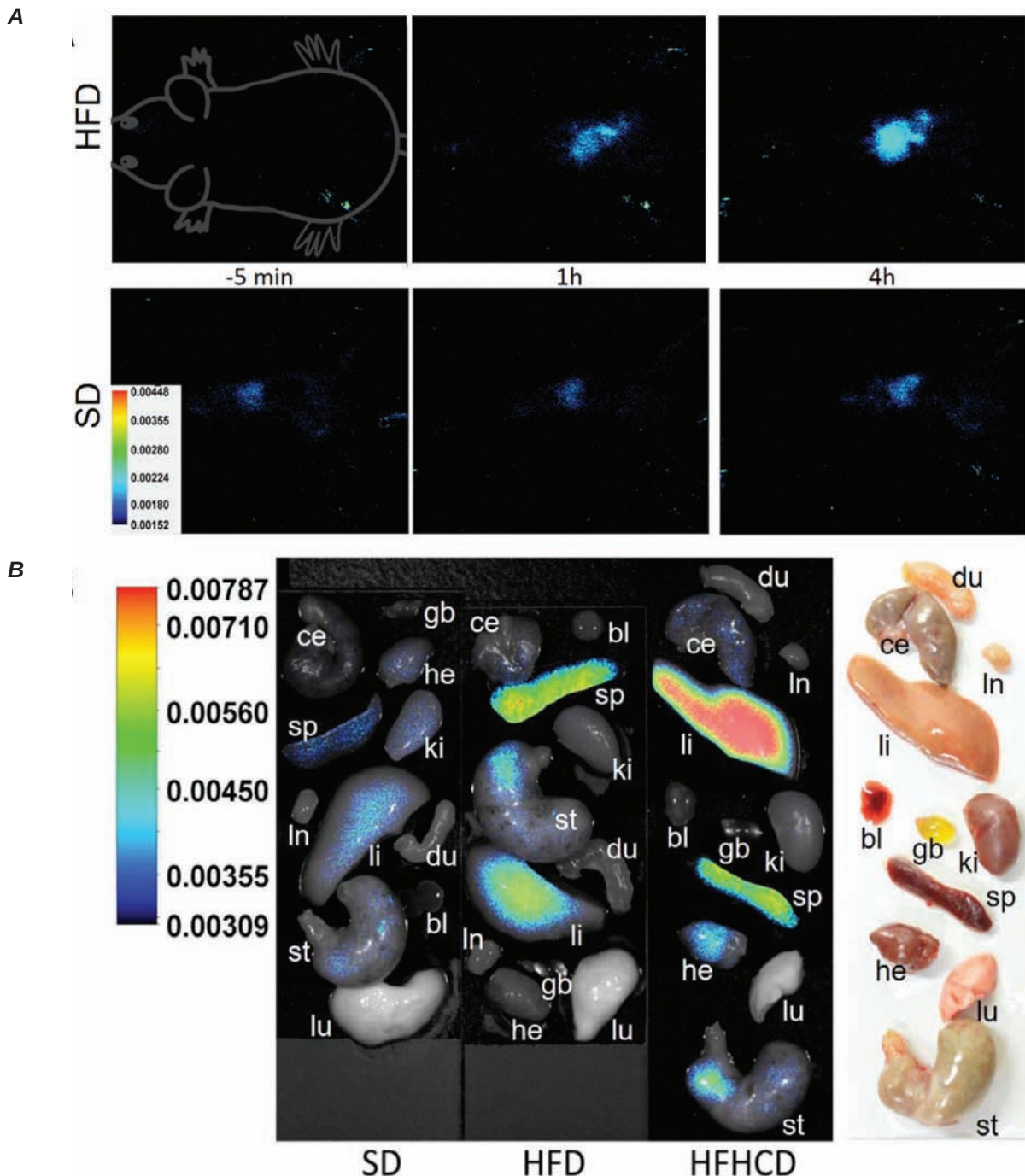


Fig. 3. Neutrophil elastase activity in the body (A) and organs (B) of mice under SD, HFHCD and HFD. A. Visualization was done using IVISence Neutrophil Elastase 680 FAST fluorescent probe at one hour and four hours postinjection. B. After four hours, organs of mice were imaged to demonstrate systemic NE activation in liver, spleen and heart under both HFHCD and HFD. sp – spleen, lu – lung, he – heart, ln – inguinal lymph node, st – stomach, du – duodenum, ce – cecum, ki – kidney, gb – gall bladder, li – liver, bl – blood

Microscopic analysis of spleen cells, utilizing additional staining for neutrophil markers (CD11b) and extracellular DNA (SYTOX Green), confirmed the presence of numerous NETs associated with the activated probe. These NETs persisted even after neutrophil destruction during NET formation (Fig. 4). The activation of neutrophil elastase in the liver further supports the conclusion of a systemic low-grade inflammatory response induced by HFCHD and HFD.

Assessing functional state of neutrophils at low-grade inflammation caused by HFHCD. To check ROS levels at HFCHD diet, we used mice at 35 days on HFHCD or SD and induced air pouches in them. At day 40 (fifth day of air pouch formation), we injected 1 mg of cholesterol crystals into air pouch to stimulate neutrophil infiltration. Next day, we isolated air pouch lavage, pre-incubated cells with ROS sensor DCFDA and incubated half of the

sample with strong activator — peptide covered nanodiamonds [19]. Two hours later, ROS evaluation revealed that at HFHCD, ROS levels were significantly ($P < 0.05$) higher than at SD. At the same response to nanodiamonds serving as a sterile particulate stimulant, ROS was lower at HFHCD compared to SD ($P < 0.01$).

Thus, neutrophils at HFHCD possess a functional phenotype attributable to low-grade inflammatory conditions, particularly autoimmune disorders, namely higher basal ROS level with decreased activation potential.

Discussion

The study's findings shed light on the intricate relationship between dietary patterns, liver pathology and the involvement of NETs in the progression of NAFLD to NASH. The observed histological

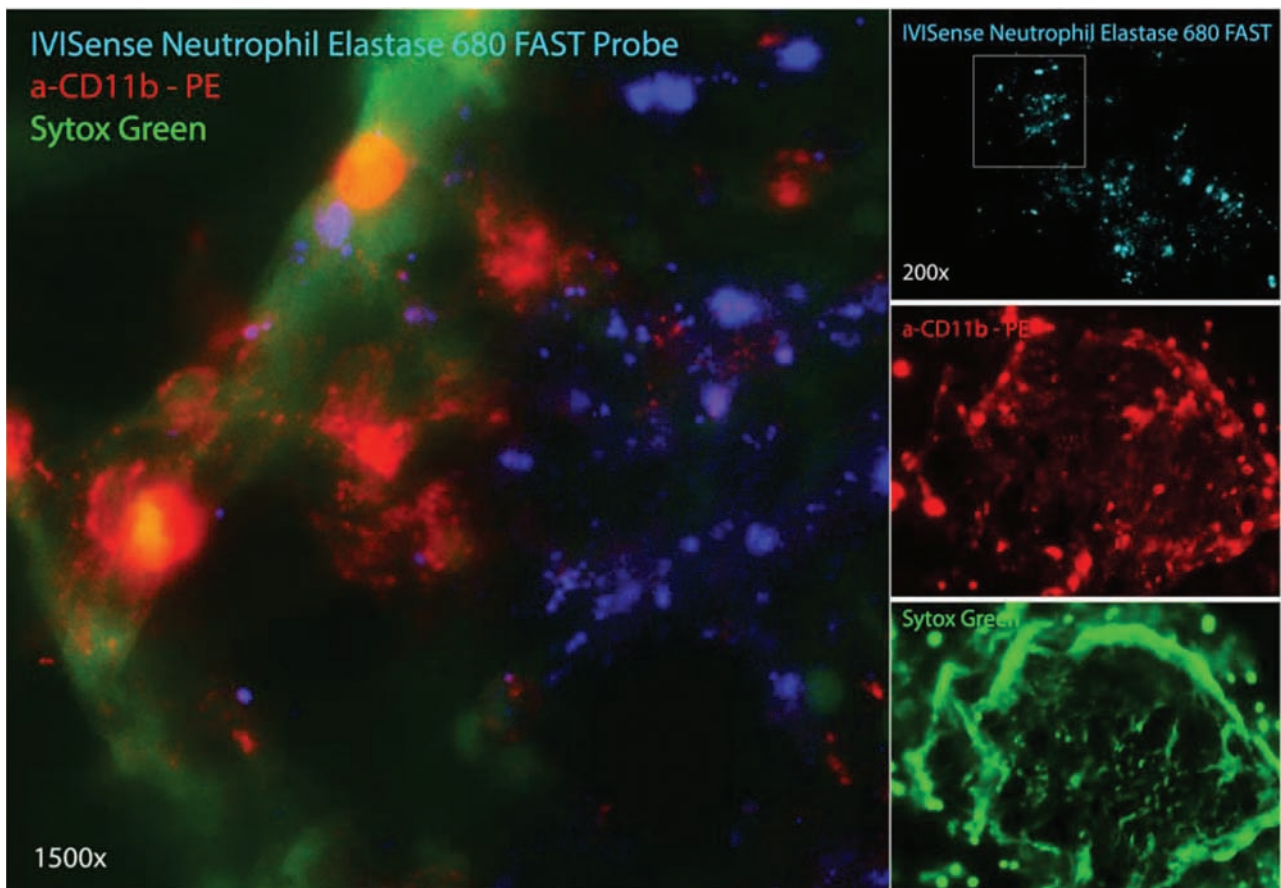


Fig. 4. Microscopic analysis of splenocyte smears from Fig. 3(B), counterstained to visualized PMN cells with a-CD11b and externalized DNA with SYTOX Green. Neutrophil elastase is colocalized with CD11b-positive granules and externalized DNA, suggesting proof of NETs formation in studied splenocytes. IVISence Neutrophil Elastase 680 FAST fluorescent probe was imaged using custom IR filter from Omega Filters and pseudo-colored cyan. The center image is the merged enlargement of the area shown at top left for individual channels. The purple color results from the signal overlaps from a-CD11b with IVISence NE FAST probe

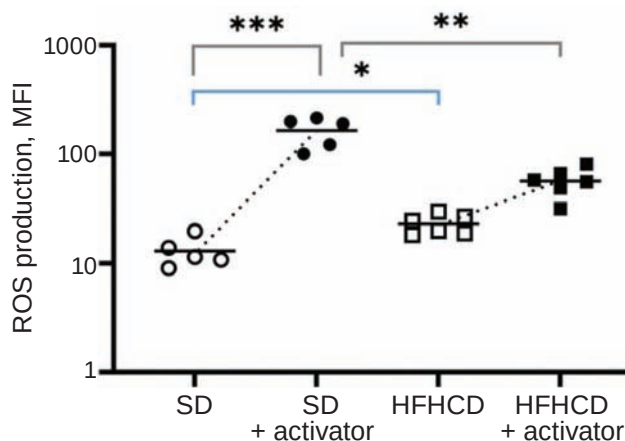


Fig. 5. ROS production in neutrophils under SD and HFHCD. PMN-reach air pouch lavage was incubated with ROS sensor DCFDA and measure for ROS production in basal state and upon the contact of 200 $\mu\text{g/ml}$ of sterile peptide-coated nanodiamonds, as positive activator control. ROS levels were analyzed as MFI of corresponding cell population

changes in the liver, such as macrosteatosis and microsteatosis, validate the selected mouse model as representative of NASH. The pronounced increase in NE activity in the blood after six weeks under HFHCD and HFD points toward a systemic impact of NETs in liver inflammation.

The *in vivo* imaging of NE activity further strengthens the notion that NETs are not confined locally but affect multiple organs. The persistence of NETs, even after neutrophil destruction, highlights the complexity of the inflammatory response induced by these diets. The study's innovative use of fluorescence imaging techniques contributes to a real-time understanding of enzymatic activity in the liver and spleen. These findings also demonstrate systemic involvement of the inflammatory process involving neutrophils.

Taking into account an abundance of neutrophils in the tissues and their tendency to migrate (the concept of patrolling neutrophils), [22] the observed neutrophils and NETs seen in spleen are probably the cells that were in contact with areas of inflammation in the liver and returned into hematopoietic organs. Such activated neutrophils are known to live long, exhibiting many regulatory actions including conversion to myeloid-derived suppressor cells of PMN type [23]. It is widely accepted that neutrophil activation with ROS-dependent oxidative

burst is needed for resolution of inflammation [24], while insufficient neutrophil priming will result in chronification of inflammation with one of the consequence being autoimmune disorders, for example, low-grade inflammation. Thus, autoimmune conditions like arthritis [25], multiple sclerosis [26] and a set of others are often associated with ROS levels in the inactive PMN cells being higher than that in a healthy population constantly damaging tissues, but also exhausting ROS machinery and decreasing the ability to produce oxidative burst upon neutrophil priming needed to resolve inflammatory conditions [27]. Specific approaches were proposed to correct this aberrant ROS production [28]. The observed increase in ROS levels in neutrophils under HFHCD indicates a functional phenotype associated with low-grade inflammation and constant sub-activating production of ROS leading to chronic inflammation resembling conditions found in autoimmune disorders. While at the time of neutrophil priming with sterile nanodiamonds particles, their oxidative burst is lower at HFHCD compared to SD due to the exhaustion of ROS-producing machinery during the course of chronic inflammation observed at HFHCD. The observed increased of NE activity at HFHCD supports current hypothesis, while the relatively late (six weeks) appearance of detectable NE activity in serum assays can be explained by the presence of natural inhibitors of NE preventing its detection [20]. *In vivo* IR imaging of NE activity in tissues partially solves this problem.

Conclusion. This study provides compelling evidence for the significant impact of HFHCD and HFD on liver pathology, demonstrating their role in inducing NASH in a mouse model. The systemic activation of NETs and the observed increase in NE activity underscore the intricate relationship between diet, inflammation and organ-wide effects. Furthermore, the findings suggest a potential link between dietary-induced low-grade inflammation and a functional phenotype in neutrophils resembling conditions seen in autoimmune disorders. Overall, this research contributes valuable knowledge to the field and may guide the development of targeted interventions for liver diseases associated with dietary factors.

Ethical committee approval. Ethics Committee of Danylo Halytsky Lviv National Medical University, Protocol 20201221/9.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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АКТИВАЦІЯ НЕЙТРОФІЛІВ ЗА ДІЄТИ З ВИСОКИМ ВМІСТОМ ХОЛЕСТЕРОЛУ ТА ФРУКТОЗИ СПРИЧИНЯЄ ХРОНІЧНУ ЗАПАЛЬНУ ВІДПОВІДЬ У МИШЕЙ

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Неалкогольна жирова хвороба печінки, що переходить в неалкогольний стеатогепатит (НАСГ) вражає чималий відсоток населення. Це дослідження вивчає роль нейтрофільних позаклітинних пасток (НПП) у запаленні печінки, спричиненому дієтою з високим вмістом жирів і холестеролу (HFHCD) та дієтою з високим вмістом фруктози (HFD). Метою дослідження була візуалізація активності нейтрофільної еластази (NE) під час HFHCD та HFD, які створюють передумови хронічного субгострого запалення, та оцінка функціонального стану нейтрофілів. У дослідженні використовували лабораторних мишей, які перебували на HFHCD, HFD або на стандартній дієті протягом шести тижнів. Зроблено гістологічну

оцінку печінки за допомогою флуоресцентного зонда, визначено активність NE, як *in vivo*, так і в препаратах лімфоїдних органів. За допомогою флуоресцентної мікроскопії оцінювали функціональний стан нейтрофілів, а саме їхню здатність до формування оксидативного вибуху. Миші, які перебували на HFHCD та HFD, демонстрували ураження печінки, які узгоджуються з НАСГ, що було підтверджено патогістологічно. Активність NE в крові значно збільшувалась після 6 тижнів перебування на зміненому раціоні, що свідчить про системну участь НПП. Флуоресцентна візуалізація *in vivo* підтвердила активність NE в декількох органах. Мікроскопічний аналіз виявив наявність НПП навіть після руйнування нейтрофілів у селезінці, що свідчить про системну участь НПП у модуляції запальних процесів. Нейтрофіли у разі HFHCD проявляли функціональний фенотип, пов'язаний з хронічним субгострим запаленням, із вищими базальними рівнями реактивних сполук кисню та зменшеним потенціалом до активації. Це дослідження встановлює системний вплив НПП на запалення печінки, спричинене HFHCD та HFD, надаючи уявлення про функціональний стан нейтрофілів. Висновки сприяють розумінню механізмів хронічного субгострого запалення та можуть бути використані для створення стратегій терапії цих захворювань.

Ключові слова: НАСГ, дієта з високим вмістом жирів, нейтрофіли, нейтрофільні позаклітинні пастки, нейтрофільна еластаза, хронічне субгостре запалення.

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