UNIVERSITY OF CRETE

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Master's Thesis

Haemopoiesis Research Laboratory, University of Crete Medical School

"INVESTIGATION OF NEUTROPHILS' ACTIVATION IN A COHORT OF PATIENTS WITH CHRONIC IDIOPATHIC NEUTROPENIA (CIN) THAT CARRY SPECIFIC *MEFV* GENE VARIANTS"

"ΔΙΕΡΕΥΝΗΣΗ ΤΗΣ ΕΝΕΡΓΟΠΟΙΗΣΗΣ ΤΩΝ ΟΥΔΕΤΕΡΟΦΙΛΩΝ ΣΕ ΜΙΑ ΚΟΟΡΤΗ ΑΣΘΕΝΩΝ ΜΕ ΧΡΟΝΙΑ ΙΔΙΟΠΑΘΗ ΟΥΔΕΤΕΡΟΠΕΝΙΑ ΠΟΥ ΦΕΡΟΥΝ ΔΙΑΦΟΡΕΤΙΚΕΣ ΓΕΝΕΤΙΚΕΣ ΠΑΡΑΛΛΑΓΕΣ ΣΤΟ *MEFV* ΓΟΝΙΔΙΟ"

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ABSTRACT

MEFV gene mutations are associated with Familial Mediterranean Fever (FMF), an autoinflammatory disease whose pathogenesis is based on neutrophils' activation with entry in autophagy and extrusion of Neutrophil Extracellular Traps (NETs) decorated with the cytokine IL-1b, creating a vicious cycle of excessive inflammation. Such mutations were recently found in increased frequency in a cohort of patients with Chronic Idiopathic Neutropenia (CIN). Here, we investigated an association between *MEFV* mutations in CIN patients and neutrophils' activation evident with increased NETosis, as in the case of FMF. The experimental procedure we followed was to co-culture healthy PMNs with serum from patients and either use the supernatants of the cultures to quantify NETs with sytox green staining for extracellular DNA and dsDNA/MPO complexes ELISA or to use the treated cells for NETs immunostaining using confocal microscopy. In addition, PMNs directly isolated from patients were used to detect their direct response as regards to spontaneous NETosis induction. After comparison of CIN patients with likely pathogenic *MEFV* mutations, CIN patients negative for *MEFV* mutations and healthy individuals we concluded that NETosis isn't significantly increased in patients with *MEFV* mutations. Ex vivo culture of patients PMNs and microscopic observation gave us an indication that CIN patients, regardless of their *MEFV* variations, present an increase in NETosis as compared to healthy individuals. However, NETosis is only one process that indicates neutrophils' activation. So, based on FMF's molecular mechanism, neutrophils should also be studied for autophagy and production of inflammatory cytokines. Then, their activation status will be clear and we could conclude whether *MEFV* mutations affect CIN patients' phenotype and if not, whether there is a counterbalancing effect of CIN and FMF' mechanisms co-existence. This information will not only characterize the particular cohort of patients and potentially change the categorization criteria and follow up approaches, but will also shed light to CIN's pathogenesis mechanism which is not clearly defined yet.

ΠΕΡΙΛΗΨΗ

Οι μεταλλάξεις του *MEFV* γονιδίου σχετίζονται με τον Οικογενή Μεσογειακό Πυρετό (Familial Mediterranean Fever/ FMF), μια αυτοφλεγμονώδη ασθένεια της οποίας η παθογένεια βασίζεται στην μη ελεγχόμενη ενεργοποίηση των ουδετεροφίλων. Αυτό οδηγεί σε ενεργοποίηση αυτοφαγικών μονοπατιών και εξωκυττάρωση παγίδων χρωματίνης από τα ουδετερόφιλα (Neutrophil Extracellular Traps/NETs) οι οποίες φέρουν προφλεγμονώδη ιντερλευκίνη 1β (IL-1β) και τελικά δημιουργείται ένας φαύλος κύκλος φλεγμονής. Τέτοιες μεταλλάξεις πρόσφατα βρέθηκαν σε υψηλή συχνότητα σε ασθενείς με Χρόνια Ιδιοπαθή Ουδετεροπενία (Chronic Idiopathic Neutropenia /CIN). Σε αυτή τη μελέτη, διερευνήσαμε την συσχέτιση των *MEFV* μεταλλάξεων στους ασθενείς με CIN, με την ενεργοποίηση των ουδετεροφίλων τους και την αυξημένη παραγωγή NETs, όπως συμβαίνει στον FMF. Η πειραματική διαδικασία που ακολουθήσαμε ήταν να συγκαλλιεργήσουμε υγιή ουδετερόφιλα με ορούς ασθενών και είτε να χρησιμοποιήσουμε τα υπερκείμενα των καλλιεργειών για να ποσοτικοποιήσουμε τα NETs με sytox green χρώση για το εξωκυττάριο DNA και DNA/MPO ELISA είτε να χρησιμοποιήσουμε τα κύτταρα για ανοσοιστοχημεία και συνεστιακή μικροσκοπία. Επιπλέον, για να ερευνήσουμε την αυθόρμητη παραγωγή NETs απομονώσαμε ουδετερόφιλα απευθείας από ασθενείς. Μετά από σύγκριση CIN ασθενών με μεταλλάξεις, CIN ασθενών αρνητικών για μεταλλάξεις και υγειών ατόμων καταλήξαμε ότι η παραγωγή NETs δεν αυξάνεται σημαντικά σε ασθενείς με μεταλλάξεις. Οι καλλιέργειες των κυττάρων των ασθενών *ex vivo*, μας έδωσε μια ένδειξη ότι στα κύτταρα των CIN ασθενών επάγεται μεγαλύτερη παραγωγή NETs, ανεξάρτητα από τις μεταλλάξεις στο γονίδιο *MEFV*. Βασιζόμενοι στον μοριακό μηχανισμό του FMF, εκτός από την παραγωγή NETs, τα ουδετερόφιλα των ασθενών αυτών θα πρέπει να διερευνηθούν για την αυτοφαγία και την παραγωγή κυτταροκινών. Αφού μελετηθούν και αυτοί οι παράγοντες, θα μπορέσουμε να καταλήξουμε σε ένα συμπέρασμα για το επίπεδο ενεργοποίησης των ουδετερόφιλων στους προς μελέτη ασθενείς, το κατά πόσο οι μεταλλάξεις επηρεάζουν τον κυτταρικό φαινότυπο και εάν οι μηχανισμοί της ουδετεροπενίας αλληλεπιδρούν/εξουδετερώνουν τους μηχανισμούς του Μεσογειακού Πυρετού. Αυτές οι απαντήσεις όχι μόνο θα οδηγήσουν σε πιο προηγμένη κατηγοριοποίηση και παρακολούθηση των ασθενών με ουδετεροπενία, αλλά και σε διαλεύκανση του παθογενετικού μηχανισμού της Χρόνιας Ιδιοπαθούς Ουδετεροπενίας, ένα πεδίο προς μελέτη.

1. INTRODUCTION

1.1 NEUTROPHIL

Neutrophils are the most abundant leukocytes in the blood as they make up the 50-70% of them and they constitute the biggest part of granulocytes or polymorphonuclear cells (PMNs), also consisting of basophils and eosinophils. Neutrophils are the first cells of the innate immune system to migrate and induce inflammation in case of disease or trauma. They sequester in the spleen and the pulmonary vascular bed (neutrophil pool) and they can migrate trans endothelially to sites of infection or tissues either following the transcellular road through endothelial cells or the paracellular road between the endothelial cells. Moreover, similar to M1 and M2 macrophages, neutrophils are grouped to two subpopulations: the N1 (CD49d+ , CD11b-) with pro-inflammatory properties and the N2(CD49d- , CD11b+) with anti-inflammatory, with their polarization to depend on environment signals (Tsuda et al., 2004). They have short life span and the production of cytokines and growth factors such as G-CSF and CXCR1, CCL2 and CXCL10 are responsible for the regulation of their maturation (Borregaard, 2010). The tightly regulated process of neutrophils' maturation or granulopoiesis is described below.

1.1.1 GRANULOPOIESIS: NEUTROPHILS' MATURATION

Hemopoietic Stem Cells (HSCs) are the first common precursor cells that give rise to all blood cells. They are capable of proliferation, self-renewal and differentiation and are controlled by the Bone Marrow microenvironment or HSC niche. The multipotent long-term HSC (LT-HSC) is a predominantly quiescent cell which is occasionally divided to self-renew and give rise to multipotent short-term HSCs (ST-HSCs), whose capacity to self-renew is limited. The ST-HSCs give rise to the non-self-renewing, multipotent progenitor (MPP) that develops further into either a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP), both of which are oligopotent progenitor cells. The pathway of granulopoiesis begins with the differentiation of CMPs to granulocyte/macrophage progenitors (GMPs), which express the CD34 antigen on their surface. In early stages, granulocyte/monocyte-colony-forming units (CFU-GM) give rise to granulocyte-colony-forming units (CFU-G). In later stages, Myeloblasts (MBs) are formed which are the first cells of granulocytic lineage expressing CD34 and CD33 surface antigens. Next to the maturation sequence are the Promyelocytes (PMs; CD34-CD33+) and Myelocytes (MCs; CD34-CD33+), which give rise to Metamyelocytes (MMs), cells with kidney-shaped nucleus, Band Cells (BCs; CD33-CD15+) with a band-shaped nucleus and finally Neutrophils (NPs) or Polymorphonuclear granulocytes (PMNs), named from their segmented nucleus (Andrews et al., 1983; Cowland & Borregaard, 2016; Terstappen et al., 1990).

1.1.2 FUNCTIONS AND GRANULES

Neutrophils have very important functions for the immune system regulation and defense such as chemotaxis migration, phagocytosis, NETosis, exocytosis. Neutrophils functions depend on their interactions with other cells like DCs, monocytes, endothelial cells and on the produced cytokines and chemokines in their proximal or distant environment. Their presence in lymphoid organs can modulate adaptive immune responses, by mainly suppressing $CD4^+$ T cells. They also carry different kinds of granules with specific proteins responsible for various functions and signals. Based on their loaded proteins, neutrophil granules are classified into three distinct subsets: primary (azurophil) granules (myeloperoxidase [MPO]), secondary (specific) granules (lactoferrin), and tertiary (gelatinase granules) (gelatinase). Granules can be liberated into phagosomes or out of the cell/exocytosis or release their protein load extracellularly or on NET structures. Neutrophils' functionality is altered after their migration from the circulation to a tissue and the investigation of these changes in each case and in association with disorders is an open field of research (Nauseef & Borregaard, 2014).

1.2 NEUTROPENIA

Neutropenia is defined as a condition of persistent low counts of neutrophils in the peripheral blood and it is divided in different categories depending on its varying pathological mechanisms, genetic background, age of onset and severity. These disorders are divided into congenital and acquired neutropenia.

Non-Immune Chronic Idiopathic Neutropenia of Adults (NI-CINA) is characterizing the subset of patients that has no evidence of immune-mediated disease, underlying autoimmune disease, nutritional deficiency or myelodysplasia and have normal marrow cytogenetics. The marrow findings vary from hypoplastic to hyperplastic myeloid series and the clinical course is quite benign (Papadaki, Palmblad, et al., 2001). The pathogenesis mechanisms are poorly understood and more information will be given below as Chronic Idiopathic Neutropenia (CIN) is the disorder under investigation in our study.

1.3 CHRONIC IDIOPATHIC NEUTROPENIA (CIN)

CIN is an isolated neutropenia without any known congenital or acquired cause. It is a benign disorder of granulopoiesis belonging to the spectrum of Bone Marrow (BM) failure syndromes in

a mild form. CIN has an age onset in late childhood to adulthood and it mainly affects women. It is described as the unexplained and prolonged reduction in the number of circulating neutrophils under the lower limit of the normal distribution in a defined ethnic population.

The diagnosis of CIN is based on excluding criteria, examining evidence that indicate underlying diseases such as primary BM diseases, nutritional deficiencies (vitamin B12, folic acid, copper), LGL-proliferative disease, autoimmune diseases, infections and hypersplenism, the absence of history of exposure to irradiation, use of chemical compounds or intake of drugs, and a normal BM karyotype (Palmblad et al., 2016). In addition, Benign Ethnic Neutropenia, should be excluded via DARC/ACKR1 genotyping for the single-nucleotide polymorphism rs2814778 (-46T > C) (Fragiadaki et al., 2020; Reich et al., 2009).

Based on the number of circulating neutrophils, neutropenia can be categorized to mild with a neutrophils' rage from 1000 to 1800 per ul of blood, moderate with a range from 500 to 1000 per ul and severe when the count is below 500 per ul of blood (Papadaki, Palmblad, et al., 2001).

The clinical manifestations of the disorder include infections where granulocyte colony-stimulating factor (G-CSF) is administered and it is effectively increasing the circulating neutrophils of the patient, splenomegaly, anemia and osteopenia and osteoporosis. Also, apart from neutropenia the disease is characterized by lymphopenia and dysfunction of the BM and leukocyte trafficking.

1.3.1 PATHOGENESIS

In CIN, BM microenvironment has an inhibitory effect of granulopoiesis and this phenomenon is attributed to an oligoclonal population of activated T cytotoxic lymphocytes (Papadaki et al., 2005; Spanoudakis et al., 2010) and monocytes (Bizymi et al., 2019; Velegraki et al., 2012), low proportion of BM PMNs-MDSCs leading to sustained T cell suppression and inflammation in the BM (*Bizymi N et al, ASH 2020*). There is also production of proinflammatory and proapoptotic cytokines and mediators (Spanoudakis et al., 2010), such as TNF-α, IL-1β, TGF-β1, IL-6 as well as IFN-γ, and FasL. This leads precursor cells of the CD34+/CD33+ granulocytic progenitors' compartment to apoptosis through mechanisms involving the pathways of FAS/FasL (Papadaki et al., 2003) and CD40/CD40L in the presence of TNF-a (Mavroudi & Papadaki, 2011).

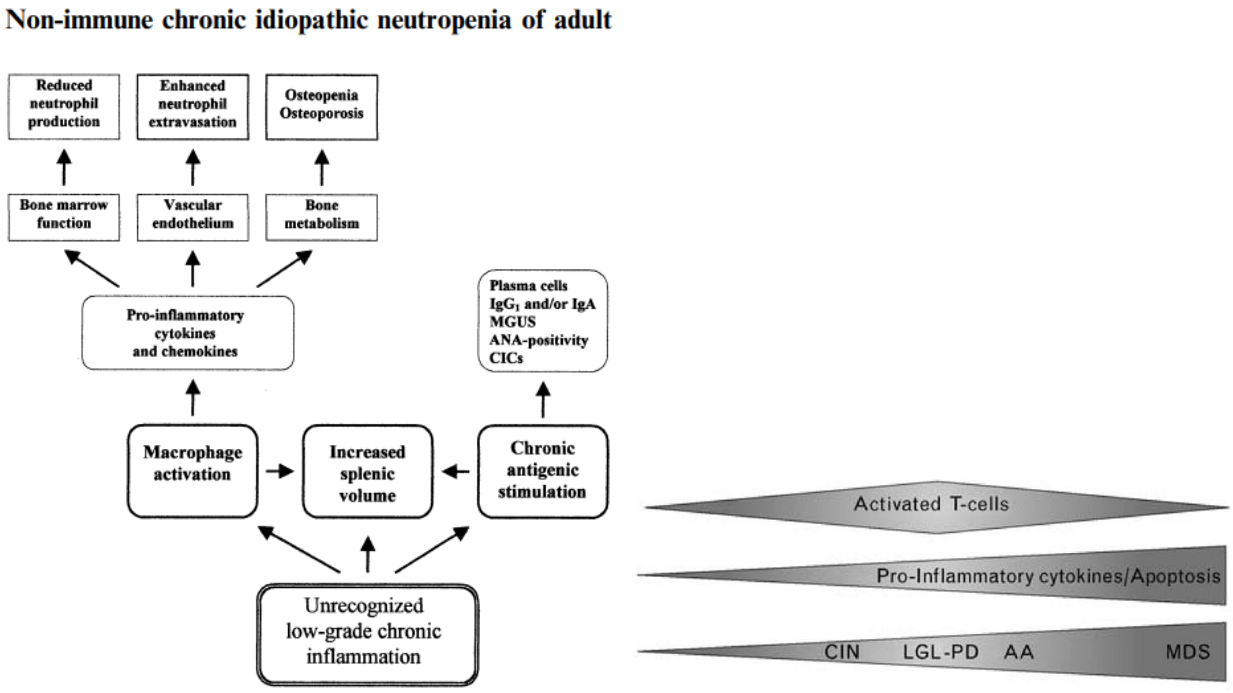
There is a low-grade inflammatory environment in the BM of these patients, leading to both chronic antigenic stimulation and activation of macrophages. Antigenic stimulation is indicated by increased serum IgG1 and/or IgA, the increased frequency of monoclonal gammopathy of undetermined significance (MGUS), the increased incidence of antinuclear antibodies (ANA) and the increased levels of circulating immune complexes (CICs). Macrophage activation is indicated by the increased levels of macrophage-derived proinflammatory cytokines and chemokines and

the increased concentrations of stromal cell derived cytokines in long-term bone marrow culture supernatants (Papadaki, Palmblad, et al., 2001). As a result of these phenomena, reduced neutrophil production in the BM occurs.

In addition, low counts of circulating neutrophils can also be explained by enhanced neutrophil extravasation to the spleen as increased splenic volume has been observed in these patients, reflecting the hyperplasia of splenic macrophages and endothelial cells as a result to the elevated levels of proinflammatory cytokines and adhesion molecules found in the serum (Papadaki, Palmblad, et al., 2001; Papadaki & Eliopoulos, 2009) (Figure 1.1.)

Finally, there is a report for genetic predisposition for CIN development associated with a particular HLA allele. The study which was carried out for a cohort of a genetically homogenous population in Crete, Greece showed that of all the alleles tested (HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1), only the HLA-DRB1∗1301 allele was significantly elevated in CIN patients (Papadaki, Eliopoulos, et al., 2001).

A. **B.**



Eur J Haematol 2001: 67: 35–44, Papadaki et al. *Palmblad J & Papadaki HA. Curr Opin Hematol 2008;15:8-14*

Figure 1.1. (A**)** Schematic representation of a mechanism possibly operating in the pathogenesis of CIN. **(B)** Chronic idiopathic neutropenia in the spectrum of bone marrow failure syndromes associated with inflammatory bone marrow microenvironment.

1.4 FAMILIAL MEDITERRANIAN FEVER (FMF)

Familial Mediterranean fever (FMF) is the most common monogenic autoinflammatory disease (AID) over the world. The most common clinical features of FMF include self-limited inflammatory attacks of fever and polyserositis along with high acute phase response, while a significant complication of is amyloidosis, and it is responsible for long-term morbidity and mortality. Other features are abdominal pain, peritonitis, pleuritis, serositis, arthritis, dermal manifestations and renal complications.

Diagnosis is based on major and minor criteria of the clinical phenotype of each patient and a genetic testing for the *MEFV* gene can be done to support the clinical criteria. Booty et al showed that screening of the most common mutations of the *MEFV* gene is sufficient for diagnosis rather than sequencing the whole gene (Booty et al., 2009).

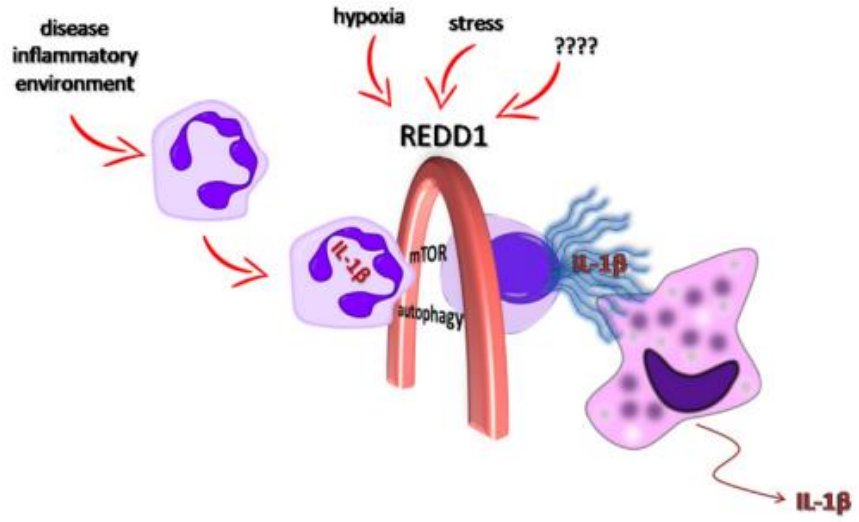
As regards the inheritance of FMF, it is not an ordinary autosomal recessive disease as is the case in other monogenic Mendelian disorders, but the inheritance, penetrance, and pathogenesis patterns of FMF are more complicated. Since there are heterozygous patients with clinical phenotype of FMF, a dominant condition with low penetrance is indicated. A recent study determined that FMF-like symptoms increased from patients carrying a single low penetrance mutation toward patients with two high-penetrance mutations suggesting a “dose effect” of mutations. Heterozygotes’ FMF clinical manifestation could also be explained by the synergistic effect of serum amyloid A (SAA) gene with *MEFV* gene mutations. Finally, apart from genetics, the environment has an effect on the disease’s phenotype as much as epigenetics and microbiota factors (Alghamdi, 2017; Özen et al., 2017).

1.4.1 PATHOGENESIS

The main role of pyrin is to regulate inflammation, which function is lost upon gain-of-function mutations resulting in the activation of the NRLP3 inflammasome and IL-1β secretion. Another important function of pyrin is its action as a receptor for the autophagy-driven degradation of components of the NLRP3 inflammasome, resulting in the inhibitor of inflammation (Skendros et al., 2019).

Based on these functions of pyrin, the molecular pathogenic mechanisms that underlie autoinflammation was investigated with focus on autophagy and Neutrophil Extracellular Traps (NETs) formation in FMF patients. On the one hand, significantly lower levels of basal autophagy in neutrophils of FMF patients in remission compared to healthy individuals was demonstrated,

indicating the association of the mechanism with Target Of Rapamycin (mTOR) and autophagy dysregulation(Kimura et al., 2017). On the other hand, it was shown that neutrophils release large amounts of NETs decorated with bioactive IL-1β, in an autophagy-dependent manner. The conclusion of these studies is that the autophagy/NET axis is tightly involved in the pathogenesis of FMF autoinflammation (Apostolidou et al., 2016). More evidence about this mechanism emerged with a study that links the stress-induced regulated in development and DNA damage responses 1 (REDD1) protein with the activation of the mTOR/autophagy pathway leading to inflammatory IL-1β-bearing NETs. REDD1 activation in neutrophils would be the "second hit or NETotic hit" of the disease after the "first hit" which is the priming inflammatory environment of the disease leading to the production of IL-1b by neutrophils (Skendros et al., 2017) (Figure 1.2).



Panagiotis Skendros et al, Journal of Autoimmunity, https://doi.org/10.1016/j.jaut.2019.102305

Figure 1.2. REDD1 induction as a gateway to autophagy-driven NET-formation in autoinflammation.

1.5 THE MEDITTERANEAN FEVER GENE (*MEFV)*

Mediterranean Fever gene *(MEFV)*, is the gene whose mutations are associated with Familial Mediterranean Fever (FMF), a recessively inherited systemic auto-inflammatory disease. *MEFV* gene is located on chromosome 16q, it is about c long and it consists of 10 exons. The encoded protein is pyrin or alternatively marenostrin; TRIM20, a 781 amino-acids protein/ 86kDa, which normally functions as an innate immune sensor that can trigger formation of an inflammasome, allowing the production of inflammatory mediators during infection.

1.5.1 *MEFV* GENETIC ALTERNATIONS AND CLASSIFICATION

There are many identified variants of the *MEFV* gene which are located throughout the coding sequence of the gene. Variants can affect the gene expression regulation either on the mRNA level (post-transcriptionally) or on the protein level (post-translationally). Also, there might be alternations on the splicing of the immature RNA leading to different isoforms. Different forms of pyrin can be found in different kinds of cells like in monocytes and granulocytes but generally dysregulation alters the function of inflammasome and leads to uncontrolled inflammation (Grandemange et al., 2011).

Recent studies, have reveal that *MEFV* mutations are not loss-of-function mutations confirmed by the finding that FMF-associated pyrin variants detect Rho GTPase inhibition. Furthermore, functional investigation of *MEFV* mutations' consequence in FMF patients indicated that pyrin variants trigger increased inflammasome responses to low concentrations of a Pyrin-activating stimulus in comparison to NLRP3- or NLRC4-activating stimuli. This demonstrates that FMF mutations are gain-of-function mutations(Chae et al., 2011), although, it was additionally shown that FMF mutations are not classical gain-of-function mutations leading to a constitutively active inflammasome, but rather hypermorphic mutations that specifically decrease the activation threshold of the Pyrin inflammasome. However, it is currently unclear whether the increased activation of pyrin results from loss of a negative regulation or from a facilitated activation mechanism, but it is hypothesized that the microtubule-dependent step required to activate the Pyrin inflammasome might play a role (Van Gorp et al., 2016). Finally a gene-dosage effect is indicated due to higher responses of patients bearing two *MEFV* exon 10 mutations in comparison to patients bearing a single *MEFV* exon 10 mutation (Jamilloux et al., 2018).

1.5.2 *MEFV* POPULATION GENETICS WITH FOCUS ON GREEK AND CRETAN POPULATIONS

There are two genetic studies, where the whole *MEFV* coding sequence was examined, that demonstrate the FMF risk of *MEFV* alternations in the populations of both Greece and Crete.

First, Giaglis et al studied a cohort of Greek FMF patients in comparison to Greek healthy controls, using non-isotopic RNase cleavage assay (NIRCA) and sequencing which allowed mutational and haplotypic analysis of the entire coding sequence of *MEFV*, subjected to population genetics analysis software. The results showed that 83.6% of the patients carried at least one known mutation, with the most common mutations identified to be M694V (38.1%), M680I (19.7%), V726A (12.2%), E148Q (10.9%) and E230K (6.1%) and 16.9% of FMF patients carried no *MEFV* mutation. The carrier rate of common *MEFV* mutations among Greek healthy controls was low (0.7%) and thus the diagnostic significance of their presence, even in heterozygosity, is very pronounced. It was reported that the R202Q heterozygosity is quite common among healthy individuals and causes no harm, but the relative absence of R202Q homozygosity among healthy individuals may reflect a potential dosage-dependent deleterious effect. In addition, half of the

FMF patients that didn't carry other *MEFV* mutations were homozygous for R202Q and were associated with “atypical FMF”. Finally, the aberration of the Hardy–Weinberg principle among FMF patients, but not among healthy controls, indicate that R202Q homozygosity might be disease related (Accetturo et al., 2020)(Giaglis et al., 2007).

Fragouli et al, studied a cohort of Cretan FMF patients in comparison to Cretan healthy controls by performing a molecular analysis focused on the 12 most frequent FMF-associated mutations. The results indicated that 83.1% of FMF patients had at least one *MEFV* mutation, of whom about 8% were homozygotes and 92% heterozygotes for FMF-associated mutations. No mutations were detected in 16.9% of the patients. The first most common mutation found was M694V which was shown to be disease related even in heterozygous state, and then E148Q (14%), followed by M694I (7%) and V726A (4.9%). None of the patients was found to be a carrier for the M680I mutation which is very common in Greeks, while lower frequencies of E148Q and M694I, where reported in Greeks compared to Cretans. In contrast, F479L and V726A mutations were found rare in Crete but frequent in Cypriot Greece and finally the rare mutations K695R and A744S were also detected in the Cretan cohort. Population genetics analysis showed an FMF carrier frequency in healthy Cretan population of approximately 6% (1:17) a fact that leads to the classification of the population as ‘high risk’ in regards to FMF prevalence. Finally, the position of the Cretans in the Western European sub-tree and the Greeks closer to other Eastern populations indicates different gene flow (Fragouli et al., 2008).

Finally, a meta-analysis study based on 16,756 chromosomes from FMF patients and normal individuals from 14 affected populations have shown that *MEFV* mutations are distributed non-uniformly along the Mediterranean Sea area. The most frequent mutations detected in FMF patients are M694V (39.6%), V726A (13.9%), M680I (11.4%), E148Q (3.4%), and M694I (2.9%), while 28.8% of chromosomes carry unidentified or no mutations. Also, the mean overall carrier rate is 0.186 with peak values in Arabs, Armenians, Jews, and Turks with Jews to be considered the candidate population for founder effects in *MEFV*. The concluding remark was that the *MEFV* mutation pattern is non-uniform regarding distribution, phenotypic expression, neutrality and population genetics characteristics (Papadopoulos et al., 2008).

Table 1
Mutations in the *MEVF* gene, allele frequencies and total number of mutations per patient detected in FMF patients from mainland Greece (n = 152) and Crete (n = 71) (data from refs 29, 30).

Mutations	Mainland Greece	Crete
	Number of alleles of patients (%)	
M694V	80 (26.3)	39 (27.5)
M680I	39 (12.8)	0 (0)
V726A	21 (6.9)	7 (4.9)
E148Q	19 (6.3)	20 (14.1)
E230K	9 (3)	0 (0)
K695R	4 (1.3)	1 (0.7)
M694I	8 (2.6)	10 (7)
A744S	3 (1)	1 (0.7)
E148V	2 (0.7)	0 (0)
T267I	2 (0.7)	0 (0)
E167D	2 (0.7)	0 (0)
M680L	1 (0.3)	0 (0)
R761H	1 (0.3)	1 (0.7)
F479L	0 (0)	2 (1.4)
S702C	0 (0)	1 (0.7)
Number of mutated alleles per patient	Number of patients (%)	
2	65 (42.8)	24 (33.8)
1	62 (40.8)	35 (49.3)
0	25 (16.4)	12 (16.9)

Skendros P et al, Journal of Autoimmunity 104 (2019) 102305

Figure 1.3. Table representing the allele frequencies of the *MEFV* gene mutations in Greek and Cretan populations

1.6 THE ENCODED PROTEIN PYRIN

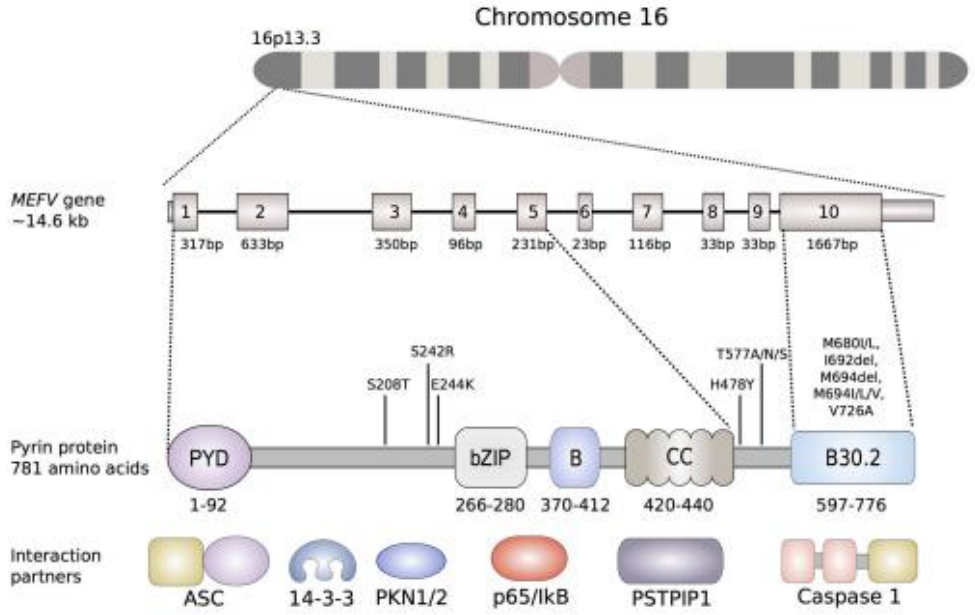
1.6.1 STRUCTURE AND FUNCTION

Since pyrin is a sensor of pathogens' infection, it can detect pathogen/ danger-associated molecular patterns (PAMPs/DAMPs) and so it belongs to the group of cytosolic pattern recognition receptors (PRRs). Due to its role, pyrin is mostly found in innate immune cells such as neutrophils and monocytes and its expression can be upregulated by various cytokines IFN-γ, LPS, TNF-α, IL-4, and IL-10.

Human pyrin is identified to consist of four functional units, the pyrin domain (PYD), a zinc finger domain (bBox), a coiled coil (CC) domain and a B30.2/SPRY domain (Figure 1.4). C-terminal B30.2 domain together with the B-Box and coiled-coil domains, constitutes a tripartite motif, which is contained in proteins involved in viral recognition and this is also the reason for the alternative name of the protein, TRIM20. The lack of the ring domain classifies pyrin as an "incomplete TRIM protein" which is accompanied by the loss of E3 ubiquitin ligase activity conferred by the ring domain. Nevertheless, it has been proposed that pyrin can recognize specific substrates and

assemble a direct precision autophagy machinery (Schnappauf et al., 2019)(Heilig & Broz, 2018)(Grandemange et al., 2011).

Via its PYD domain, the protein binds to the inflammasome adaptor protein, apoptosis-associated speck like protein with a caspase recruitment domain (ASC), which subsequently causes caspase-1 mediated production of IL-1β. The B-box and the α-helical, coiled-coil domain may play a role in the oligomerization of pyrin which should have a role in the inflammasome formation. The C-terminal B30.2 domain of pyrin is of particular importance since most of the FMF-associated mutations are clustered in this domain and it is therefore essential for the molecular mechanisms leading to FMF. Several studies have shown that this domain directly interacts with caspase-1, and inflammasome components such as NLRP3, NLRP1 (Schnappauf et al., 2019).



Oskar Schnappauf et al., Front. Immunol.,2019, doi: 10.3389/fimmu.2019.01745

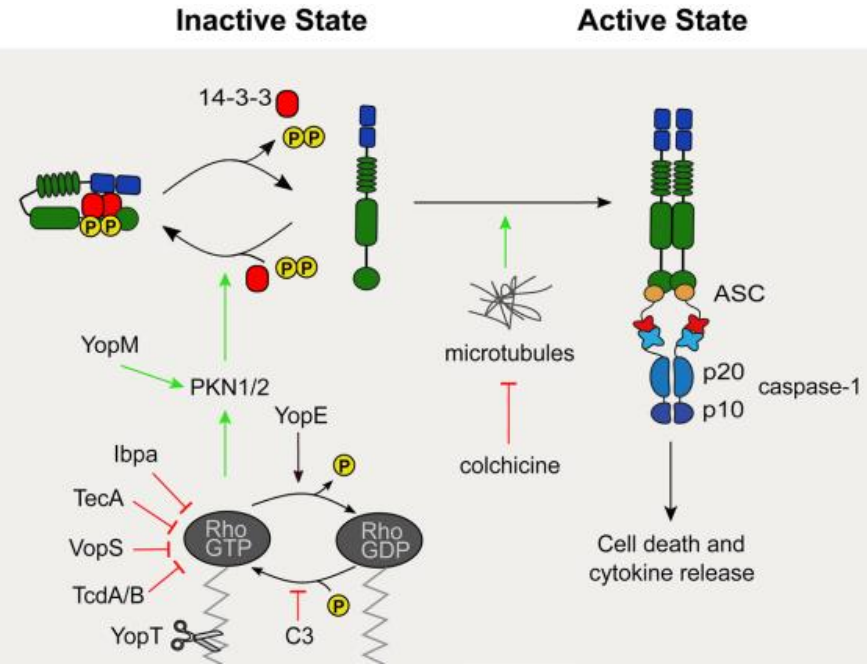
Figure 1.4. Schematic representation of the MEFV gene and the encoded pyrin protein.

1.6.2 ACTIVATION AND INACTIVATION MECHANISM

One of the prime bacterial targets is the RhoA GTPase, a molecular switch that can convert between an active GTP- and an inactive GDP-bound state.

In an inactive state, pyrin is kept autoinhibited by 14-3-3ε which is bound to the phosphorylated pyrin. The phosphorylation is maintained by kinases (PKN1/2) which in turn are activated and phosphorylated by the activated RhoA GTPase.

Upon inactivation of RhoA by bacterial effector proteins, such as Ibpa, TecA, VopS, TcdA/B, YopT, YopE, and C3, PKN1/2 is dephosphorylated. As a consequence, pyrin phosphorylation is reduced resulting in dissociation of 14-3-3ε and relieving pyrin autoinhibition. This allows the assembly of the pyrin inflammasome and initiation of the pro-inflammatory cell death pyroptosis (Heilig & Broz, 2018) (Figure 1.5).



Rosalie Heilig and Petr Broz, Eur. J. Immunol. 2018. 48: 230–238

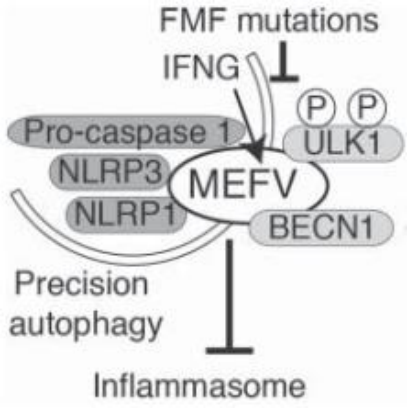
Figure 1.5. Mechanism of pyrin activation.

1.6.3 MECHANISM OF ACTION

Upon activation, pyrin, assembles its own inflammasome, which mediates activation of caspase-1, which in turn promotes the maturation of the proinflammatory cytokines IL-1β and IL-18 and the induction of inflammatory cell death (pyroptosis). Pyroptosis is morphologically different from apoptosis in that it involves cell swelling and lysis. Moreover, pyroptosis involves caspase-1 mediated cleavage of gasdermin D (GSDMD), subsequent translocation of the N-terminal pore-forming domain to the cellular membrane and release of pro-inflammatory cytokines (Schnappauf et al., 2019).

In addition, *MEFV*/TRIM20 acts as a receptor for selective autophagy of inflammasome components, contributing to autophagy stimulated in response to IFN-γ. Specifically, it recognizes inflammasome components, brings ULK1 and BECN1 to NLRP3-*MEFV* receptor-target

recognition complexes, and directs assembly of autophagosomes to sequester and degrade the target. As a result, inflammasome components, and *MEFV* itself, are subjected to autophagic degradation. Through this process, MEFV suppresses CASP1 activation and IL1B production. This is also an example of the fact that precision autophagy prevents excessive inflammation. Additionally, ATG16L1, a component of the autophagy E3-like complex that regulates LC3 conjugation and autophagosome formation, is also present in MEFV protein complexes. FMF-associated variants of *MEFV* reduce autophagic activity due to a diminished ability to form protein complexes with ULK1. As a result, FMF-associated variants of *MEFV* display a reduced capacity to downregulate inflammasome components, which results in excessive IL1B production(Kimura et al., 2017) (Figure 1.6).



Tomonori Kimura et al, J Cell Biol 2015; 210(6):973–989; http://dx.doi.org/10.1083/jcb.201503023

Figure 1.6. MEFV/TRIM20, mutated in familial Mediterranean fever, targets the inflammasome components for autophagic degradation.

1.7 NEUTROPHIL EXTRACELLULAR TRAPS (NETS)

Neutrophil Extracellular Traps (NETs) is a quiet recent finding described by Brinkmann et al in 2004, as a form of innate response that binds microorganisms, prevents them from spreading, and ensures a high local concentration of antimicrobial agents to degrade virulence factors and kill bacteria. They indicated that upon activation, neutrophils release granule proteins and chromatin that together form extracellular fibers that bind Gram-positive and -negative bacteria (Brinkmann et al., 2004). NETs contribute to immobilize invading microorganisms such as bacteria, fungi and viruses but also in response to sterile triggers. NET release can be instigated by many triggers and via a multitude of distinct pathways with often unknown interdependence. Except for their role in immune defense, NETs play important detrimental or beneficial roles in inflammation, autoimmunity and other pathophysiological conditions (Boeltz et al., 2019).

1.7.1 NETS FORMATION

NETs release is mainly connected to a cell death process called NETosis or Lytic NET formation. This process starts with neutrophils actin dynamics' arrest and depolarization and continues with disassembly of the nuclear envelope, and decondensation of the nuclear chromatin into the cytoplasm of intact cells, mixing with cytoplasmic and granule components. Then the plasma membrane permeabilizes, and NETs expand into the extracellular space 3–8 hours after neutrophil activation (Fuchs et al., 2007). Specifically, Lytic NET formation is a NADPH-oxidase dependent procedure, where activated NADPH-oxidase via PKC and Raf-MEK-ERK signaling pathway results in ROS generation. In addition, peptidylarginine deiminase 4 (PAD4) is activated and citrullinates arginine on histones causing chromatin decondensation. Myeloperoxidase (MPO) and neutrophil elastase (NE) are released from cytoplasmic azurophilic granules and then translocated to the nucleus. All together contribute to chromatin unfolding. NE also cleaves gasdermin D (GSDMD) in the cytosol to its active form (GSDMD-NT) which besides forming pores in the plasma membrane also mediates pore formation in nuclear and granule membranes, enhancing NE and other granular content release.

Alternatively, there is the non-lytic NETosis, where there is absence of cell death and NETs are released within minutes of exposure to the stimulus, reported during systemic S. aureus infection and mounted by the first neutrophils to arrive at sites of infection (Yipp et al., 2012). Non-lytic NETosis is initiated through Toll-like receptor 2 (TLR2), TLR4 or complement receptors independent of NAPDH oxidase activation (Castanheira & Kubes, 2019; Papayannopoulos, 2018).

1.7.2 NETOSIS REGULATION

NETosis must be tightly regulated to prevent pathology. Mainly the regulation depends on two factors, the size of the invading microorganisms and the expression of virulence factors. Regarding the microorganisms' size, larger ones initiate NETosis from neutrophils while the smaller ones are taken up into phagosomes, fusing with azurophilic granules and sequestering NE away from the nucleus. This process blocks chromatin decondensation and further NETosis which indicates the competition between the two responses (Metzler et al., 2014). Another aspect of NETosis regulation comes with the clearance mechanisms of NETs which can trigger further NETs release from neutrophils. NETs can be removed either with phagocytosis by myeloid DCs or by monocyte-derived macrophages. C1q opsonized NETs were shown to be removed by macrophages in an immunologically silent manner (Farrera & Fadeel, 2013). Finally, NETs are degraded by DNase I (Hakkim et al., 2010).

1.7.3 NETOSIS VS OTHER TYPES OF CELL DEATH

Since NETosis is a type of cell death it shares common characteristics with other types of cell death like apoptosis and necrosis. However, there are also differences that makes it possible to distinguish one from another especially when studying one of these forms of cell death. Specifically, DNA fragmentation, phosphatidylserine exposure, and caspase activation are characteristics of apoptosis that are not observed in NETosis. In contrast, disintegration of the nuclear envelope and mixing of nuclear and cytoplasmic material, loss of internal membranes, and disappearance of cytoplasmic organelles are hallmarks of NETosis, not associated with apoptosis. On the other hand, in NETosis perforation of membranes is induced just like in necrosis which allows intracellular proteins to leak outside the cells. This common characteristic associate both NETosis and necrosis with DAMPs release and subsequent inflammatory responses induction. In this way, it is more challenging to distinguish NETosis and necrosis in terms of an experimental study (Fuchs et al., 2007; Y. Kobayashi, 2015).

1.7.4 NETOPATHIES

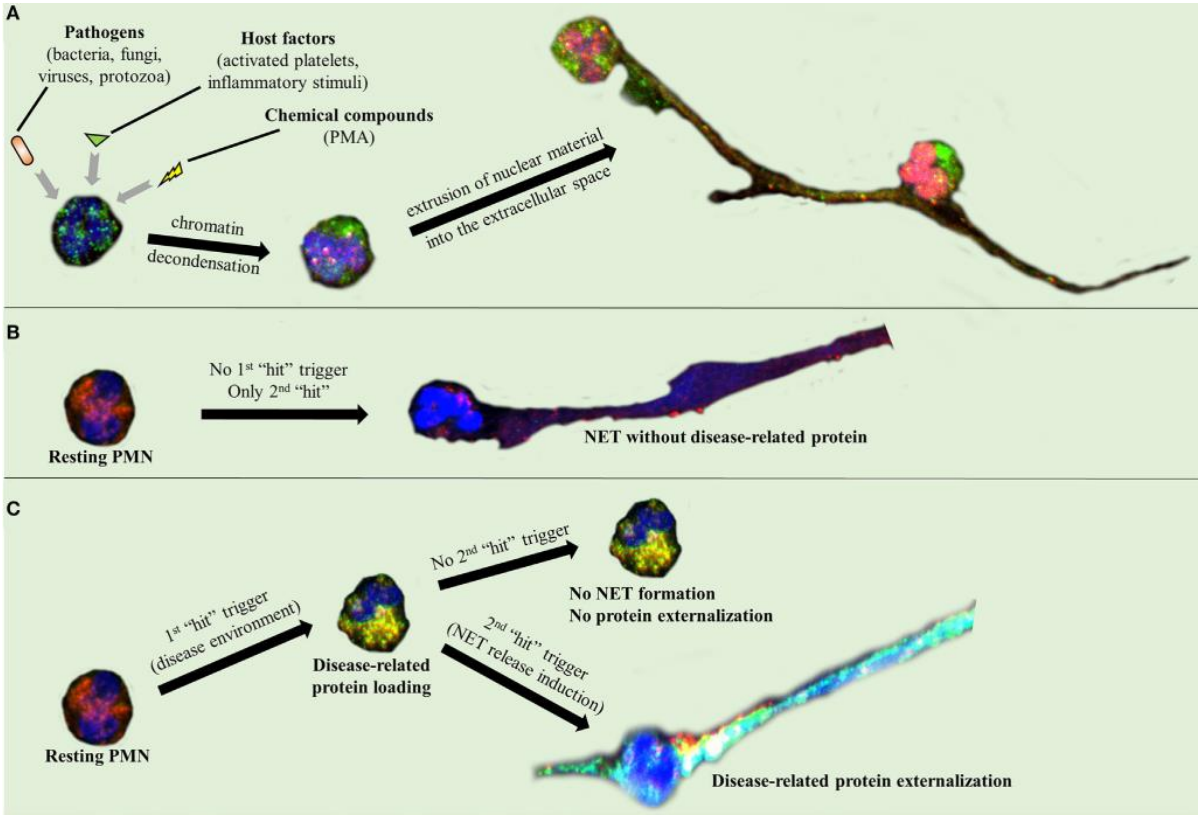
Besides the role of NETs in infection, NETs also have a role in the pathophysiology of non-infectious diseases, such as thrombosis (Kambas et al., 2012), autoimmune diseases (Hakkim et al., 2010), genetically driven autoinflammatory (Apostolidou et al., 2016), and other inflammation-related diseases, metabolic disorders, lung diseases, fibrosis, and cancer (Demers et al., 2012).

Recent evidence elucidate NLRP3/PAD4-induced NETosis in the absence of infection, linking the canonical inflammasome and NETosis in sterile environment (Münzer et al., 2021).

Independently of the stimulus, NETs are composed of DNA, citrullinated histone 3 (cit-H3), NE, and MPO (Kolaczkowska et al., 2015). Apart from these proteins NETs can be found decorated with various other proteins which could be associated to and define the pathology of non-infectious diseases. As described in FMF's pathological mechanism, the "two hit" process can explain the different pathways of NETs formation in infection and in other diseases. The first "hit" in this process is the disease-specific environment that primes neutrophils to express disease-associated protein. A second "hit" is then required for the induction of NET formation. In the case of infection though, only the second "hit" is present so naïve neutrophils are primed to release NETs following the basic pathway of NETosis. As a result, such disorders can be characterized as NETopathies due to the fact that they are ruled by the bioactive disease-related neutrophil extracellular trap (NET) proteins (Mitsios et al., 2017) (Figure 1.7).

Finally, as long as NETs and their disease related proteins are identified they can have not only prognostic and diagnostic roles but also therapeutic ones. Targeting NETs formation or integrity, or specific NET proteins, promises novel therapeutic strategies for various disorders. Examples of

such targeting are the administration autophagy, ROS, IFN-a, PAD4 inhibitors that blocks NETs formation (SLE, RA), DNases that destructs NETs integrity (Fibrosis, Thrombosis) and Antibodies targeting specific bioactive NET proteins like IL-17 and IL-1β (Psoriasis, FMF) (Mitsios et al., 2017).



Alexandros Mitsios et al, Front. Immunol. 7:678. doi : 10.3389/fimmu.2016.00678

Figure 1.7. Neutrophil extracellular trap (NET) formation and protein decoration. (A) NET formation mechanism and (B, C) the two-step process through which the disease-related protein is externalized.

2. HYPOTHESIS

Based on previously reported increased frequency of mutations of the *MEFV* gene in patients with idiopathic inflammatory conditions other than FMF (Boursier et al., 2019; Manthiram et al., 2017), the existence of common *MEFV* mutations was investigated in a well characterized cohort of 50 CIN patients (Skendros et al., 2021).

Genetic alterations were detected in 44% of the patients (22/50), with 20% (10/50) of patients to carry mutation on exons 10 and/or exon 2 identified as pathogenic. This frequency (20%) is significantly higher in comparison to the carrier rate of common *MEFV* mutations in the healthy Greek population which is 0.7%. NIRCA and NGS analysis were used and indicated: one patient with heterozygous I720M mutation, two with heterozygous A744S and one homozygote, one

with heterozygous M694V, one with heterozygous K695R, one with heterozygous M680I, four homozygous R202Q and two heterozygous R202Q combined with heterozygosity of I720M and A744S. In addition, 12 patients carried the R202Q heterozygosity (Table 1.2).

Table 1.1 Mutations found in the CIN patients (Skendros et al., 2021)

Patients (n)	**Exon 10**	**Exon 2**
1	I720M hetero	-
2	A744S hetero	-
1	A744S homo	-
1	M694V hetero	-
1	K695R hetero	-
1	M680I hetero	-
4	-	R202Q homo
1	I720M hetero	R202Q hetero
1	A744S hetero	R202Q hetero
1	A744S homo	R202Q homo

Patients that were found to carry mutations in the *MEFV* gene, had no clinical manifestation of FMF or more severe neutropenia, compared to the patients negative for *MEFV* mutations. The only difference was the laboratory finding of more elevated levels of serum IgG in the first group.

The question raised from this study, is **whether patients who carry the *MEFV* genetic alterations, have a pathogenetic/modifying effect in the inflammatory responses associated with CIN or if they represent atypical cases of FMF. As a first step to elucidate that, in the present study we investigated the Neutrophil Extracellular Trap (NET) formation in CIN patients with different *MEFV* variants to clarify their potential effect in patients' immune dysregulation.**

3. METHODS

Human Subjects

We studied 20 patients (17 female and 3 male subjects) aged 27 to 78 years with a diagnosis of Chronic Idiopathic Neutropenia according to previously established criteria. The patients had an absolute neutrophil count < 1.8 x 109 /L (mean, 1262.5 ± 132.629 x 109/L; median, 1400 x 109/L;

range, 200-1790 x 109/L) for a period lasting >4 months, had no clinical/laboratory evidence of underlying disease associated with neutropenia, absence of history of exposure to irradiation, use of chemical compounds or intake of drugs that might cause neutropenia, negative antineutrophil antibodies in granulocyte immunofluorescence and agglutination test results, inconclusive results of BM biopsy and aspiration for a specific diagnosis with absence/rare dysplastic features, and normal BM karyotype. Benign ethnic neutropenia, currently characterized as "typical neutrophil count with Fy(a-b-) status," was excluded by DARC/ACKR1 genotyping for the single-nucleotide polymorphism rs2814778 (-46T> C).

Six patients harbored at least one *MEFV* mutation, as seen in Table 1.2, based on previously reported data (Skendros et al., 2021), 7 patients were negative for *MEFV* mutations and 6 patients were carriers of the heterozygous benign R202Q polymorphism. We also studied healthy donors age and sex matched with our patients.

Table 1.2 Human subjects used for the purposes of our study.

Patient	**Gender**	**Age**	**NEU(*/ul)**	**Exon 10**	**Exon 2**
M1	F	54	1500	A744S homo	R202Q homo
M2	F	75	500	I720M hetero	R202Q hetero
M3	F	31	600	-	R202Q homo
M4	F	53	1400	K695R hetero	-
M5	F	36	300	A744S hetero	R202Q hetero
M6	F	68	1700	M694V hetero	R202Q hetero
N1	F	46	1200	neg	neg
N2	F	78	1790	neg	neg
N3	M	49	1400	neg	neg
N4	F	85	1700	neg	neg
N5	M	27	200	neg	neg
N6	F	54	1000	neg	neg
N7	M	29	800	neg	neg
N8	F	68	1700	neg	R202Q hetero
N9	F	34	1790	neg	R202Q hetero
N10	F	59	1400	neg	R202Q hetero
N11	F	74	1500	neg	R202Q hetero
N12	F	59	1600	neg	R202Q hetero
N13	F	33	900	neg	R202Q hetero
N14	F	68	1250	neg	R202Q hetero

Isolation and culture of blood neutrophils

Human peripheral blood neutrophils were isolated with double gradient ficoll centrifugation, washing with PBS and lysis of RBCs with water for injection. Cell viability was measured at 99% by trypan blue dye exclusion. Neutrophils were cultured with no phenol red RPMI medium (Gibco, 11835030) supplemented with 1% v/v heat-inactivated FBS and 10mm Hepes (Gibco, 15630080) in a humidified 37°C/5% CO2 incubator.

Generation, isolation and quantification of NETs

Neutrophils (1×106) from healthy individuals were seeded in 12-well tissue culture plates and cultured with no phenol red RPMI (Gibco) supplemented with 1% v/v heat-inactivated FBS and 10mm Hepes (Gibco), for 3 hours in a humidified 37°C/5% CO2 incubator. Serum from CIN and healthy individuals was used at 15% v/v final concentration in these short-term neutrophils' cultures. Supernatants were discarded, cells were carefully washed once with pre-warmed medium, and NETs-containing supernatants were vigorously collected and spun at 150g for 5 minutes at 4°C to obtain cell-free supernatants. PMA (100nM Sigma Aldrich, P1585) was used as positive control and untreated cells as negative control. To quantify NET-containing supernatants, we used the SYTOX green Nucleic Acid Stain kit (Invitrogen, S7020) according to the manufacturer instructions, MPO/dsDNA ELISA and Immunofluorescence where decondensed nuclei or filamentous DNA structures stained for both DAPI and NE were considered as NETs, to calculate the proportion of NETs-releasing cells out of the total number of cells on a coverslip.

SYTOX GREEN assay

SYTOX® Green nucleic acid stain (S7020) is a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes and yet will not cross the membranes of live cells. After brief incubation with SYTOX® Green nucleic acid stain, the nucleic acids of extracellular space fluoresce bright green when excited with 450–490 nm spectral line. NETs-containing supernatants coated in 96-well plates, were incubated with SYTOX® Green stain for about 15 minutes and then absorbance was measured at 450nm. Background signal was subtracted. All samples were assessed in duplicates.

MPO/dsDNA ELISA

NET-associated MPO/dsDNA complexes were quantified by a modified capture ELISA. Specifically, 1μg/ml of mouse anti-human MPO antibody (mAb 266-6K1, Hycult Biotech) was coated overnight to 96-well microtiter plates. After blocking with 5% w/v BSA, human serum and NETs-containing supernatants were added together with a peroxidase-labeled anti-DNA monoclonal antibody of the Cell Death ELISAPLUS kit (1:25, Roche, 11544675001). The plate was incubated for 2 ½ hours, shaking at 300 rpm at room temperature. After 3 washes with PBS,

peroxidase substrate (ABTS) was added to incubate at room temperature in the dark for 20 minutes. Absorbance at 450 nm wavelength was measured. Background signal was subtracted. All samples were assessed in duplicates.

Immunofluorescence

Neutrophils were seeded on coverslips coated with 0.001% poly-l-lysine (Sigma-Aldrich, P8920) and cultured for 3 hours at 37°C/5% CO2. Cells were fixed with 4% paraformaldehyde (PFA), blocked with 5% w/v BSA/PBS buffer and permeabilized with 0.2% triton X-100 for 4 minutes. DNA was stained with 300nM DAPI (Sigma Aldrich, D9542). NET protein staining was performed using Rabbit Monoclonal anti-NE (abcam [EPR7479] ab131260, 1:100 dilution) primary antibody for 1.5 hour at room temperature, followed by 1-hour incubation with Goat Anti-Rabbit IgG H&L Alexa488-conjugated (abcam, ab150077, 1:1000 dilution) secondary antibody. Three washes with 0.5% w/v BSA/PBS were performed in-between all stainings. After staining, coverslips were mounted on Mowiol 4-88 (Sigma-Aldrich, 81381) and were observed with confocal microscopy (40X objective, Leica SP8). Confocal images were analyzed with the FIJI software.

Statistical Analysis

Data were analyzed using GraphPad Prism 8 software. Results are presented as the means ± SEM (standard error of the mean) and/or dot-plots. Statistical analysis for comparison of multiple conditions/treatments was conducted with One-way ANOVA for multiple comparisons. Two-tailed p-value < 0.05 was considered statistically significant.

4. RESULTS

To determine the effect of *MEFV* variants on NETs formation from peripheral neutrophils of patients with CIN we conducted different functional assays. The first step was to isolate neutrophils from peripheral blood of healthy donors and culture them for a short time with patients' serum to induce NETosis. Then mechanical forces were applied to the cell cultures in order for the formed NETs to be cut off and isolated in the supernatant of the culture. Using these supernatants, we conducted Sytox Green assay, a fluorescence staining for the extracellular DNA which gave us a good indication of the NETs quantity in each culture condition. Sytox Green staining on its own can be considered as an indication of NETosis because extracellular DNA can also come from apoptotic or necrotic cells in the supernatants of cell cultures. So, we also proceeded with a modified capture ELISA for dsDNA/MPO complexes which are specifically

associated with NETs. Finally, in addition to these fluorescence quantification methods we conducted immunofluorescence staining in combination with confocal microscopy to both count the cells directly and to determine the quality of the formed NETs in each condition.

INVESTIGATION OF NETS FORMATION IN A CIN PATIENT WITH TWO HOMOZYGOUS *MEFV* VARIANTS

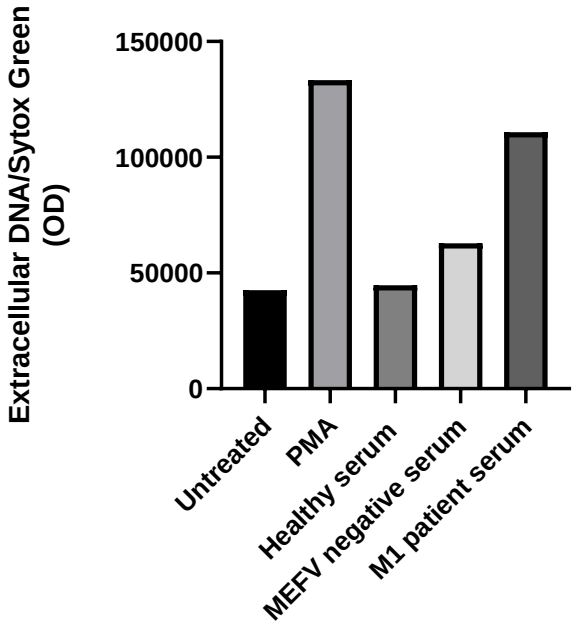
We sought to determine the effect of serum from the patient, who carries in homozygosity two different *MEFV* variants (A744S homo and R202Q homo), patient M1. This patient was considered to most likely have an altered NETosis status based on the particular genetic status on the *MEFV* gene locus.

Following a three-hour of co-culture of isolated healthy PMNs with 5% diluted patient's serum we isolated NETs in the supernatant and conducted Sytox Green assay. This condition was compared to PMNs treated with 5% healthy serum and PMNs treated with 5% serum from a CIN patient negative for *MEFV* mutations. Untreated PMNs supplemented with RPMI-FBS medium were used as a negative control and PMA treated PMNs as a positive control. The comparison of the conditions from the Sytox Green assay indicated a higher level of NETosis in PMNs treated with M1's patient serum compared to PMNs treated with healthy serum or serum from *MEFV* negative patient. The NETosis levels of the last two were comparable to the levels of untreated PMNs/negative control **(Figure 4.1 A)**.

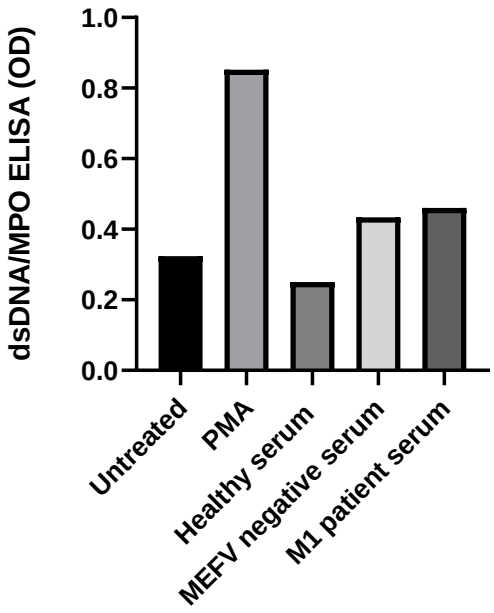
To verify this result and to specifically detect NETs in the supernatants of the cultures we used the same culture conditions as mentioned above, but instead of the Sytox green assay, we conducted ELISA for dsDNA/MPO complexes for culture supernatants. The results indicated an increase of NETosis in CIN patients both positive and negative for *MEFV* mutations in comparison to healthy individuals **(Figure 4.1 B)**.

Finally, we used the same culture conditions/treatments of PMNs for immunofluorescence staining with anti-NE antibody and DNA staining with DAPI. The co-localization of these two extracellularly indicated NETs as they were imaged using confocal microscopy. This gave us an impression of NETs quality in each condition and a semi-quantification approach to validate the previous experiments' results. We detected different shapes of NETs as the PMA robustly induced big clusters of cells to extrude NETs which seemed cloudy shaped compared to the serum treated PMNs which appeared to be more linear. We could also conclude that PMNs treated with patients' serums formed more NETs compared to PMNs treated with healthy serum **(Figure 4.1 C)**. Based on these experiments we got the indication that specific *MEFV* mutations can be associated with higher levels of NETosis in patients carrying them and as a result differences in NETosis could be associated with an altered immune response in this cohort of CIN patients.

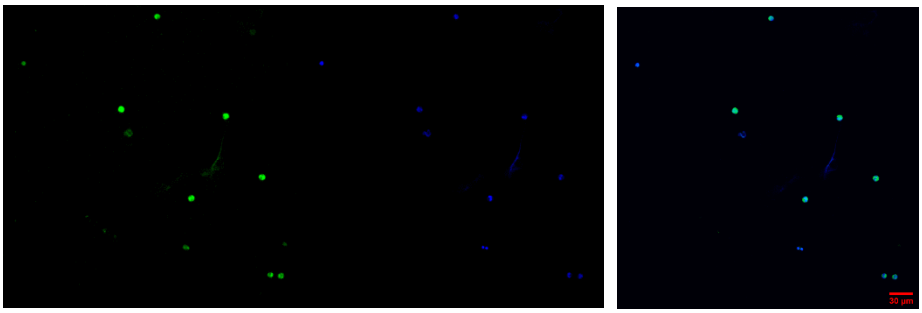
A.



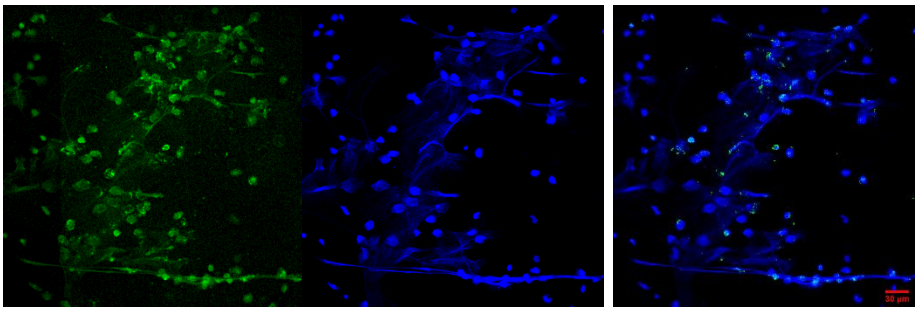
B.



C.



Control PMNs/Untreated



Control PMNs/PMA treated

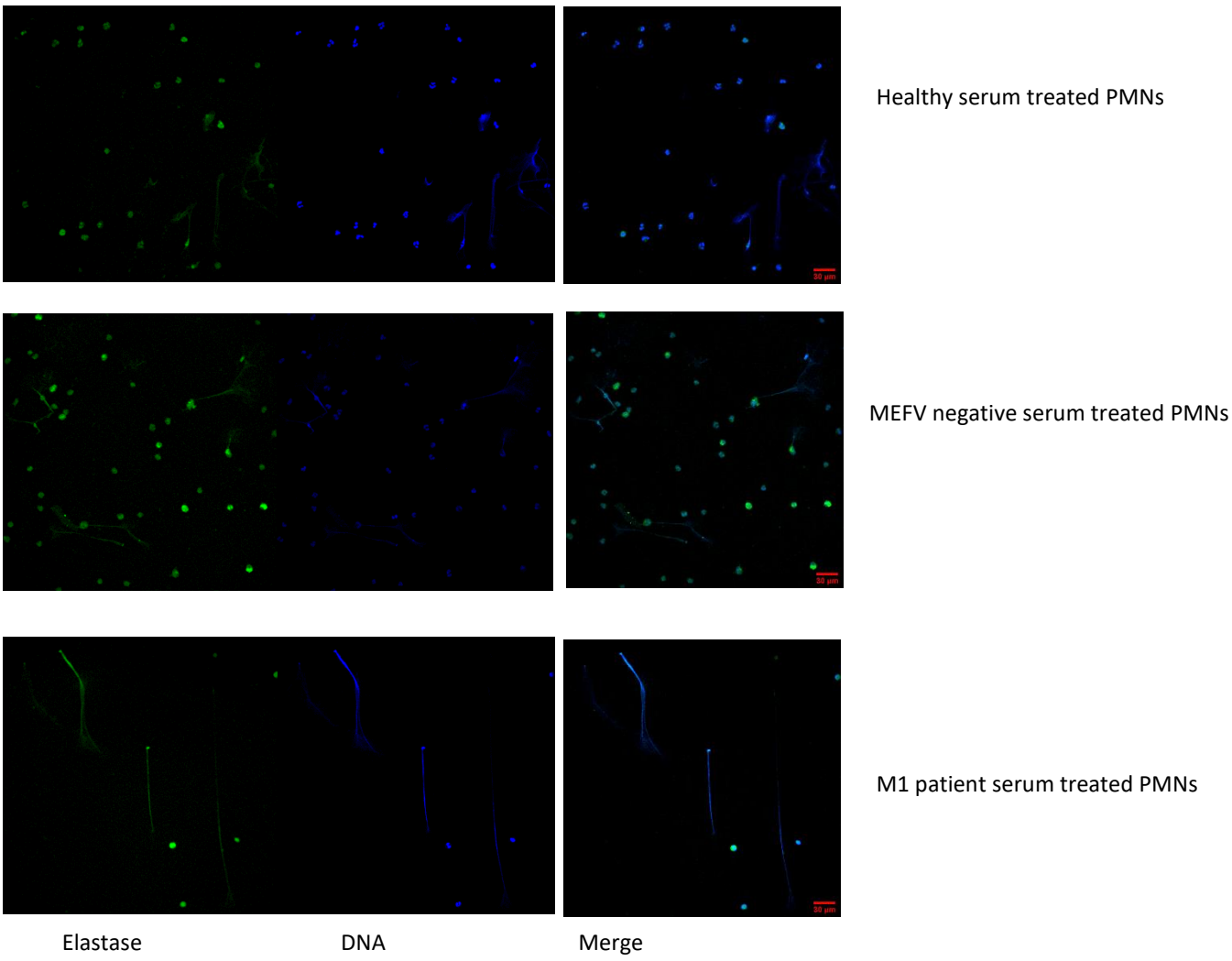


Figure 4.1. Stimulation of healthy PMNs with serum from M1 patient who is considered to carry *MEFV* pathogenic variants in comparison to treatment with serums from a healthy individual and a *MEFV* negative CIN patient. **(A)** Extracellular DNA measurement with Sytox Green staining as an indication for NETs release from PMNs stimulated with different serums. **(B)** dsDNA/MPO complex levels specifically associated with NETs release from stimulated PMNs as measured with ELISA. **(C)** Healthy PMNs treated with different serums for 3 hours and then stained with anti-Neutrophil Elastase (NE), and DAPI for DNA. Representative confocal images (scale bar 30μm, z stack analysis, 40x magnitude) show the characteristic co-localization of NE (green) and DNA (blue) on NET structures.

SCREENING FOR NETOSIS IN ALL PATIENTS CARRYING LIKELY PATHOGENIC *MEFV* VARIANTS

Following the same experimental plan as in the first part of our study where we investigated the NETosis status in one patient, we proceeded in the screening of patients carrying different *MEFV*

mutations as described previously. These patients (n=6) were compared to *MEFV* negative patients and healthy controls. Stored serums from PB collected at different time points of the follow-up of these patients were used for the conducted experiments. This series of experiments was repeated up to five times, which means that control PMNs were isolated from five different healthy donors.

The isolated PMNs were cultured for 3 hours with different serums from patients with or without *MEFV* mutations or healthy individuals. NETs were cut off in the supernatants. Firstly, we conducted Sytox Green assay with supernatants coming from the following conditions: 3 healthy donors' serums, 9 *MEFV* negative patients' serums and 9 *MEFV* mutated patients' serums originating from 6 patients and different time points.

The analysis took place for each patient of interest of the 6 mutated patients, combining the five independent experiments' data (mean ± SD represented). Comparisons were made between the Mutated patient's serum treatment and healthy serums or *MEFV* negative patients' serums **(Figure 4.2 A-F)**. None of the patients who carry *MEFV* mutations was presented to induce a robust response of NETs formation by neutrophils. In addition, statistical analysis was conducted for this set of data from the Sytox Green assay **(Table 1)**. Multiple comparisons one-way ANOVA was used to compare the following categories: healthy individuals, *MEFV* negative patients and *MEFV* mutated patients. No statistically significant difference was observed between these categories of interest: healthy individuals, *MEFV* negative patients and *MEFV* mutated patients (for p-value < 0.05). This analysis is also represented by a dot-plot graph where data from all the experiments are grouped in the three categories for comparison. Each dot represents a value from one experiments' condition (mean ± SD represented) **(Figure 4.2 G)**.

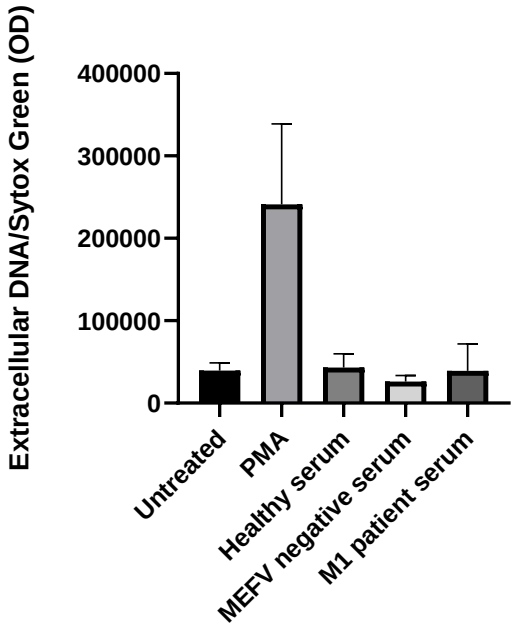
Table 1. Statistical analysis with One-way ANOVA test of data from Sytox Green assay. Five independent experiments with cells from five different healthy donors treated with serums from 6 *MEFV* mutated patients, 9 *MEFV* negative patients and 3 healthy individuals.

One-way ANOVA test: multiple comparisons	**Mean Diff.**	**95.00% CI of diff.**	**Significance (p-value)**
Untreated vs. PMA treated	-158514	-196315 to -120713	**** <0.0001
Untreated vs. Healthy individuals	8034	-27519 to 43587	ns
Untreated vs. *MEFV* negative patients	15408	-16044 to 46861	ns

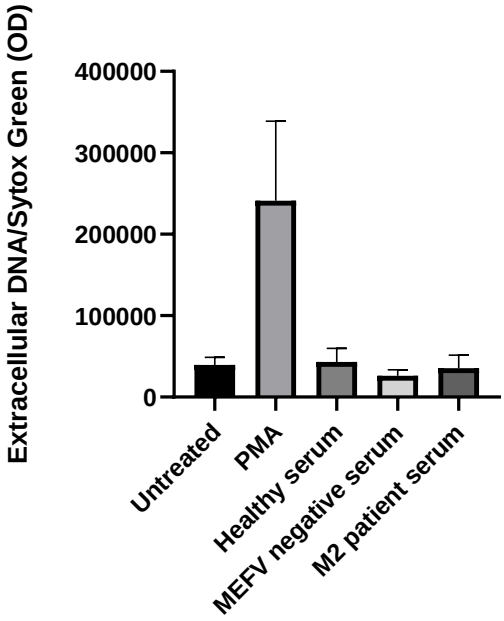
Untreated vs. Mutated patients	10922	-19386 to 41230	ns
PMA vs. Healthy individuals	166548	130995 to 202102	**** <0.0001
PMA vs. *MEFV* negative patients	173923	142470 to 205375	**** <0.0001
PMA vs. Mutated patients	169436	139128 to 199744	**** <0.0001
Healthy individuals vs. *MEFV* negative patients	7374	-21338 to 36086	ns
Healthy individuals vs. Mutated patients	2888	-24566 to 30342	ns
MEFV negative patients vs. Mutated patients	-4487	-26371 to 17398	ns

After the Sytox Green experiments in cultures' supernatants, we proceeded to the detection of NET specific DNA/MPO complexes with ELISA either for culture supernatants or directly for patients' serums. As regard the ELISA for serums it was conducted for 3 healthy individuals' serums, 3 serums from *MEFV* negative and 6 serums from *MEFV* mutated patients. Comparison of the 3 categories indicated no significant difference in NETs levels directly in the serums **(Figure 4.2 H)**. DNA/MPO ELISA was also conducted for supernatants coming from short term cultures of PMNs from one healthy donor treated with 3 serums from healthy individuals, 9 from *MEFV* negative patients and 9 from *MEFV* mutated patients. A representative dot-plot graph indicates the different categories for comparison which have no significant differences one from another (mean ± SD represented) **(Figure 4.2 I)**.

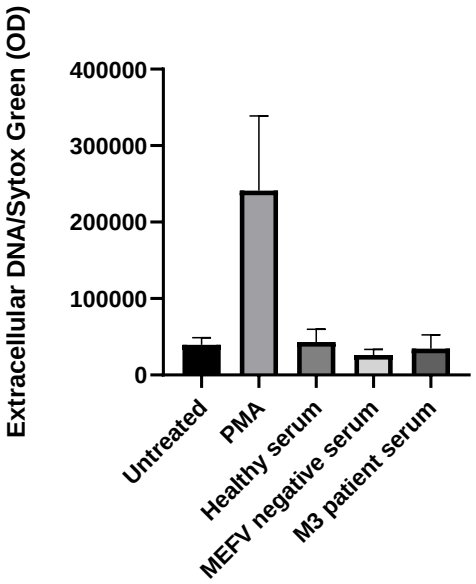
A.


Extracellular DNA/Sytox Green (OD)
400000
300000
200000
100000
0
Untreated
PMA
Healthy serum
MEFV negative serum
M1 patient serum

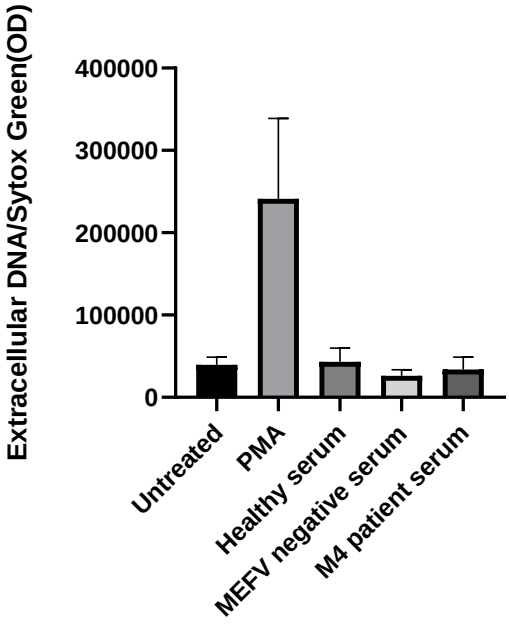
B.


Extracellular DNA/Sytox Green (OD)
400000
300000
200000
100000
0
Untreated
PMA
Healthy serum
MEFV negative serum
M2 patient serum

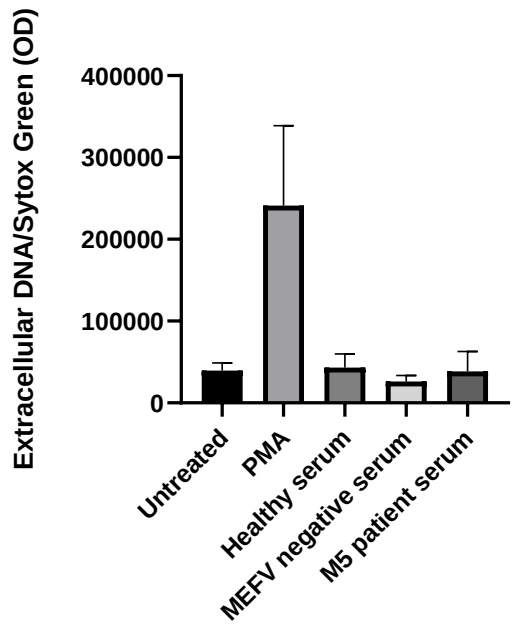
C.


Extracellular DNA/Sytox Green (OD)
400000
300000
200000
100000
0
Untreated
PMA
Healthy serum
MEFV negative serum
M3 patient serum

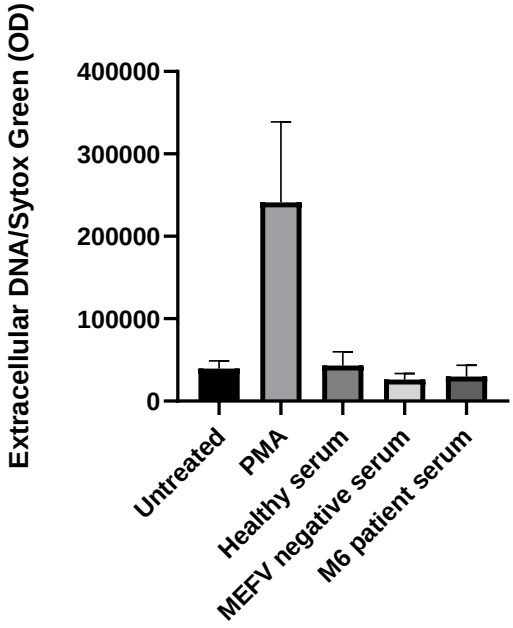
D.


Extracellular DNA/Sytox Green(OD)
400000
300000
200000
100000
0
Untreated
PMA
Healthy serum
MEFV negative serum
M4 patient serum

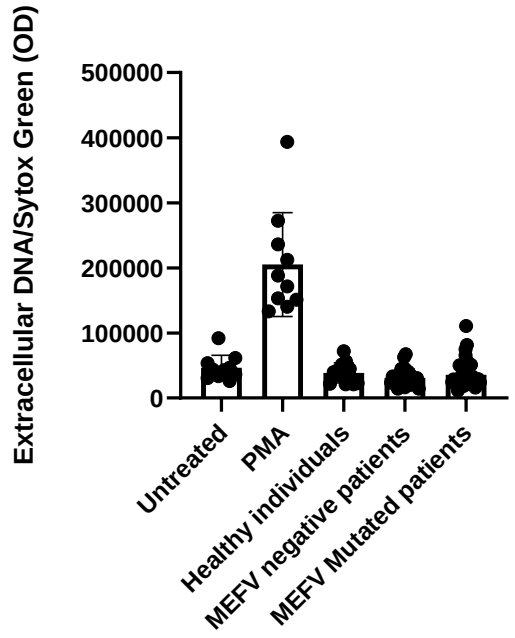
E.


Extracellular DNA/Sytox Green (OD)
400000
300000
200000
100000
0
Untreated
PMA
Healthy serum
MEFV negative serum
M5 patient serum

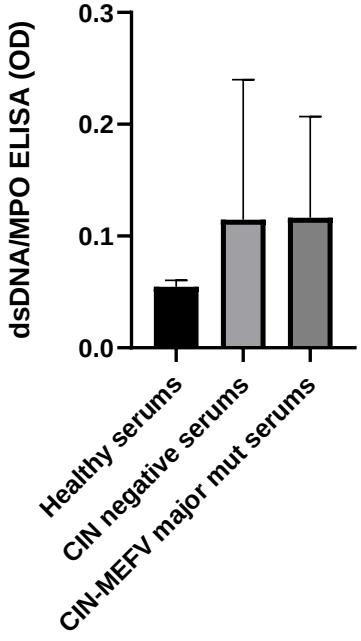
F.


Extracellular DNA/Sytox Green (OD)
400000
300000
200000
100000
0
Untreated
PMA
Healthy serum
MEFV negative serum
M6 patient serum

G.


Extracellular DNA/Sytox Green (OD)
500000
400000
300000
200000
100000
0
Untreated
PMA
Healthy individuals
MEFV negative patients
MEFV Mutated patients

H.


dsDNA/MPO ELISA (OD)
0.3
0.2
0.1
0.0
Healthy serums
CIN negative serums
CIN-MEFV major mut serums

I.

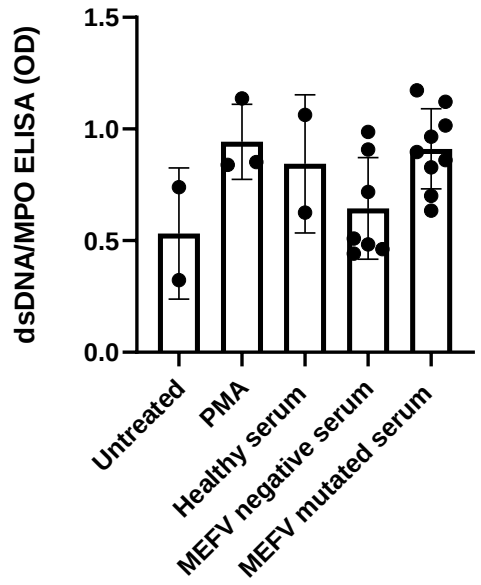
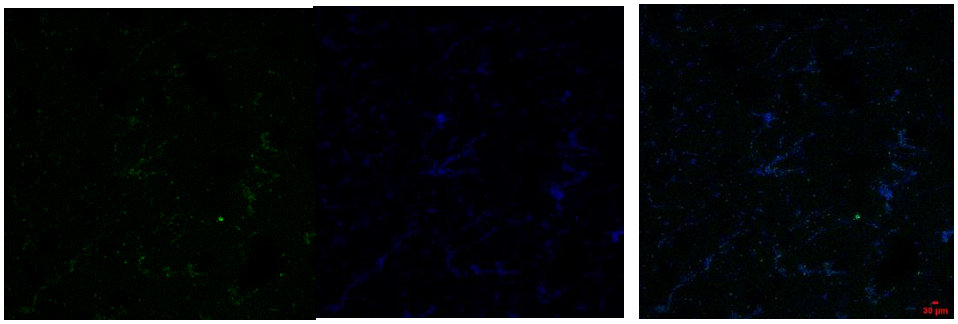
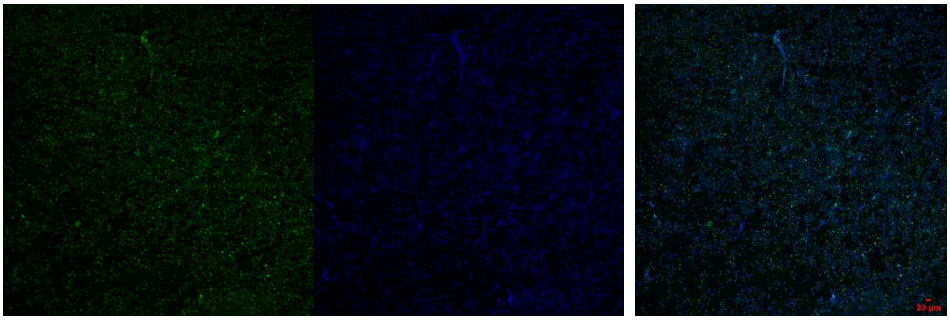


Figure 4.2. Screening of different serums from CIN patients positive and negative for *MEFV* mutations and comparison of NETosis induction between them and healthy individuals' serums. **(A-F)** Extracellular DNA measurement with Sytox Green staining as an indication for NETs release from control PMNs stimulated with serums from the 6 *MEFV* mutated patients M1-M6, respectively. Each patient's serum (M1-M6) was compared to healthy controls and *MEFV* negative patients. Five independent experiments were conducted for every serum (mean ± SD represented). **(G)** Total analysis of the previous data for the Sytox Green assay, presenting the categories for comparison in a dot-plot graph. **(H)** ELISA for the detection of DNA/MPO complexes directly in serums from *MEFV* positive and negative patients and healthy individuals (mean ± SD). **(I)** dsDNA/MPO complexes levels specifically associated with NETs release from stimulated control PMNs as measured with ELISA. One independent experiment represented in a dot-plot graph (mean ± SD).

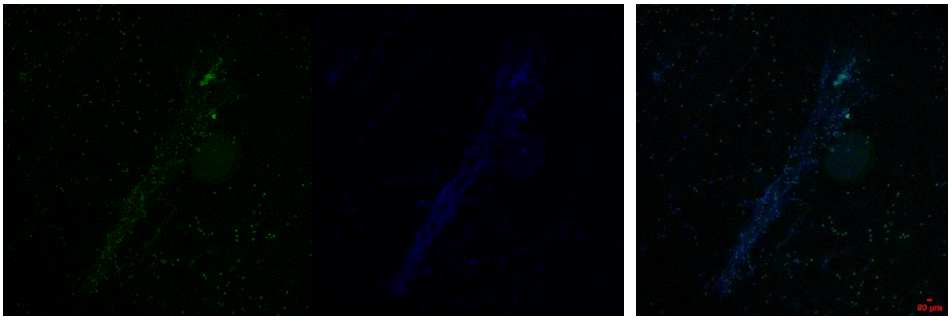
In addition to the Sytox and ELISA experiments, we cultured control PMNs on coverslips for immunostaining with anti-NE antibody and DAPI. In this way, NETs extruded from PMNs treated with serum, were visualized with confocal microscopy. For the short-term culture healthy PMNs were treated with serum from patients: M1 and M2 who carry *MEFV* mutations and patients N1 and N2 who are negative for *MEFV* mutations. Also, PMNs were treated with autologous healthy serum. As controls were used untreated and PMA treated cells. Confocal microscopy was conducted in 10x magnitude with z-stack analysis and 30μm scale. Green represents NE and blue the DNA. A general increase of NETosis in CIN patients both positive and negative for *MEFV* mutations was indicated as compared with healthy serum treated cells **(Figure 4.3)**.



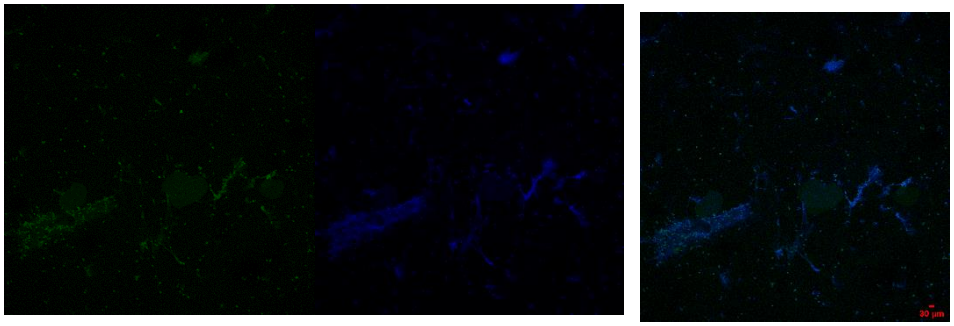
Control PMNs/Untreated



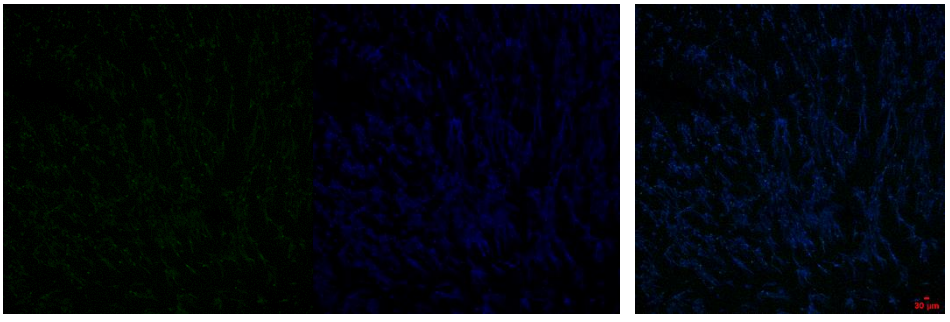
Control PMNs/PMA treated



PMNs treated with MEFV mutated patient's serum M1



PMNs treated with MEFV mutated patient's serum M2



PMNs treated with MEFV negative patient's serum N1

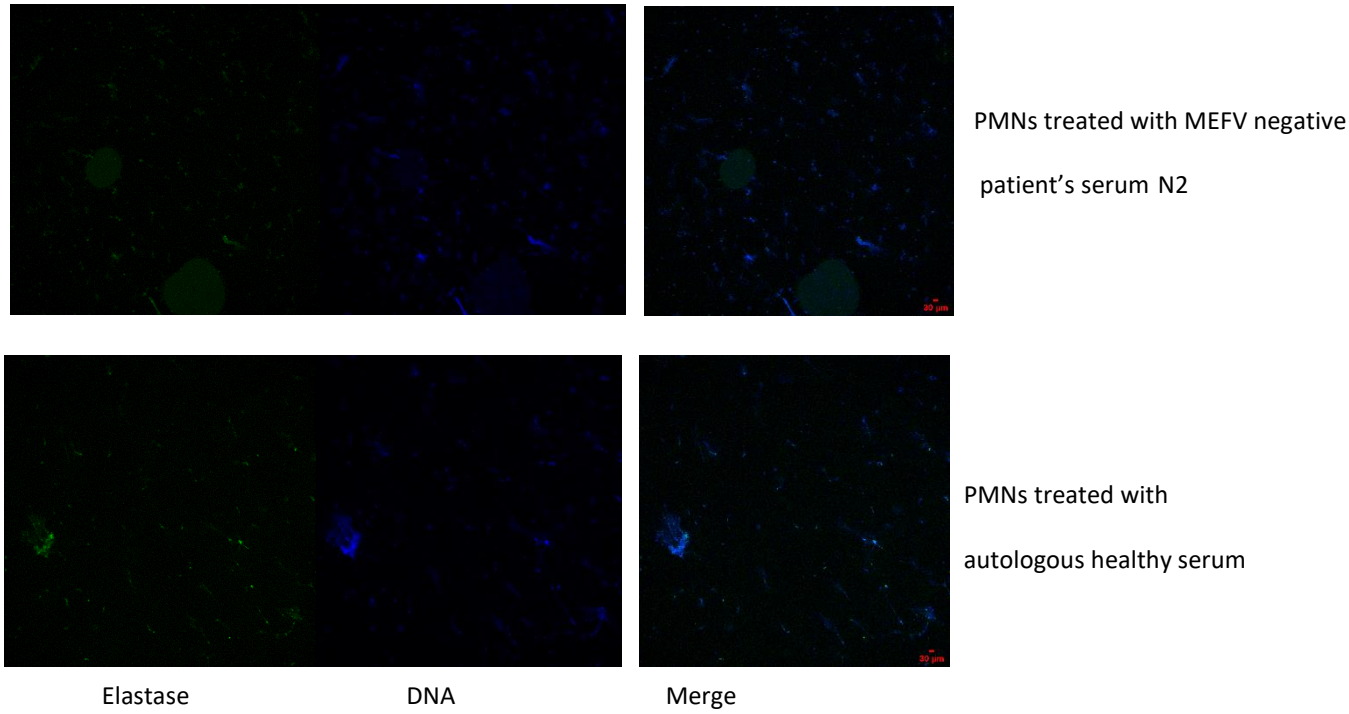


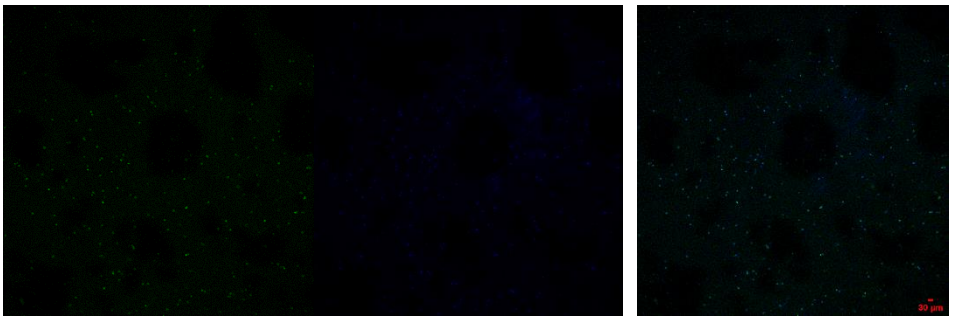
Figure 4.3. Immunostaining of serum treated control PMNs to compare NETosis in *MEFV* negative and *MEFV* mutated patients. Healthy PMNs treated with different serums for 3 hours and then stained with anti-Neutrophil Elastase (NE), and DAPI for DNA. Representative confocal images (scale bar 30μm, z stack analysis, 10x magnitude) show the characteristic co-localization of NE (green) and DNA (blue) on NET structures.

We also counted the formed NETs in an attempt to calculate the proportion of NET releasing cells among the total number of cells on each coverslip. Since the activated cells in some cases create clusters and it is difficult to detect the exact number of cells and NETs, we decided to divide the observed NETs in three classes and measure NETs in each class separately. NETs projecting clearly from one cell only were grouped as class I. NETs connected to many cells creating clusters of approximately 5-50 cells, were considered as class II and finally clusters of many more than 50 cells were grouped as class III. The quantification verified the microscope observation that serum from CIN patients induce increased NETosis in control PMNs as compared with healthy serum **(Table 2)**.

Table 2. Quantification of the released NETs from control PMNs when treated with serum from healthy donor or CIN patients with or without *MEFV* mutations.

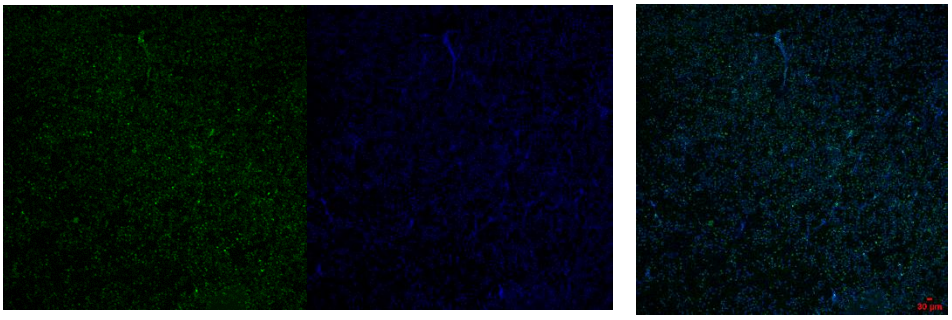
Healthy PMNs	Untreated	PMA treated	Autologous (healthy) serum treated	M1 patient's serum treated	M2 patient's serum treated	N1 patient's serum treated	N2 patient's serum treated
Class I	125	350	285	242	226	280	140
Class II	7	71	27	22	30	32	67
Class III	3	5	3	2	1	1	1

Finally, to get a view of the result of every *MEFV* mutated patient's serum on control PMNs and NETosis, we treated PMNs with serums from patients M1-M6. Then we stained for NE and DNA and took images at the confocal microscope. No excessive NETosis was detected in the conditions where healthy PMNs were treated with patients' serums in comparison to treatment with healthy serum or to the negative control, the untreated cells. The representative images are presented at **Figure 4.4**.



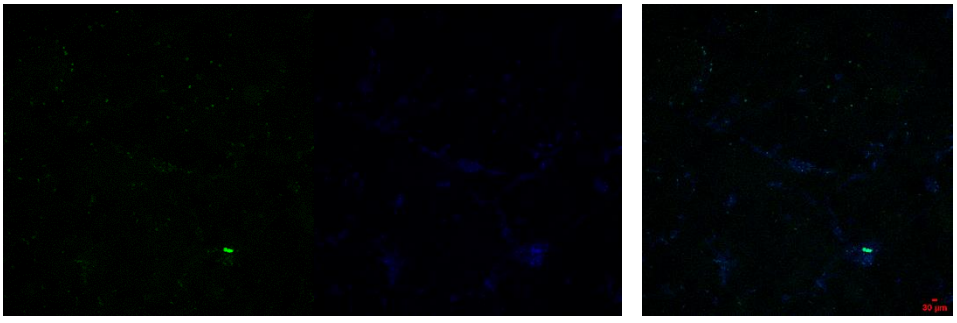
Control PMNs

Untreated



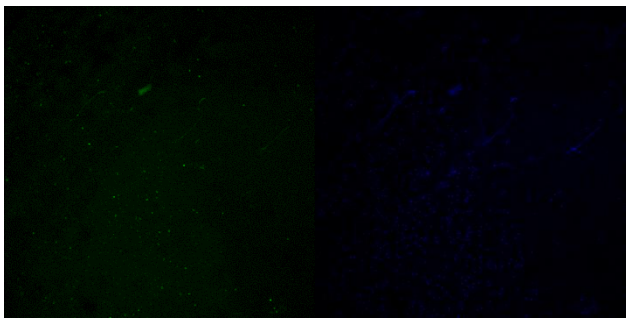
Control PMNs

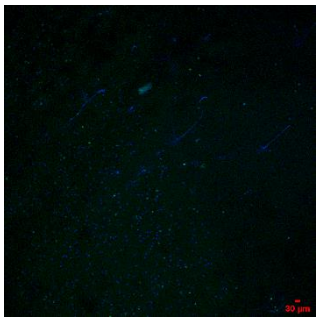
PMA treated



Control PMNs

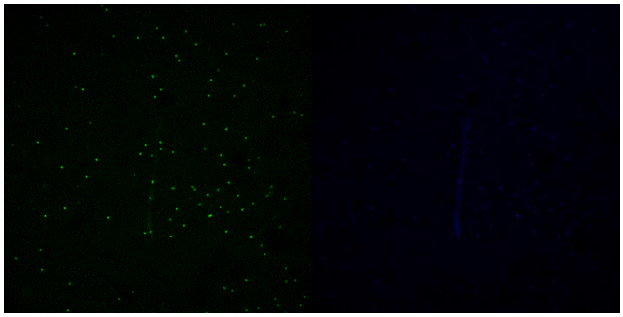
Healthy serum treated

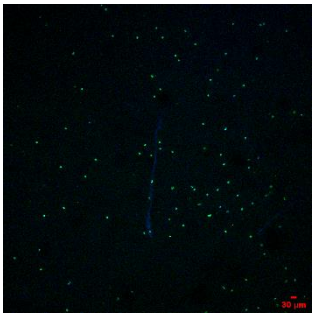




Control PMNs

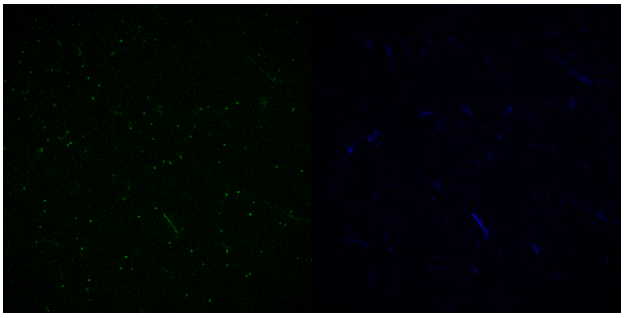
M1 patient serum treated

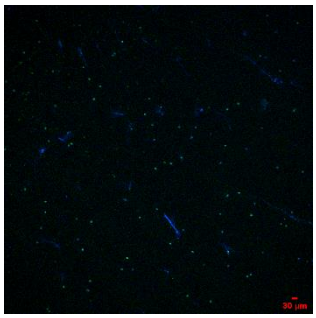




Control PMNs

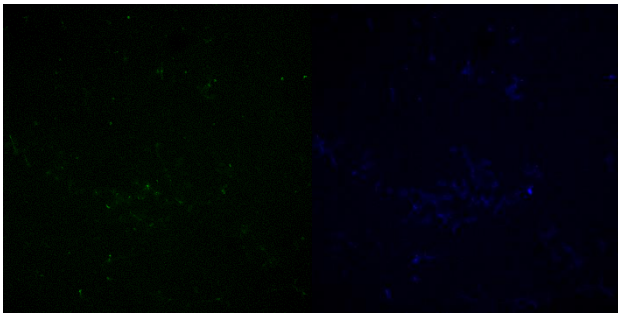
M2 patient serum treated

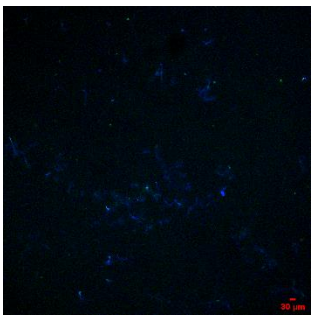




Control PMNs

M3 patient serum treated





Control PMNs

M4 patient serum treated

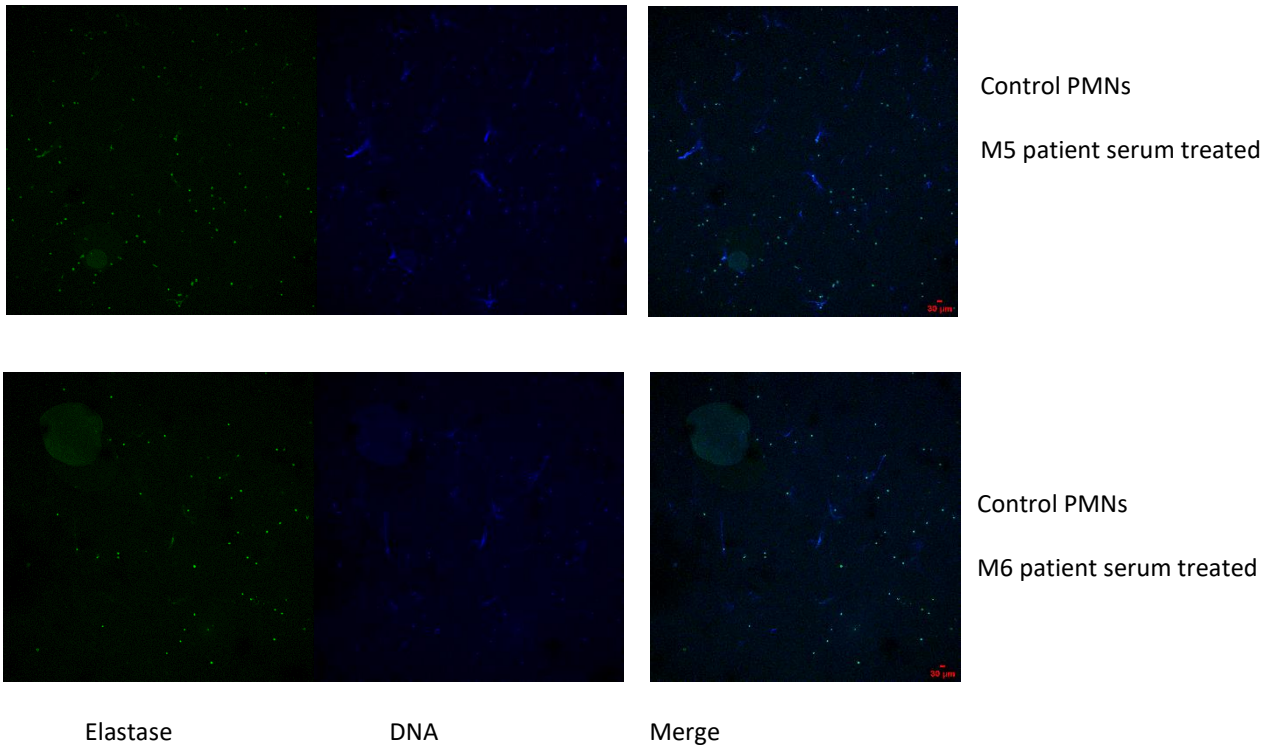


Figure 4.4. Immunostaining of control PMNs treated with serum from all the *MEFV* mutated patients of the cohort. Staining with anti-Neutrophil Elastase (NE), and DAPI for DNA. Representative confocal images (scale bar 30μm, z stack analysis, 10x magnitude) are presented foe each condition.

EX VIVO CULTURE OF PATIENTS' NEUTROPHILS TO DIRECTLY DETECT THE SPONTANEOUS NETOSIS

From the in vitro experiments, as described above we tried to elucidate the role of *MEFV* mutations in the inflammation and inflammatory cytokines' levels in the serum of blood of the CIN patients carrying them by means of NETosis detection. The hypothesis is that *MEFV* mutations enhance the inflammatory environment in the blood and this in turn changes the immune responses of patients, with increased NETosis to be one important change. The previous experiments are based on serums from patients but of high importance is also to investigate the functionality and activation of patients' neutrophils directly.

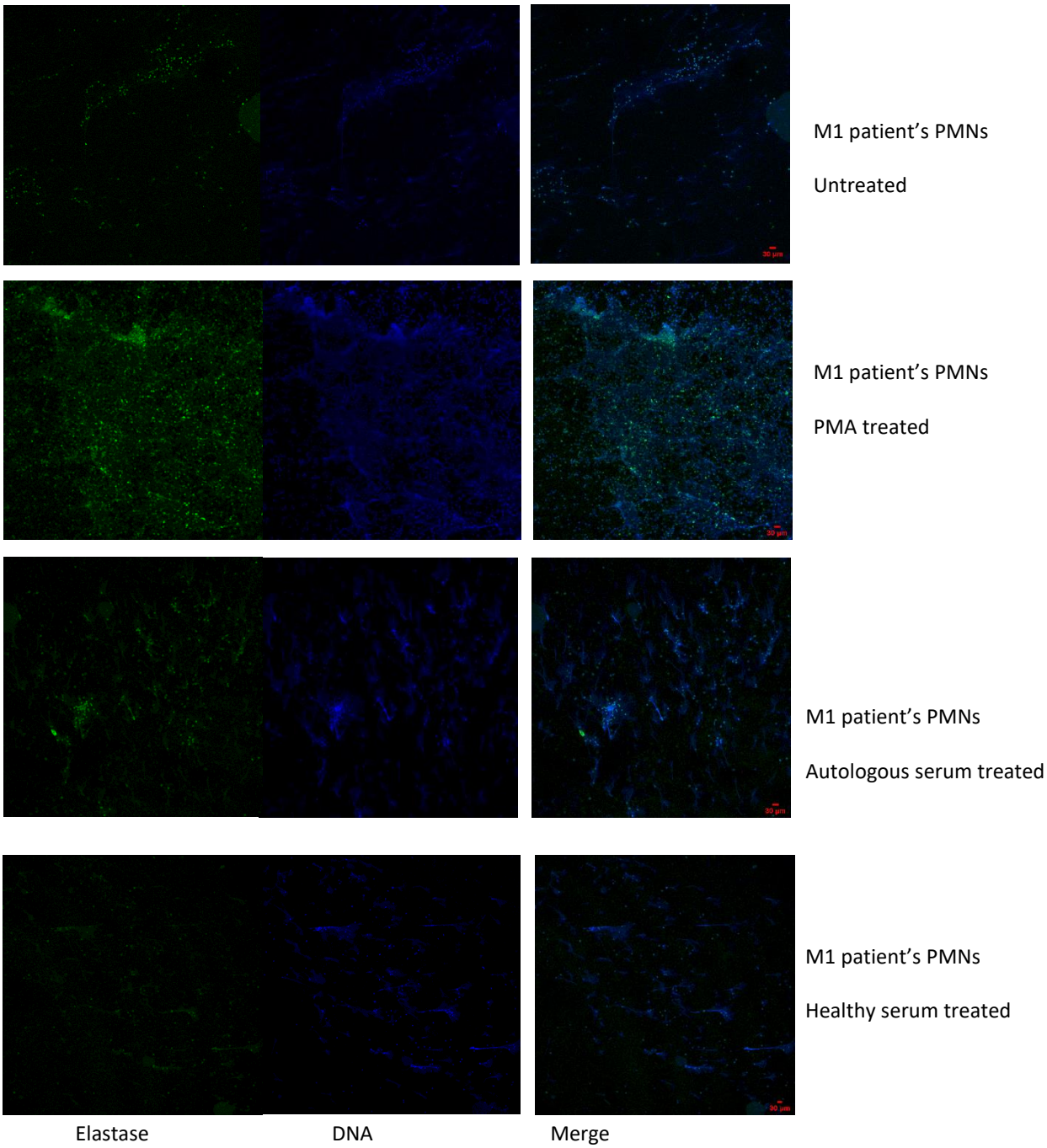
We isolated PMNs from patients that carry or not *MEFV* mutations and cultured them directly on coverslips to detect the spontaneous NETosis they may undergo due to their previous activation in vivo. We supplemented with RPMI medium and 1% FBS and cultured for three hours. After that we stained for NE and DNA and took images in the confocal microscope (scale bar 30μm, z stack analysis, 10x magnitude). In correlation with the immunostaining results from the in vitro co-culture of control PMNs with patients' serums (Figure 4.3), the patients' neutrophils were

more activated in comparison to healthy cells regardless their *MEFV* genetic status. PMNs from each patient were treated either with healthy serum or with autologous serum, but no significant difference was observed between the two conditions **(Figure 4.5 A-D)**. In addition to the observations coming from the images of the PMNs stained for NETs, we also counted the NETs released from activated neutrophils with the method described in the previous part of the results. The results from this counting are represented in **Table 3** and verify the microscope observations.

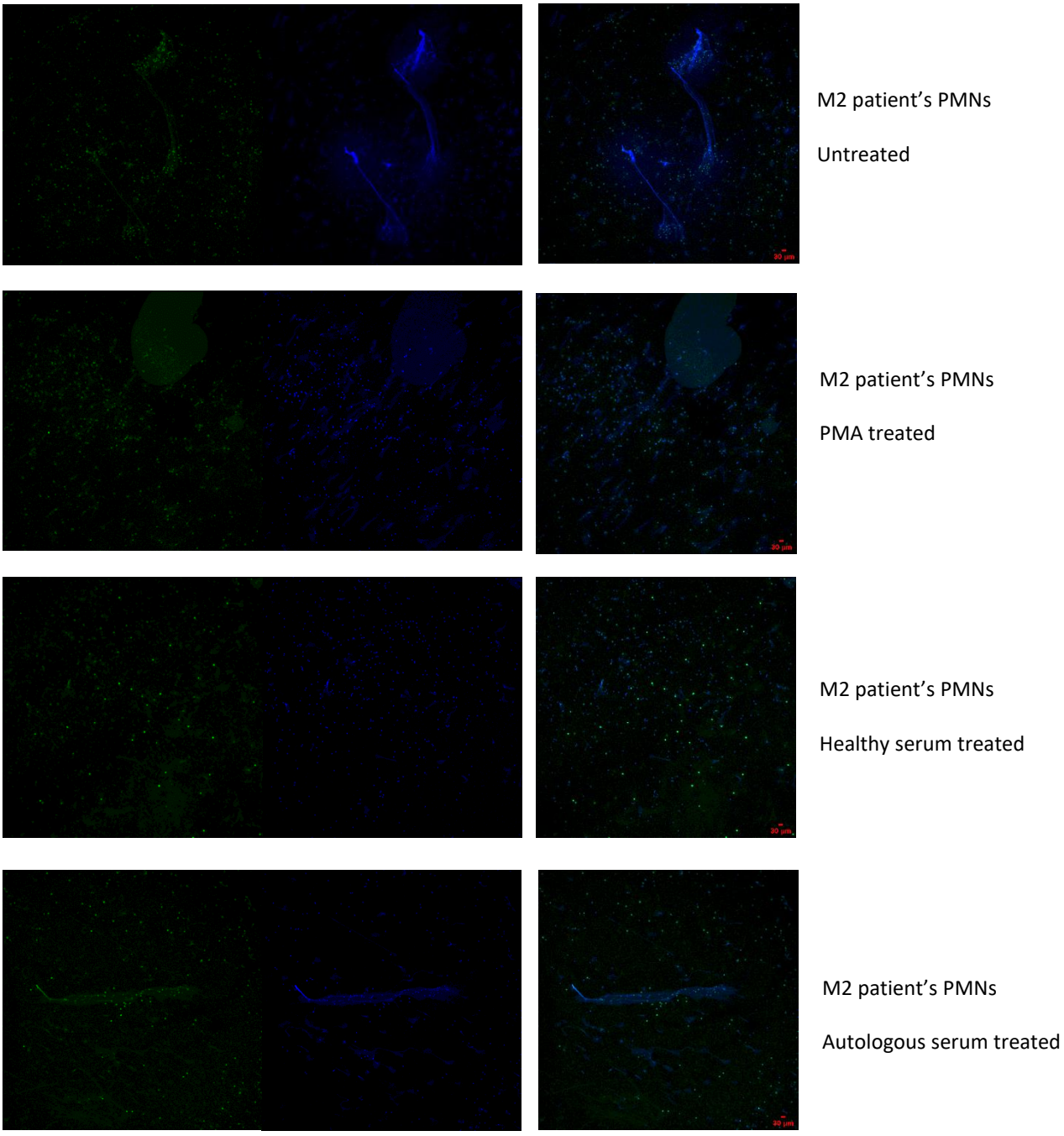
Table 3. Quantification of the released NETs from Neutrophils from patients and healthy donor cultured ex vivo for three hours with either autologous or healthy serum.

	Untreated PMNs	**PMA treated**	**Autologous serum treated**	**Healthy serum treated**
M1 patient				
Class I	223	200	182	235
Class II	27	44	43	27
Class III	3	3	1	-
M2 patient				
Class I	289	176	149	99
Class II	10	1	21	4
Class III	4	-	-	-
N1 patient				
Class I	320	91	300	277
Class II	31	44	9	19
Class III	4	3	1	1
N2 patient				
Class I	250	300	143	192
Class II	50	60	100	49
Class III	-	12	-	-
Healthy individual				
Class I	134			
Class II	8			
Class III	3			

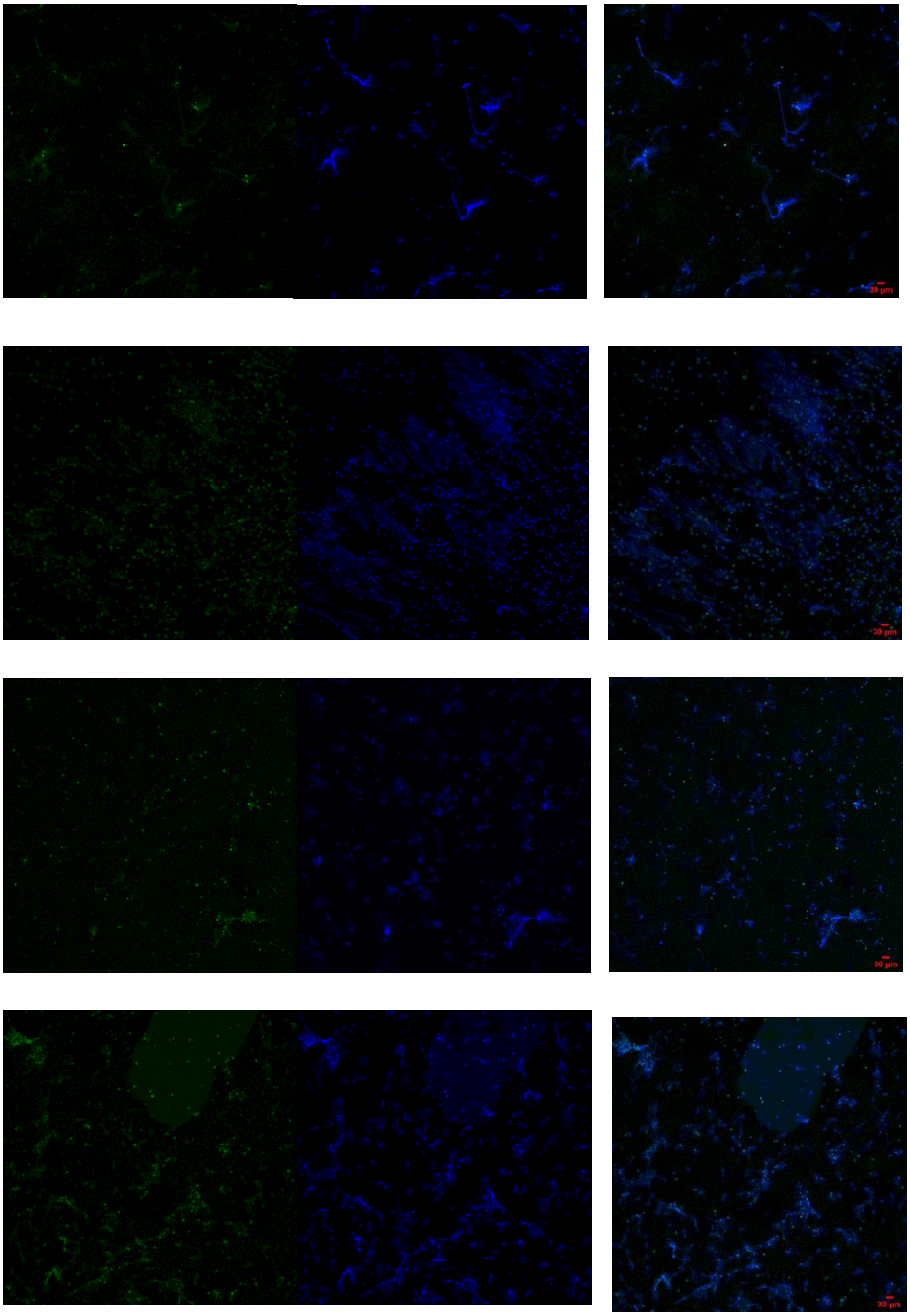
A.


M1 patient's PMNs
Untreated
M1 patient's PMNs
PMA treated
M1 patient's PMNs
Autologous serum treated
M1 patient's PMNs
Healthy serum treated
Elastase
DNA
Merge

B.


M2 patient's PMNs
Untreated
M2 patient's PMNs
PMA treated
M2 patient's PMNs
Healthy serum treated
M2 patient's PMNs
Autologous serum treated
Elastase
DNA
Merge

C.


N1 patient's PMNs
Untreated
N1 patient's PMNs
PMA treated
N1 patient's PMNs
Healthy serum treated
N1 patient's PMNs
Autologous serum treated
Elastase
DNA
Merge

D.

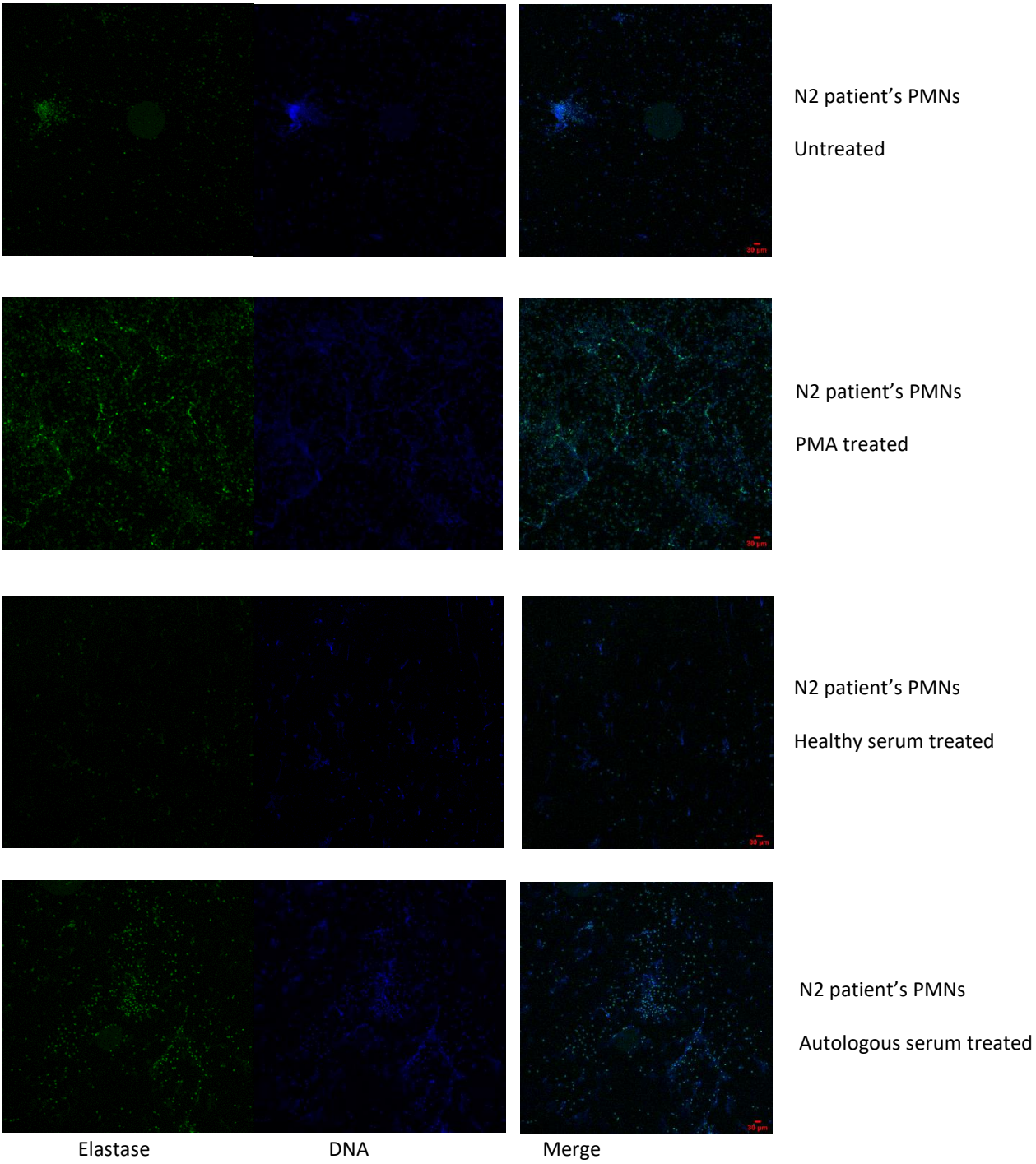


Figure 4.5. Direct ex vivo culture of patients' PMNs treated either with autologous or with healthy serum to elucidate the levels of spontaneous NETosis. (A-D) Immunostaining for NETs with anti-NE antibody for Elastase and DAPI for DNA of neutrophils isolated from two *MEFV* mutated patients (M1, M2) and two *MEFV* negative patients (N1, N2). Representative confocal images (scale bar 30μm, z stack analysis, 10x magnitude).

5. DISCUSSION

NETosis is a neutrophils' function which constitutes one of the main defense mechanisms of these cells and wasn't elucidated until 2004 (Brinkmann et al., 2004). Since then, intense research in the field of NETs indicated among other things, NETs deleterious effect in autoinflammatory and autoimmune diseases. Increased NETs formation and defective NETs removal leads to increased levels of NETs and consistent NETosis via a positive feedback mechanism of activation of the process. NETs formation in the context of diseases follows a different mechanism than in normal immune defense, as the extruded NETs are not only higher in number but also decorated with proteins specifically defective for the particular disorder. Such disorder is proven to be FMF, where the attack periods are characterized by excessive NETs formation by neutrophils that have entered the state of autophagy. This is attributed to the inflammatory environment of these patients and other undetermined factors. The produced NETs are extruded decorated with IL-1b, which activates monocytes to produce IL-1b and again prime neutrophils to undergo NETosis, which constitutes a vicious cycle. Despite the characterized mechanism of the disease, FMF patients are highly heterogenic as regards their genetic background, as they carry many different Single Nucleotide Variants (SNVs) on the *MEFV* gene locus. The genetic aberrations have to do mainly with the exons 2 and 10, which encode two domains with important functions in autophagy and immune responses, the PYD and the B30.2 domain. Such mutations in the *MEFV* gene were found in a cohort of CIN patients (Skendros et al., 2021), who interestingly had no signs of the clinical phenotype of FMF. The question rose from these observations is whether these patients have dysregulations in their immune system responses and if they share common characteristics with FMF patients on a cellular and molecular level, despite their lack of clinical manifestations of FMF. Another question would be whether the molecular mechanisms underlying CIN counterbalance the FMF attack mechanisms.

Disease-related *MEFV* mutations in exons 2 and 10 were detected with nonisotopic RNase cleavage assay (NINCA) and the results were confirmed with NGS analysis. Likely pathogenic mutations (A744S, M694V, K695R, I720M, M680I) were found in 20% of the tested population whereas 24% was found carrier of the R202Q polymorphism in exon 2 in heterozygosity whose association with FMF in the Greek population is controversial. The rest 56% of the patients was negative for the tested *MEFV* variants.

In the case of *MEFV* gene, there is no consensus in the classification of variants regarding clinical information. Also, only 55.7% of the variants could be successfully classified with almost a third of them being variants of uncertain significance (VOUS). A five-tier classification of genetic variants is provided by guidelines, reported by the American College of Medical Genetics (ACMG) and the Association for Molecular Pathology (AMP) categorizing variants in pathogenic, likely pathogenic, VOUS (or VUS), likely benign and benign. Due to differential classification of variants

in different databases and the complexity of the inheritance of different mutations, the need for improvement of classification of many unsolved, unclassified or of uncertain significance *MEFV* gene variants emerged. For FMF, a well-curated locus-specific database is available at INFEVERS (https://infevers.umai-montpellier. fr/web/) containing information for more than 500 variants.

In a recent study published in 2020, Accetturo et al used innovative bioinformatic methods of gene-specific calibration of prediction algorithms for FMF variants' interpretation. Trying to resolve the problem of miss-classification and of the high numbers of unsolved variants, they firstly mapped the variants along the *MEFV* coding sequence to confirm the pathogenic and likely pathogenic clusters on the SPRY and the PYD and the zf-B box domains, respectively. Next, they used Rare Exome Variant Ensemble Learner (REVEL), which has been demonstrated to outperform most of the available predictors, to score both classified and unclassified variants of the *MEFV* gene. Although, this method was perfect in identifying the benign and likely benign variants (97.2%) while only 13 pathogenic/likely pathogenic variants (31.7%) demonstrated a score above the threshold for pathogenicity. Therefore, they computed a novel REVEL threshold for pathogenicity that allowed the proposal of a novel classification for all the *MEFV* variants previously considered as VOUS or unsolved. They also indicated that *MEFV* pathogenic variants are non-randomly distributed. Even with this novel tool for classification, the need for the establishment of functional assays to experimentally validate variants interpretation is urgent (Accetturo et al., 2020).

A classification of the *MEFV* variants found in the CIN cohort of patients, was conducted based on different databases and classification according to the bibliography.

Table 4. Classification of the *MEFV* variants under investigation, based on different databases.

	A744S	**M694V**	**K695R**	**I720M**	**M680I**	**R202Q**
INFEVERS	VOUS	Pathogenic	Likely Pathogenic	VOUS	Pathogenic	Benign
CLIN VAR	Conflicting	Conflicting	Conflicting	VOUS	Conflicting	Benign
UNIPROT	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Benign
ACGM classification	VOUS	Pathogenic	Pathogenic	-	Pathogenic	Benign

*VOUS= Variant of Undetermined Significance

For the evaluation of the significance of each variant in the pathogenesis of a disease more factors should be considered. Such factors are the hetero- or homo- zygous state of the variant, the combination of different *MEFV* variants in one patient and the possible interaction of the *MEFV* variants with mutations carried in other genes. So, a global evaluation of the genetic

background of each patient should be done and any speculation should be tested with functional experiments.

In our study, we grouped the patients of the cohort in two categories: CIN patients that carry likely pathogenic *MEFV* mutations and CIN patients negative for *MEFV* mutations. The aim is to determine any differences in NETosis between these categories.

Patients that were found to carry the R202Q polymorphism in heterozygosity could form another category of comparison and evaluation of its significance in CIN. The conflict around the association of the R202Q polymorphism with FMF is based on its classification as benign and the hypothesis that it might be disease-related in the Greek population since an aberration of the Hardy–Weinberg principle was detected among Greek FMF patients, but not among healthy controls. It was also suggested that the polymorphism could have a potential dosage-dependent deleterious effect depending on its frequency in homozygosity or heterozygosity in healthy individuals and patients (Giaglis et al., 2007). So, basically heterozygotes could be considered benign since it is a common variant in the general population, but homozygotes could have a pathogenic effect. Here, we analyzed our results considering the R202Q heterozygous patients a different category for comparison with the *MEFV* mutated and *MEFV* negative patients. As expected, there was no difference of these patients as compared to *MEFV* negative patients in terms of NETosis. So, we decided to include these patients to the *MEFV* negative category.

The technique we used to detect the number of NETs in the supernatants of serum-treated PMNs was the Sytox green assay. Sytox green stains the extracellular DNA as it can only penetrate the compromised plasma membranes. Apart from NETs, extracellular DNA comes from apoptotic and dead cells. So, it can be considered as an indication of NETs quantity and be used for the comparison of different conditions, as the number of dead cells in each case could vary depending on the health state of the donor the particular time point, the time of inactivity between the point the blood was taken and the beginning of the experiment and the manipulation of the particular sample by the investigator. Though, there are newest, high-throughput methods that can exclude this parameter and quantify NETosis based on both the extruded NETs and the cells undergoing NETosis in early stages, distinguishing NETosis from apoptosis. Such method is the IncuCyte ZOOM platform, a real-time quantification tool which is based on live imaging and an analysis software with many possibilities that can make accurate and selected calculations to improve specificity and sensitivity of NETosis detection (Gupta et al., 2018).

Another technique that we broadly used in our study is immunofluorescence microscopy, which is the gold standard method for quality and quantity assessment of structures and processes that can be visualized with fluorescence staining. The drawback of this methods though, is the subjective evaluation by the researcher. The difficulty of discriminating all forms of NETosis in

the microscope is more complex when it comes to NETs investigation. A recent publication in Nature portfolio, explains the different morphologies of NETs and classifies NETs in early and late phase NETs based on the shape of the cell, the nucleus lobes, the membrane projection and the MFI of NE and histone HA2 while the later ones are also sub-divided in web like and non-web like NETs based on their plasma membrane projection length. This extended analysis of NETs structure indicates the complexity and the multi-parameters' evaluation that should take place to minimize errors when analyzing NET related results (Thubthed et al., 2022). The IncuCyte live imager platform can also optimize this method, since all the events of NETosis can be captured, even in early stages and make automated calculations.

Our results are divided in three groups of experiments. First, we determined the NETosis status of one patient who carries two pathogenic *MEFV* variants in homozygosity and was the most possible to have a dysregulated immune system response. The results indicated an increase in NETosis induction and NETs formation in the particular patient as compared with a *MEFV* negative patient and a healthy individual. After that we categorized the *MEFV* mutated patients in one group and conducted a screening for NETosis induction compared to a group of *MEFV* negative patients and a group of healthy individuals. The statistical analysis indicated no significant differences between the three groups. Finally, we did ex vivo experiments where we isolated neutrophils directly from patients to observe the spontaneous NETosis their neutrophils were undergoing and as a result their activation level due to the patients' inflammatory environment. Again, the three categories were compared based on immunostaining experiments and NETs were quantified in each condition. Microscopy observations indicated an increase in NETs levels in CIN patients independent of their *MEFV* status as compared with healthy individuals, but with no statistical significance.

The general conclusion of the study is that *MEFV* mutations are not considered significant in terms of NETosis induction in CIN patients who carry them. However, it is important to evaluate other factors implicated in neutrophils' activation status, before concluding that the immune system of CIN patients carrying *MEFV* mutations is not dysregulated like in FMF patients. Such factors are the investigation of the autophagy status of neutrophils in these patients. In FMF attacks, neutrophils enter an autophagic state through the REDD1 elevated expression and the activation of the mTOR/autophagy pathway. For this purpose, we are currently isolating RNAs from our patients to conduct RNA arrays and qPCRs specific for autophagic genes. Another factor indicative of neutrophils' activation is the production of pro-inflammatory cytokines. Detection of elevated levels of such molecules in the serum of patients would indicate persistent inflammation, such as IL-1b which is specifically produced in the case of FMF.

In conclusion, although we detected no differences in CIN patients with *MEFV* mutation in terms of NETosis, the molecular and clinical characterization of this cohort of CIN patients would be

completed with the investigation of neutrophils' activation state as described above. Neutrophils' activation mechanism in FMF patients (Skendros et al., 2017) and part of the molecular mechanism of CIN (Papadaki et al., 2003, 2005; Papadaki & Eliopoulos, 2009) are already known, but whether *MEFV* mutations have a clinical significance in CIN is an open field of research. The significance of this study is indicated by the potential change in the categorization of CIN patients and their follow-up approach in the future. Since CIN is a highly heterogenic disease and its diagnosis is based on the exclusion criteria, it is crucial to investigate every new finding in these patients to come closer to the elucidation of the molecular mechanism of CIN which is not clearly defined yet and constitutes an open field of research.

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