UNIVERSIDADE DE SÃO PAULO
INSTITUTO DE QUÍMICA
Programa de Pós-Graduação em Ciências Biológicas (Bioquímica)

GABRIELLY ALEXANDRIA DE MOURA FREITAS

A MAP OF NEUTROPHIL GRANULES BY PROTEOMICS

São Paulo

Data depósito: 31/03/2022

(Versão corrigida em 27/06/2022)

GABRIELLY ALEXANDRIA DE MOURA FREITAS

MAPA DOS GRÂNULOS DO NEUTRÓFILO POR PROTEÔMICA

Dissertação apresentada ao Instituto de Química da Universidade de São Paulo para obtenção do Título de Mestre em Ciências (Bioquímica).

Orientadora: Profa. Dra. Graziella Eliza Ronsein.

São Paulo

2022

Ficha Catalográfica elaborada eletronicamente pelo autor, utilizando o programa desenvolvido pela Seção Técnica de Informática do ICMC/USP e adaptado para a Divisão de Biblioteca e Documentação do Conjunto das Químicas da USP

Bibliotecária responsável pela orientação de catalogação da publicação:
Marlene Aparecida Vieira - CRB - 8/5562

A374m

Alexandria, Gabrielly
Mapa dos grânulos do neutrófilo por proteômica / Gabrielly Alexandria. - São Paulo, 2022.
65 p.

Dissertação (mestrado) - Instituto de Química da Universidade de São Paulo. Departamento de Bioquímica.
Orientador: Ronsein, Graziella Eliza

1. Neutrófilos. 2. Grânulos. 3. Fracionamento. 4. Proteômica. I. T. II. Ronsein, Graziella Eliza, orientador.

Universidade de São Paulo
Instituto de Química

"Mapa dos neutrófilos por proteômica"

GABRIELLY ALEXANDRIA DE MOURA FREITAS

Dissertação de Mestrado submetida ao Instituto de Química da Universidade de São Paulo como parte dos requisitos necessários à obtenção do grau de Mestra em Ciências obtido no Programa Ciências Biológicas (Bioquímica) - Área de Concentração: Bioquímica.

_____

Profa. Dra. Graziella Eliza Ronsein

(Orientadora e Presidente)

APROVADO(A) POR:

_____

Profa. Dra. Sayuri Miyamoto

IQ – USP

_____

Prof. Dr. Giuseppe Palmisano

ICB - USP

_____

Profa. Dra. Leda Quercia Vieira

UFMG

SÃO PAULO

27 de maio de 2022

Dedico este trabalho à minha avó Maria do Socorro Alexandria de Moura.

AGRADECIMENTOS

Tenho muitas pessoas incríveis que me permitiram e ajudaram a chegar ao fim desta árdua jornada que foi meu mestrado e, por isso, já sou muito grata. Quero começar agradecendo aos meus pais, Suzana e Renato, por apoiarem meu sonho e me ajudarem de todas as formas que eles podiam para que ele se realizasse. Obrigada imensamente por todo o amor, cuidado, carinho e apoio que me dão e por sempre tentarem entender meu ponto de vista, mesmo que as vezes seja difícil. Não posso deixar de agradecer também à minha irmã, Grasiella, por todo o carinho, conversas e memes compartilhados que alegraram minha rotina e me permitiram sorrir até nos momentos mais solitários durante o longo isolamento social resultante da pandemia. Eu amo vocês e tenho plena consciência que não seria quem sou ou estaria onde estou sem a presença de vocês na minha vida. Para finalizar esse pequeno núcleo familiar, mesmo que ele nunca possa compreender essa mensagem, não posso deixar de agradecer ao meu lindo gatinho resgatado, meu filho de quatro patas, Thackey Binx, que foi minha única companhia física durante meus 5 meses de isolamento social completo, e que foi minha âncora para não enlouquecer. Pode parecer bobo, mas ele foi (e é) minha luz na escuridão e uma fonte de amor e carinho inesgotável.

Quero agradecer agora à minha incrível orientadora, Graziella Ronsein, que meu deu uma chance após uma breve conversa e topou me guiar nesse trabalho incrível que pudemos desenvolver. Você é uma pesquisadora incrível, de grandes ideias e cujo entusiasmo pela ciência é simplesmente cativante e inspirador. Além de tudo isso, você ainda é uma mulher forte e com um coração enorme com quem sempre senti que podia contar e fez com que eu me sentisse segura. Você fez de tudo para me dar um lar longe do meu e sou imensamente grata por isso. Nesses breves 3 anos pude aprender tanto com você que não sou capaz de colocar em palavras o quão engradecer tudo foi para mim. Obrigada por me ensinar e por me ouvir, por permitir sempre que eu trouxesse ideias a você e por confiar tanto em mim. Sentirei saudades

de nossas conversas e dos nossos longos dias lado a lado na bancada, fazendo experimentos até tarde.

Não posso deixar também de agradecer aos meus colegas de laboratório, a quem muito tagarelei sobre filmes, séries, músicas e tudo mais. Como todos que me conhecem, sabem, sou uma grande tagarela e sou capaz de falar sobre, basicamente, qualquer assunto por tempo demais. Douglas Ricardo, Lorenna Rocha, Amanda Ribeiro, Rafaela Nascimento, Stella Boutris, Rebeca Bonfim e André Luiz obrigada por terem me recebido de braços e corações abertos e pela amizade tão incrível de vocês. Obrigada por todas as conversas, risadas e por ouvirem minhas reclamações e tristezas quando os experimentos falhavam. Obrigada também por toda ajuda e pelo tanto que me ensinaram, nada disso também seria possível sem vocês. Vou sentir imensas saudades de nossas conversas diárias e nossas trocas de comidas e doces. Quero também agradecer demais às técnicas Agda Bertolucci, Mariana Massafera, Fernanda Manso Prado e Izaura Nobuko por todo o apoio nesses anos de trabalho e ajuda. Um agradecimento especial à Mariana, com quem trabalhei mais diretamente, e que me ensinou TANTO sobre proteômica e métodos de digestão. Obrigada Mari por embarcar nas minhas perguntas loucas de "por quê a gente usa essa coisa X? O que é essa coisa Y?". Obrigada pelas conversas sobre história política e sobre a razão dos nomes das coisas.

Nosso lab, no entanto, sempre foi muito plural e sempre "trocamos muitas figurinhas" com outros laboratórios e assim pude conhecer um montão de gente incrível que fez meu trabalho ser possível e com quem compartilhei ótimos momentos. Desta forma, quero agradecer aos seguintes colegas: Bianca Vargas, Amanda Fahart (Fox), Hellen Valerio, Pablo Reis, Felipe Fuzita, Adriano Paulo Oliveira, Marcelo Donegá, Nathália Miranda, Matheus Favonoretto e Arthur Migliatti. Um agradecimento especial aos meus primeiros amigos no IQ-USP, Isaac Araujo e Cheila Gomes, obrigada por fazerem eu me sentir bem-vinda e compreender e compartilhar das minhas dificuldades de adaptação a São Paulo. Por fim, não posso deixar de

agradecer aos amigos que fiz na disciplina de bioquímica avançada, nosso laço indescritível foi forjado através de longas horas de estudo, reclamação e sofrimento: Ana Maria, Larissa Diniz, Lohana Lopes, Julian Serra, Lara Guimarães, Rafaela Naresi e Lucas Guerra. Por fim, quero agradecer a todos meus colegas representantes discentes por todos os debates e, principalmente, para minha parceira de COOPERA-IQ e congregação, Roberta Vincenzi. Todos vocês permitiram que minha jornada fosse mais leve, feliz e tranquila. Quero agradecer também aos professores cujo input ajudou muito a melhorar meu trabalho e que me permitiram usar seus equipamentos para experimentos: Marisa Gennari, Paolo Di Mascio, Ohara Augusto, Daniela Truzzi, Maurício Baptista e Flávia Meotti. Por último, mas jamais menos importantes, quero agradecer aos técnicos que muito me ensinaram Edlaine Linhares e, especialmente, Fernando Coelho. Fernando, você é uma pessoa de luz extremamente paciente e divertido e OBRIGADA por me ensinar tudo de gel-2D e western blot. Obrigada por sempre estar disponível para me ajudar a compreender melhor meus resultados e debater comigo como melhorar o resultado de cada blot.

Não posso deixar de agradecer aos amigos que, mesmo de longe, me mantiveram motivada e me ajudaram a ter forças para seguir em frente. Daniel Galinis, obrigada por sua parceria e por ter falado comigo quase que diariamente mesmo que fosse só para mandar um meme bobo e alegrar meu dia. Eu acho que jamais serei capaz de explicar o quanto eu sou grata por isso. Beatriz Alexandre, eu sou extremamente grata por te ter na minha vida. Você é uma mulher incrível, de coração gigantesco e que sabe tanto me aconselhar quanto puxar minha orelha quando necessário. Obrigada por sempre atender o telefone ou responder rápido as minhas mensagens, por ter me auxiliado a passar pelos momentos de tristeza e imensa saudade do meu lar, Niterói. Tamara Marques e Ítalo Faustino por sempre me surpreenderem com seu carinho imenso e companheirismo. Lucas Perne, na verdade já é meu irmão, que está ao meu lado há mais de 15 anos, e quem muito houve minhas reclamações e broncas. Obrigada pelo seu apoio

e por estarmos sempre crescendo juntos. Não posso deixar de mencionar meus amigos da época do Nação HP, que me acompanharam em tantas fases da vida, Amanda Souza e Beto Ferreira, que me acompanharam e apoiaram nessa nova etapa. Mandinha, obrigada pelo ano que dividimos em São Paulo e obrigada por me apresentar amigos e fazer eu me sentir mais querida pelos paulistanos. Beto, obrigada por ter tirado um final de semana para ir me visitar! Você não faz ideia do quão importante foi para mim e o quanto eu precisava daquilo e nem sabia. Preciso agradecer também aos amigos paulistas que fiz, através da Mandinha: Isa, Peter, Will, Doug e Marinara. Obrigada por toda e cada conversa e risada. Por fim, quero agradecer a querida Tah Brito, minha prima emprestada com quem pude reacendar a amizade da infância ao ir para São Paulo. Obrigada por partilhar das mesmas saudades que eu tinha, em especial a saudade de guaravita. Eu sou muito grata por todos vocês na minha vida!

Agora vou reservar um espaço para agradecer ao meu namorado, Bernardo Miranda. Meu amor, obrigada por acreditar em mim até quando eu mesma não acredito mais. Obrigada por sempre me apoiar e por me ensinar uma nova forma de amar, todo dia, me fazendo descontruir diversos pensamentos que a sociedade nos impõe de que amor é co-dependência. Obrigada por aceitar minhas manias, mesmo que não entenda, e por tudo o que construímos no nosso relacionamento nesses 7 anos. Sei que nem sempre sou boa expressando isso e reclamo muito, talvez mais do que deveria, mas eu te amo imensamente, sou muito grata por tê-lo em minha vida e sei o quão sortuda eu sou.

Quero agradecer aos órgãos que permitiram que esse projeto fosse feito, CAPES e FAPESP, pela bolsa de mestrado e pelo apoio financeiro durante esses 3 anos.

Para finalizar, por sei o quanto já estou me estendendo, quero agradecer à minha avó, Maria do Socorro, a quem dedico essa dissertação, que infelizmente faleceu em março de 2020. Vovó, sei o quanto você sempre valorizou o saber científico e acadêmico, e me dói intensamente você não poder estar presente na minha defesa de mestrado. Obrigada pela mulher inspiradora que

você foi e por me ensinar, já pequena, a expressar meus sentimentos através da poesia. Escrever foi, por muitas vezes, meu refúgio e hoje é minha ligação com você. Eu te amo vovó e sinto muito a sua falta. Para te homenagear, deixo aqui um singelo poema escrito por você para que seja imortalizado aqui junto ao meu trabalho.

Pensamento X

As palavras se organizaram, criaram asas, vestiram-se liricamente, se evadiram da prisão do pensamento e, ganharam o mundo para mostrar a força da poesia. Infiltraram-se em mentes e corações dando-lhes acesso as mais lindas e puras fantasias.

-Marsoalex

“Se no mundo houvesse mais loucos, haveria menos violência”

(Raphael Montes)

RESUMO

Neutrófilos são leucócitos polimorfonucleares que possuem papel fundamental na defesa do organismo. Essas células desempenham diversas ações a fim de assegurar a eliminação de um patógeno e, além disso, orquestram a resposta imune inata e adaptativa. O conjunto composto pelos grânulos de armazenamento e as vesículas secretórias compõe a principal estrutura fisiológica dos neutrófilos. Estes componentes são essenciais desde a ativação celular, participando de todas as funcionalidades desta célula. Os grânulos são subdivididos em azurófilos, específicos e gelatinase. Eles podem ser distinguidos por meio de seu conteúdo proteico e, como são importantes na funcionalidade dos neutrófilos, identificar quais proteínas são armazenadas nestas organelas é imprescindível para entender melhor essa célula como um todo. Algumas proteínas, estão presentes de forma abundante e, portanto, são utilizadas como marcadores dos grânulos. Tais proteínas são mieloperoxidase (MPO) para os grânulos azurófilos, gelatinase de neutrófilo associada a lipocalina (NGAL) e lactoferrina (LTF) para os específicos, metaloproteinase de matrix 9 (MMP9) para os grânulos de gelatinase e fosfatase alcalina (AP) para as vesículas secretórias. Isolar estas estruturas, no entanto, é desafiador visto que os protocolos existentes na literatura utilizam grandes volumes de amostra, cerca de 400 mL de sangue ou 3×10^8 neutrófilos, para apenas um isolamento, impedindo a realização de replicatas técnicas e biológicas. Desta forma, o objetivo do presente estudo foi desenvolver um protocolo miniaturizado de isolamento dos grânulos neutrofílicos e utilizar métodos bioquímicos, de proteômica e *machine learning* para investigar o conteúdo proteico destas estruturas celulares. Para isto, 40 mL de sangue periférico de três voluntários aparentemente saudáveis foi coletado. Os neutrófilos foram então isolados, lisados com cavitação de nitrogênio e o fracionamento subcelular foi realizado baseado em um gradiente descontínuo de 3 camadas de Percoll. O método de isolamento foi avaliado através da investigação dos marcadores utilizando western blotting (WB), zimografia de gelatina e ensaios enzimáticos em cada fração coletada. O isolamento demonstrou-se eficiente e permitiu uma ótima separação dos grânulos

em um gradiente menor que 1 mL, cerca de 37 vezes menor que os métodos atualmente descritos na literatura. Além disso, a análise proteômica foi capaz de identificar 369 proteínas presentes em pelo menos 3 das 5 réplicas investigadas e, utilizando ferramentas de *machine learning*, 140 proteínas foram classificadas como pertencentes a um dos tipos de grânulos ou vesícula secretória com alto nível de confiabilidade. Por fim, o presente estudo foi o primeiro a investigar o proteoma dos grânulos utilizando replicatas técnicas e biológicas, criando e fornecendo uma base de dados robusta que poderá ser utilizada em estudos futuros. Conclui-se, portanto, que a metodologia miniaturizada desenvolvida é eficaz, reprodutível e mais barata, além de permitir estudos mais complexos e profundos sobre o proteoma dos grânulos dos neutrófilos em diferentes momentos celulares, tais como quando ativados via estímulos distintos.

Palavras-chave: neutrófilos, grânulos, fracionamento, proteômica

ABSTRACT

Neutrophils are polymorphonuclear leukocytes that play a key role in the organism defense. These cells enroll in a range of actions to ensure pathogen elimination and orchestrate both innate and adaptative immune responses. The main physiological structures of neutrophils are their storage organelles that are essential since the cell's activation and participate in all their functions. The storage organelles are divided into 2 types: granules and secretory vesicles. The granules are subdivided into azurophilic, specific and gelatinase. The granules are distinguished by their protein content, and since they play an important role on the neutrophil function, the knowledge of the proteins stored in these organelles can help to better understand these cells. Some proteins are present in high abundance and are used as markers for each storage organelle. These proteins are myeloperoxidase (MPO) for azurophil granules, neutrophil gelatinase associated with lipocalin-2 (NGAL) and lactoferrin (LTF) for specific granules, matrix metalloproteinase-9 (MMP9) for gelatinase granules and alkaline phosphatase (AP) for secretory vesicles. The isolation of neutrophil's granules, however, is challenging and the existing procedures rely on large sample volumes, about 400 mL of peripheral blood or 3×10^8 neutrophils, not allowing for multiple biological and technical replicates. Therefore, the aim of this study was to develop a miniaturized neutrophil granule's isolation method and to use biochemical assays, mass spectrometry-based proteomics and a machine learning approach to investigate the protein content of the neutrophil's storage organelles. With that in mind, 40 mL of the peripheral blood of three apparently healthy volunteers were collected. The neutrophils were isolated, disrupted using nitrogen cavitation and organelles were fractionated with a discontinuous 3-layer Percoll density gradient. The presence of granule's markers in each fraction was assessed using western blot , gelatin zymography and enzymatic assays. The isolation was proven successful and allowed for a reasonable separation of all neutrophil's storage organelles in a gradient of less than 1 mL, about 37 times smaller than the methods

described in the literature. Moreover, mass spectrometry-based proteomics identified 369 proteins in at least 3 of the 5 samples, and using a machine learning strategy, the localization of 140 proteins was predicted with confidence. Furthermore, this study was the first to investigate the proteome of neutrophil granules using technical and biological replicates, creating a reliable database for further studies. In conclusion, the developed miniaturized method is reproducible, cheaper, and reliable. In addition, it provides a resource for further studies exploring neutrophil granule's protein content and mobilization during activation with different stimuli.

Keywords: neutrophil, granules, fractionation, proteomics

SUMMARY

1. INTRODUCTION

1.1. Neutrophils and their role in the immune system

Neutrophils are the most abundant leukocytes in the peripheral blood circulation, and they are essential for the immune response. These cells are recruited to the inflammatory site and enroll in different actions to eliminate pathogens. Examples of such actions are the phagocytosis, the production of reactive oxygen species (ROS), degranulation of potent antimicrobial peptides (1,2) and release of extracellular DNA traps, called NETs (Neutrophil Extracellular Traps, (3)). In addition, neutrophils are capable of orchestrating the immune response due to the secretion of inflammatory cytokines and chemokines that not only stimulate and modulate dendritic cells and, therefore, T cells, but also recruit and stimulate other leukocytes (4).

Neutrophils in the peripheral blood circulation are in a resting state, which means they are not very responsive to stimuli from the environment. Neutrophil's full activation is a two-step process involving first priming and then, activation. The priming occurs in the bloodstream and is responsible for changes in the cells that makes them fully responsive to their environment. It also induces neutrophil's diapedesis to the inflammatory sites (5). In the inflammatory sites, the neutrophil is activated and will play a variety of actions to eliminate the pathogen, that may lead to degranulation (6).

1.2. Neutrophil's granules

The neutrophil's granules play a major role in all the possible actions that the cell may enroll to eliminate a pathogen, including neutrophil's own activation process (7). Traditionally, the granules were divided into two types: peroxidase-positive, also called azurophil granules, and peroxidase-negative, the so-called specific granules (8). However, the peroxidase-negative granules were found to be heterogenous in content and function, hence not represented by just

one type of granule, but two subsets: specific and gelatinase granules (9). In addition to the granule subsets, the secretory vesicles, a different type of storage organelle, is also present in neutrophils (10).

The heterogeneity of neutrophil's granule subsets can be explained through the targeting-by-timing hypothesis. This hypothesis postulates that during granulopoiesis, distinct granules are formed in different maturation stages in the bone marrow (11,12), by the diverging of the constitutive secretory pathway into storage particles (12). Thus, the protein composition of each granule subset is determined by the stage of maturation of the cell at which the granule proteins are synthesized. Each granule subset is unique regarding its structure, content, function, and ability (or not) for exocytosis (9,13,14). The stages in which each storage particle is formed are shown in **Figure 1**. Briefly, azurophilic granules are formed in the promyelocyte stage, the specific granules during myelocyte phase, and the gelatinase granules formed in the metamyelocyte stage. After the metamyelocyte stage, the granules formation stops. Secretory vesicles are formed through endocytosis (15,16) during the band or polymorphonuclear stage, when neutrophils are already heading to the bloodstream (12,13,17).

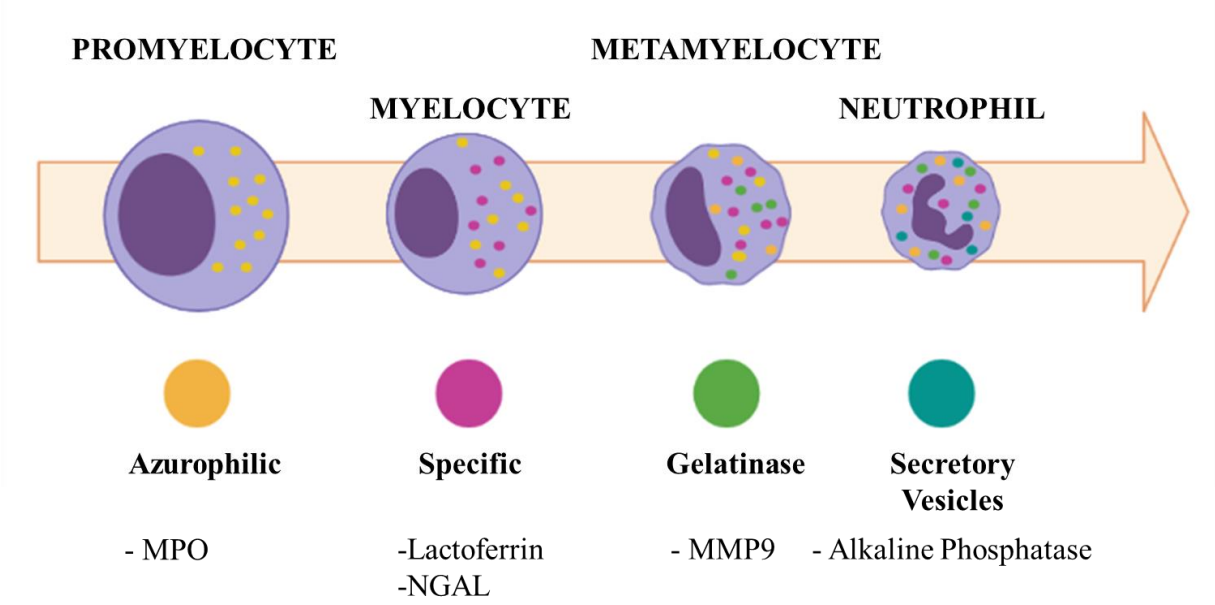


Figure 1. Adapted from Lawrence, SM; Corriden, R & Nizet, V. The ontogeny of a neutrophil: Mechanisms of granulopoiesis and homeostasis. Microbiol Mol Biol Rev, 2018 (18). Image created with BioRender.

The protein composition of the granules is key to understanding their functions and, therefore, the neutrophil's actions in the organism. Although the complete set of proteins belonging to each specific granule is not yet known, some proteins are used as classical markers of each granule due to their high abundance (**Figure 1**). Thus, myeloperoxidase (MPO) is used as a marker for azurophil granules, neutrophil gelatinase associated with lipocalin or lipocalin 2 (NGAL) and lactoferrin for specific granules, gelatinase B for gelatinase granules, and alkaline phosphatase (AP) and albumin (ALB) for secretory vesicles (12,17).

The control of the neutrophil's degranulation process that occurs during priming and pathogen elimination is not completely understood, however it is known there are different set points of exocytosis for different granule types (6,13). During neutrophil priming, secretory vesicles membranes fuse with plasma membrane, and their content is completely secreted. This event is what furnishes the cellular membrane with the receptors for the diapedesis and activation processes, due to the fusion of the vesicles membrane, full of receptors and important transmembrane proteins, and cell membrane (13). In contrast, the granules subsets are not

completely secreted at this stage and the percentage of secretion between them varies not only in this moment but also after the full neutrophil activation (13,20). Azurophil granules degranulation, for example, are most commonly directed to the phagosome vacuole (20) and the secretion varies with different stimuli, but the maximum percentage observed was 20% (21).

The degranulation process can be divided into two main events: mobilization of the storage organelle and fusion with the cell membrane. The microtubules and dynamics of actin polymerization/depolymerization are known as important factors for the mobilization of granules. The microtubules direct the azurophil granules to the phagosome (20), while the actin dynamics appears to be involved in the active transport of granules to the plasma membrane, due to the role of Rac-dependent F-actin formation in the cytoplasm to promote primary granules exocytosis (22). The abundance of actin in the storage organelle also appears to be associated with the mobilization process; therefore the secretory vesicles, highly enriched in actin, are the first to be mobilized (23). In neutrophils, the process of fusion of organelle's membrane with plasmatic membrane has been described before. Thus, the proteins VAMP-2 and SCAMP are associated with the attachment of the granule to the cell membrane and the membrane fusion, respectively, and their abundance may be decisive for the propensity of fusion (14,24).

1.3. Subcellular fractionation

Isolation of the neutrophil granules is necessary for a deep understanding of the composition and function of these particles, and subcellular fractionation plays a central role in this process. Subcellular fractionation is a method that is based on ultracentrifugation for the isolation of cellular components. Nowadays, a Percoll gradient is the most common strategy to isolate neutrophils granules, since this type of gradient showed improved resolution on separating the different granules when compared with a sucrose gradient (25). Percoll is a

colloidal medium of silica particles coated with polyvinylpyrrolidone (PVP) that has become a classic medium for density gradients used in subcellular fractionation (26).

The first use of Percoll gradients for isolation of neutrophil granules occurred in 1983, when Borregaard *et al.* compared two types of percoll gradients, continuous and discontinuous, to the most common gradient for granule's separation used at that time, the sucrose gradient (25). Both, continuous and discontinuous Percoll gradients, showed improved resolution and capability for separating the peroxidase-positive and peroxidase-negative granules. Between these two Percoll based methods, the 2-layer discontinuous gradient showed impressive resolution for the subcellular fractionation of neutrophil granules. Almost a decade later, in 1994, a new discontinuous Percoll density gradient was described by Kjeldsen *et al.* from the same research group, founded by Niels Borregaard (27). The new 3-layer discontinuous gradient was able to separate the peroxidase-negative granule subset in two distinct population of granules, making it possible to isolate and characterize the gelatinase granules for the first time. The last update to the isolation method came in 2011, from the same laboratory, this time described by Clemmensen *et al* (28). This new method is a 4-layer discontinuous Percoll gradient that allows the separation of plasma membrane from secretory vesicles. Even though the 4-layer gradient improved the separation of neutrophil's cellular components, the 3-layer method is still the most used. All these methods have the same issue in common, the need for large amounts of sample (blood neutrophils) and gradient volumes. Thus, the granules isolation procedure, regardless of which of these protocols is used, needs at least 3 x 10^8 neutrophils, isolated from 400 mL of blood, and a gradient volume of about 37 mL. The large number of cells, and volume of peripheral blood to obtain them, makes the fractionation procedure expensive and hard to replicate in any laboratory. Because a single isolation procedure requires the maximum amount of blood that can be obtained from a volunteer, it is impossible to perform technical and biological replicates (at least in the same day or month). The large amount of

sample required also prevents carrying out concomitant neutrophil stimuli experiments, in order to understand what happens with each granule upon distinct stimuli.

1.4. Mass spectrometry-based proteomics

Proteomics is the global analysis of proteins and the term was coined in the 1990's from merging "protein" and "genomics" (29,30). Mass spectrometry (MS) has become the method of the choice to study proteins due to its capacity to identify and quantify a large number of proteins in a complex sample.

The mass spectrometer measures the mass-to-charge ratio (*m/z*) of ionized particles, such as proteins. It is consisted of three main parts: an ion source, a mass analyzer and a detector. Briefly, analyte ions are produced in the ion source, and then separated according to their m/z in the mass analyzer. After that, they are recorded by the detector that registers the number of ions at each *m/z* value (31). Usually, liquid chromatography (LC) or SDS-page gel (1D or 2D) are used combined to MS to increase protein identification. The proteins are separated in the LC or gel, enabling a whole series of MS spectra to be acquired in each fraction, increasing the number of proteins identified (32).

The MS-based proteomics can be divided into two types of approach: top-down and bottom-up (33). The bottom-up proteomics refers to the characterization of proteins by the analysis of the peptides released through proteolysis, whereas in the top-down, the input to the mass spectrometer is intact proteins that are fragmented inside the mass spectrometer (**Figure 2**).

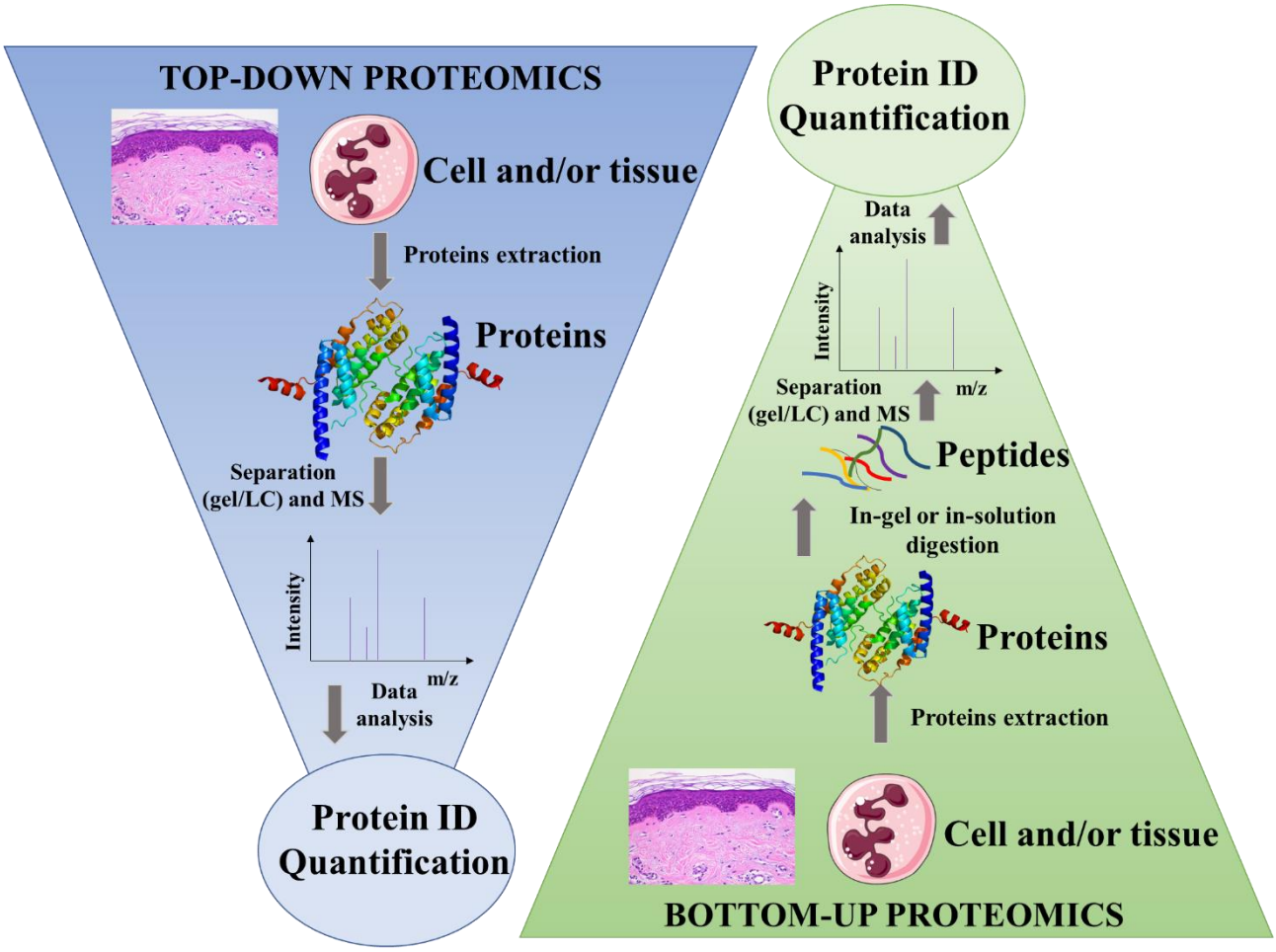


Figure 2. Figure adapted and modified from Gregorich, ZR; Chang, YH & Ge, Y. Proteomics in heart failure: top-down or bottom-up? Pflugers Arch., 2014 (34).

Shotgun proteomics is a special case of the bottom-up method, where a complex mixture of proteins is digested into peptides that are ionized and fragmented inside the mass-spectrometer, resulting in multiple ions for each peptide, like a shotgun blast. Shotgun is a semi-quantitative method and works as a global analysis of the protein mixture, because it does not focus on specific sites or proteins of interest, offering a hypothesis-free and systems-wide analysis that complements a range of targeted approaches (35). The typical shotgun proteomics workflow is shown in bottom-up part of **Figure 2**: proteins are extracted from a biological sample, enzymatically digested to peptides and the peptides are analyzed by LC-MS/MS (32). The proteins are usually digested using trypsin, which cleaves the carboxyl side of the amide

bond between arginine and lysine amino acids residues, and after the digestion the resulting peptide mixture is cleaned-up for mass spectrometry analysis (36). The cleaning procedure is a desalting method commonly performed on pipette-based devices (37).

Shotgun proteomics, also known as data-dependent acquisition (DDA), is described in detail by Aebersold & Mann, 2016, and Zhang *et al.* (36,38). In DDA approach, the mass spectra of peptide ions that co-elute at a specific point in the gradient elution (precursor ions) are recorded at the MS^1 level (full scan). The most abundant peptides are then individually fragmented in a collision cell before being recorded again at MS^2 (MS/MS) level. The MS^2 is used to determine the specific amino acid sequence of each peptide, because this type of spectrum demonstrates their fragmentation profile. The mass spectrometer alternates between full scans and MS^2 acquisitions, and as many precursor ions are isolated and fragmented within cycles of about 1 to 3 seconds. Quantification of protein levels is accomplished by spectral counting or ions intensity.

1.5. Neutrophil granule's proteomics

Knowing the proteome of neutrophil's granules is essential for the complete understanding of this cell functionality and activation. Although the importance is clear, there are few studies that tackle the subject due to the difficulties found during the isolation process, and the complexity of the analysis using mass spectrometry-based proteomics.

The first study to investigate the neutrophil granules proteome was published in 2005 by Lominadze *et al* (39), and used two proteomics approaches: 2D-gel electrophoresis followed by MALDI-TOF analysis of the spots, and protein identification with 2D HPLC (strong cation exchange and reverse phase chromatography), followed by electrospray ionization tandem mass spectrometry (ESI-MS/MS). A total of 286 proteins were identified in the 3 granules subsets combining the two methods, 87 by MALDI-TOF and 247 by ESI-MS/MS. The granule proteins

were separated using two different techniques. First, they were separated into luminal and membrane fractions by sonicating with sodium carbonate followed by ultrafiltration through 1-kDa-cutoff filters. Second, they were separated based on differential solubility in ammonium sulfate solution with the use of 2% triton X-100 to rupture the membranes. Only the proteins treated with sodium carbonate were used in 2D HPLC ESI-MS/MS, whereas the granule proteins of both methods were used in 2D-gel followed by MALDI-TOF. The identified proteins are shown in **Supplementary Table 1**.

The second, and last study addressing neutrophil's granules proteins was published almost a decade later, in 2013, by Rørvig *et al* (40). This study used SDS-page and in-gel digestion followed by nano-LC-MS/MS approach with a LTQ Orbitrap XL and identified 1292 proteins. The identified proteins were grouped according to the neutrophil's fraction in which they were more abundant (**Supplementary table 1)**. With these results, the authors suggested the existence of a new granule subset, the ficolin granule, due to the detection of large quantities of ficolin-1 in a fraction close to the gelatinase granules. However, the isolation process of the granules did not allow to conclude the presence of this granule subset, because they could not be separated from the gelatinase granules and therefore the ficolin-1 may be a possible marker for gelatinase granules as well as MMP9. The proteomic data of this study is the most recent and complete we have, to this date, of neutrophil granules and can be used as a guideline when studying neutrophil granules protein and its mobilization.

The neutrophil is an important cell in the immune system and is one of the first lines of defense in the organism. Identification and distribution profiling of granule proteins are essential for understanding the molecular mechanisms responsible for the functionally of neutrophils and therefore how they can influence in diseases, such as COVID-19 (41–43), and inflammatory processes in general. The proteome of neutrophil granules is still complex to be studied due to the challenges of the granular isolation process. Therefore, the aim of our study

is to not only further investigate the neutrophil granules protein content, but also to develop a new miniaturized isolation method that is cheaper and allows multiple biological and technical replicates.

REFERENCES

1. Hampton MB, Kettle AJ, Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood. 1998 Nov 1;92(9):3007–17.
2. Kettle AJ, Rosen H, Winterbourn CC, Nauseef WM, Klebanoff SJ. Myeloperoxidase: a front-line defender against phagocytosed microorganisms. J Leukoc Biol. 2012 Feb 1;93(2):185–98.
3. Sørensen OE, Borregaard N. Neutrophil extracellular traps - The dark side of neutrophils. Journal of Clinical Investigation. 2016 May 2; 126(5):1612-20.
4. Ludwig IS, Geijtenbeek TB, van Kooyk Y. Two way communication between neutrophils and dendritic cells. Current Opinion in Pharmacology. 2006 Aug; 6(4): 408–13.
5. Condliffe AM, Kitchen E, Chilvers ER. Neutrophil priming: Pathophysiological consequences and underlying mechanisms. Clinical Science. 1998 May; 94(5):461-71.
6. Sengeløv H, Kjeldsen L, Borregaard N. Control of exocytosis in early neutrophil activation. Journal of Immunology. 1993 Feb 15; 150(4):1535-43.
7. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. Microbes and Infection. 2003 Nov; 5(14):1317-27.
8. Bainton DF, Ullyot JL, Farquhar MG. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. J Exp Med. 1971 Oct 1;134(4):907–34.
9. Kjeldsen L, Bainton DF, Sengeløv H, Borregaard N. Structural and functional heterogeneity among peroxidase-negative granules in human neutrophils: identification of a distinct gelatinase-containing granule subset by combined immunocytochemistry and subcellular fractionation. Blood. 1993 Nov 15;82(10):3183–91.
10. Borregaard N, Miller LJ, Springer TA. Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. Science. 1987 Sep 4;237(4819):1204–6.
11. Borregaard N, Sehested M, Nielsen BS, Sengeløv H, Kjeldsen L. Biosynthesis of Granule Proteins in Normal Human Bone Marrow Cells. Gelatinase Is a Marker of Terminal Neutrophil Differentiation. Blood. 1995 Feb 1; 85(3):812-7.
12. Le Cabec V, Cowland JB, Calafat J, Borregaard N. Targeting of proteins to granule subsets is determined by timing and not by sorting: The specific granule protein NGAL is localized to azurophil granules when expressed in HL-60 cells. Proc Natl Acad Sci U S A. 1996 Jun 25;93(13):6454-7.
13. Sengeløv H, Follin P, Kjeldsen L, Lollike K, Dahlgren C, Borregaard N. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. Journal

of Immunology. 1995 Apr 15;154(8):4157–65.

14. Brumell JH, Volchuk A, Sengelov H, Borregaard N, Cieutat AM, Bainton DF, et al. Subcellular distribution of docking/fusion proteins in neutrophils, secretory cells with multiple exocytic compartments. Journal of Immunology. 1995 Dec 15;155(12):5750–9.

15. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood. 1997 May 15;89(10):3503–21.

16. Borregaard N, Kjeldsen L, Rygaard K, Bastholm L,Sengeløv H, Bjerrum OW et al. Stimulus-dependent secretion of plasma proteins from human neutrophils. J Clin Invest. 1992 Jul;90(1):86–96.

17. Kjeldsen L, Bjerrum OW, Askaa J, Borregaard N. Subcellular localization and release of human neutrophil gelatinase, confirming the existence of separate gelatinase-containing granules. Biochem J. 1992 Oct 15;287(2):603–10.

18. Lawrence SM, Corriden R, Nizet V. The Ontogeny of a Neutrophil: Mechanisms of Granulopoiesis and Homeostasis. Microbiol Mol Biol Rev. 2018 Feb 7;82(1):e00057-17.

19. Borregaard N, Sørensen OE, Theilgaard-Mönch K. Neutrophil granules: a library of innate immunity proteins. Trends Immunol. 2007 Aug;28(8):340–5.

20. Tapper H, Furuya W, Grinstein S. Localized exocytosis of primary (lysosomal) granules during phagocytosis: role of Ca2+-dependent tyrosine phosphorylation and microtubules. Journal of Immunology. 2002 May 15;168(10):5287–96.

21. Xu X, Håkansson L. Degranulation of primary and secondary granules in adherent human neutrophils. Scand J Immunol. 2002 Feb;55(2):178–88.

22. Mitchell T, Lo A, Logan MR, Lacy P, Eitzen G. Primary granule exocytosis in human neutrophils is regulated by Rac-dependent actin remodeling. Am J Physiol Cell Physiol. 2008 Nov;295(5):C1354-65.

23. Jog NR, Rane MJ, Lominadze G, Luerman GC, Ward RA, McLeish KR. The actin cytoskeleton regulates exocytosis of all neutrophil granule subsets. Am J Physiol Cell Physiol. 2007 May;292(5):C1690-700.

24. Liu L, Guo Z, Tieu Q, Castle A, Castle D. Role of secretory carrier membrane protein SCAMP2 in granule exocytosis. Mol Biol Cell. 2002 Dec 1;13(12):4266–78.

25. Borregaard N, Heiple JM, Simons ER, Clark RA. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: Translocation during activation. J Cell Biol. 1983 Jul;97(1):52–61.

26. Pertoft H, Laurent TC, Låås T, Kågedal L. Density gradients prepared from colloidal silica particles coated by polyvinylpyrrolidone (Percoll). Anal Biochem. 1978 Jul 15;88(1):271–82.

27. Kjeldsen L, Sengeløv H, Lollike K, Nielsen MH, Borregaard N. Isolation and characterization of gelatinase granules from human neutrophils. Blood. 1994 Mar 15;83(6):1640–9.

28. Clemmensen SN, Jacobsen LC, Rørvig S, Askaa B, Christenson K, Iversen M, et al. Alpha-1-antitrypsin is produced by human neutrophil granulocytes and their precursors and liberated during granule exocytosis. Eur J Haematol. 2011 Jun;86(6):517–30.

29. Wilkins MR, Pasquali C, Appel RD, Ou K, Golaz O, Sanchez JC, et al. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. Biotechnology (N Y). 1996 Jan;14(1):61–5.

30. James P. Protein identification in the post-genome era: the rapid rise of proteomics. Q Rev Biophys. 1997 Nov;30(4):279–331.

31. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003 Mar 13;422(6928):198–207.

32. Matthiesen R, Bunkenborg J. Introduction to mass spectrometry-based proteomics. Methods Mol Biol. 2013;1007:1–45.

33. Chait BT. Chemistry. Mass spectrometry: bottom-up or top-down? Science. 2006 Oct 6;314(5796):65–6.

34. Gregorich ZR, Chang YH, Ge Y. Proteomics in heart failure: top-down or bottom-up? Pflugers Arch. 2014 Jun;466(6):1199–209.

35. Meissner F, Mann M. Quantitative shotgun proteomics: considerations for a high-quality workflow in immunology. Nat Immunol. 2014 Feb;15(2):112–7.

36. Zhang Y, Fonslow BR, Shan B, Baek MC, Yates JR. Protein analysis by shotgun/bottom-up proteomics. Chem Rev. 2013 Apr 10;113(4):2343–94.

37. Rappsilber J, Mann M, Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat Protoc. 2007;2(8)1896-906.

38. Aebersold R, Mann M. Mass-spectrometric exploration of proteome structure and function. Nature. 2016 Sep 15;537(7620):347–55.

39. Lominadze G, Powell DW, Luerman GC, Link AJ, Ward RA, McLeish KR. Proteomic Analysis of Human Neutrophil Granules. Mol Cell Proteomics. 2005 Oct;4(10):1503–21.

40. Rørvig S, Østergaard O, Heegaard NHH, Borregaard N. Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors. J Leukoc Biol. 2013 Oct;94(4):711–21.

41. Borges L, Pithon-Curi TC, Curi R, Hatanaka E. COVID-19 and Neutrophils: The Relationship between Hyperinflammation and Neutrophil Extracellular Traps. Mediators Inflamm. 2020 Dec 2;2020:8829674.

42. Lutfi F, Benyounes A, Farrukh N, Bork J, Duong V. Agranulocytosis Following COVID-19 Recovery. Cureus. 2020 Jul 29;12(7):e9463.

43. Cavalcante-Silva LHA, Carvalho DCM, Lima ÉA, Galvão JGFM, Silva JSF, Sales-Neto JM, et al. Neutrophils and COVID-19: The road so far. Int Immunopharmacol. 2021 Jan;90:107233.

CHAPTER 1

A miniaturized isolation method allowed a deep proteome mapping of human neutrophil granules

Gabrielly Alexandria[A], Lorenna R Reis[A], Hellen P Valerio[A], Mariana P Massafera[A], Fernando R Coelho[A], Paolo Di Mascio[A] and Graziella E Ronsein[A]*

A: Departament of Biochemistry, Institute of Chemistry, University of São Paulo, São Paulo, Brazil.

*Corresponding author: Graziella E Ronsein (ronsein@iq.usp.br)

Institutional address: Departamento de Bioquímica, Instituto de Química, Av. Professor Lineu Prestes 748, CEP 05508-900, Butantã, São Paulo, SP. Brazil. Phone: +55 11 (3091-1078)

HIGHLIGHTS

- A miniaturized neutrophil granule isolation method was developed starting with 40 mL of blood.

- A deep proteomic analysis allowed the characterization of neutrophils granules content starting from 9 x 10^6 cells.

- A great advantage of the miniaturized method is the determination of technical and biological variances.

- The isolation method is reproducible across different subjects and can therefore be used to study the granules dynamics.

1. Introduction

Neutrophils are polymorphonuclear granulocytes that play a key role in the organism's defense. These cells are the most abundant leukocytes in the bloodstream and they are the first ones to be recruited to inflammatory sites, where they enroll in a range of actions to ensure pathogen elimination (1). Examples of these actions are phagocytosis, production of reactive oxygen species (ROS), degranulation of potent antimicrobial peptides/proteins (2,3) and release of neutrophil extracellular traps (NETs) (4). In addition, neutrophils orchestrate the immune response due to the release of chemokines and cytokines that not only recruit and stimulate other leukocytes, but also modulate dendritic cells, and therefore T cells, impacting in the adaptative immune response (5).

In the peripheral blood circulation, neutrophils are in a resting state, with low responsivity to stimuli from the environment. Neutrophil's full activation is a two-step process, divided into priming and activation. The priming occurs in the bloodstream and is responsible for cell changes that enable the neutrophil to react to its environment, inducing also the diapedesis to the inflammatory sites (6). In the inflammatory sites, these cells will be fully activated and will enroll in their actions to eliminate the pathogens. The degranulation process, composed of the exocytosis and mobilization of neutrophil storage organelles, is responsible for the priming step and participates in all neutrophil functions (7,8). Therefore, neutrophil granules are these cell's main physiological structure, being important since the cell's activation.

The neutrophil granules are storage organelles and they were traditionally divided into two types: peroxidase-positive, also called azurophil granules, and peroxidase-negative, the so-called specific granules (9). Later, the peroxidase-negative granules were found to be heterogenous in content and function, hence they were further divided into two subtypes: specific and gelatinase granules (10). In addition to the granules, there is another storage organelle present in the neutrophils, the secretory vesicles (11). The heterogeneity of neutrophil

granules subsets can be explained by the targeting-by-timing hypothesis. This hypothesis postulates that each granule type is formed during a different maturation stage in the granulopoiesis of these cells in the bone marrow, due to the divergence of the constitutive secretory pathway into storage particles (12–14). The secretory vesicles, however, are formed through endocytosis during the band or polymorphonuclear stage (15,16). Each storage organelle is unique regarding its structure, content, function, and ability (or not) for exocytosis (10,17,18).

The protein composition of the neutrophil granules is the main difference among them, and therefore, the best way to identify and differentiate each storage organelle. Moreover, the understanding of the granule's content is key to comprehend their function and, as consequence, the neutrophil actions in the organism. Although the complete set of proteins belonging to each storage organelle is not yet known, some proteins are used as classical markers due to their high abundance. The enzyme myeloperoxidase (MPO) is used as marker for azurophil granules, lactoferrin (LTF) and neutrophil gelatinase associated with lipocalin or lipocalin 2 (NGAL) for specific granules, gelatinase B (MMP9) for gelatinase granules and alkaline phosphatase (AP) for secretory vesicles (12,19,20).

The knowledge of the neutrophil granule's proteome is key for the complete understanding of this cell functionality and activation. Although the importance is clear, there are few studies that tackled this subject due to its complexity, not only regarding the granular isolation process, but also in the analysis using mass spectrometry-based proteomics. The first study to investigate neutrophil granules' proteome was published in 2005 by Lominadze et al. (21), and used 2D-gel electrophoresis followed by MALDI-TOF analysis of the spots and protein identification with 2D HPLC (strong cation exchange and reverse phase chromatography), followed by electrospray ionization tandem mass spectrometry (ESI-MS/MS). In this study, a total of 286 proteins were identified in the three granules subsets combining the two methods. The second

study addressing granules' proteome was published in 2013 by Rørvig *et al*. (22), and used SDS-page and in-gel digestion followed by nano-LC-MS/MS, identifying 1292 proteins as belonging to different granules. The proteomic data of these studies combined is the most complete we have, to this date, of neutrophil granules and can be used as a guideline when studying neutrophil granules protein and its mobilization.

Isolation of neutrophil granules is essential for a deeper comprehension of the composition and function of these particles, and subcellular fractionation plays a key role in this process. Subcellular fractionation is a method that consists in using centrifugal steps for isolating cellular components and it can be incorporated with density gradients. Nowadays, the Percoll based density gradients are the most common strategies to isolate neutrophil granules, since this type of method demonstrated a better resolution of granular isolation when compared to sucrose gradients (23). The 3-layer discontinuous Percoll density gradient was first described in 1994 by Kjeldsen *et al*. (24) and it is the most commonly used gradient to this day due to the capability of isolating the three granule's subsets and the secretory vesicles. The most recent method of isolation is a 4-layer discontinuous Percoll density gradient, proposed in 2011 by Clemmensen *et al*.(25). This gradient allows for the separation of each storage organelle and the cellular membrane; however even more recent studies still use the 3-layer gradient due to its high reproductivity. These methods, although efficient, need a large number of cells, roughly 3×10^8 neutrophils, isolated from a large volume of peripheral blood, 400mL, which makes the procedure expensive and hard to replicate in any laboratory. Furthermore, a single isolation procedure requires the maximum amount of blood that can be obtained from a volunteer, making it is impossible to perform technical and biological replicates (at least in the same day or month). Therefore, the aim of this study was to develop a miniaturized neutrophil granule isolation and investigate the protein content of each of these storage organelles using mass-spectrometry based proteomics.

2. Material and methods

The study was approved by the local Ethics Committee (CAAE #60860016.5.0000.0067). The volunteers were informed about the procedure and signed a consent form before any sample collection. The mass spectrometry data are available from the corresponding author upon reasonable request.

2.1. Neutrophil isolation

The neutrophils were isolated from fresh drawn peripheral heparinized blood of three apparently healthy donors (40 mL each). The isolation process consisted of dextran sedimentation, density centrifugation and hypotonic lysis of remaining erythrocytes as previously described (26). The presence of eosinophils was determined by optic microscopy with May-Grünwald-Giemnsa stain, and the sample was considered appropriate if eosinophils were less than 5% of the cells.

2.2. Subcellular fractionation

Cells (9×10^6) were suspended in 300 µL of PBS-glucose and 1mM of 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) was added to inhibit serine proteases, followed by a centrifugation step (400 g, 10 min, (4ºC)). After centrifugation, the pellet was ressuspended in 350 µL of disruption buffer (100mM KCl, 3mM NaCl, 3.5 mM $MgCl_2$, 10 mM PIPES; pH 7,2) and cavitated as previously described (24). The cell lysis effectivity was checked using the microscope and more than 90 % of the cells were lised. The cavitated sample was centrifuged at 400 g for 30 min at 4ºC to remove unbroken cells and nuclei. Two hundred and fifty-seven µL of the supernatant containing the granules (azurophil, specific and gelatinase), secretory vesicles (SVs), and cellular membranes (CMs) were placed on top of a 3-layer Percoll density gradient. The 3-layer gradient was formed as previously described (24) but with adjustments in the volumes and proportions. These adjustments in the gradient were performed using marked density beads (Cospheric, #DMB-kit) to predict the position of each

storage organelle in the gradient and ensure the separation. The density of each specific organelle was based on that reported in Kjeldsen *et al*., 1994 (24). The final gradient was composed of 272 µL from the 1.05 g/mL solution, and 215 µL of each of these two following solutions, with densities of 1.09 and 1.12 g/mL. Therefore, the final gradient volume with the sample was 959 µL. The gradient was centrifuged at 12,000 g for 30 min at 4ºC in a Beckman Coulter Optima Max XP ultracentrifuge with the TLA 120.2 rotor. Nineteen fractions of 50 µL were collected with a Hamilton syringe from the bottom to the top of the tube. The collected fractions were centrifuged at 100,000 g for 2 h at 4ºC at the same ultracentrifuge but with TLA-100 rotor to pellet the Percoll and to remove it from the samples. The amount of protein in each fraction was quantified using Bradford method (Biorad, Cat #5000006).

2.3. Analyses of the granule's markers

Based on previous reports (11,27–30), MPO was chosen as a marker to indicate the presence of azurophilic granules, while LTF and NGAL were chosen as markers for specific granules, MMP9 for gelatinase granules and latent AP for SVs. The investigation of the azurophil and specific granule's markers was performed by Western Blot (WB). Briefly, 1 µg of protein from each fraction was loaded at 12% polyacrylamide gel well and separated using electrophoresis. After that, the proteins were transferred to a 0,45 µm PVDF membrane and the target proteins were immunoprobed. The MPO and NGAL were marked using primary rabbit polyclonal antibodies (Chemicon®, Cat: AB1224 and Invitrogen, Cat: 711280, respectively) and secondary goat anti-rabbit conjugated with HRP antibody (Thermo Fisher Scientific, Cat: 65-6120). The LTF was marked with a primary mouse monoclonal antibody conjugated with HRP (Santa Cruz Biotechnology, Cat: sc-53498). The detection with chemiluminescence was accomplished using the SuperSignal™ West Atto Ultimate Sensitivity Substrate kit (Thermo Fisher Scientific, Cat: A38555). The resulting bands were analyzed using ImageJ software.

The MMP9 was assessed using gelatin zymography (31–33). The 8% polyacrylamide co-polymerazed with 0,1% gelatin gels were casted and 0,125 µg of protein of each fraction were loaded into the wells. After electrophoresis, the gels were renatured and incubated to allow the MMP9 to hydrolyze the gelatin. The gels were further stained with Coomassie Blue R-250 and analyzed using ImageJ software.

The AP quantification was performed with a LabTest kit (Alkaline phosphatase liquiform, ref.: 79-4/30) with some modifications for 96-well microplate. The AP is present in SVs and CMs, both located in close fractions in the gradient. The identification of which AP is present at SV or CM can be done by using Triton X-100, since the AP of the CMs are in the outside part and, in the SV, the protein is stored inside the vesicle (11,34). Therefore, by repeating the assay with and without 0,2% Triton X-100 in each fraction, it is possible to subtract the AP located in the CM from the whole AP activity (with Triton X-100) and determine the amount of latent AP, i.e. the AP stored inside the SVs.

2.4. Sample digestion for mass spectrometry

Fractions identified by the biochemical analyses as containing considerable amounts of each specific granule marker were consolidated and processed for proteomics. The in-gel protein digestion was performed using 2,5 µg of protein from each storage organelle sample as previously described with some modifications (35). Briefly, the proteins were loaded into 5% polyacrylamide gels at 200 V for 5-8 minutes for sample cleaning. Then, spots were stained with Coomassie Brilliant Blue G-250 for visualization, and subsequently destained in a solution containing methanol, water and acetic acid (50:40:10, v:v:v) overnight. Gels were washed twice with water for 5 min before excision of the gel spots. The bands were further destained with 50% acetonitrile in 25mM ammonium bicarbonate three times (5-10 min each), then dehydrated with 100% acetonitrile. Disulfide bonds reduction was performed with 20mM dithiothreitol in 50mM ammonium bicarbonate for 40 minutes at 56 °C, followed by cysteine alkylation with

55mM iodoacetamide in 50mM ammonium bicarbonate for 30 minutes at 20 °C. Eighty microliters of trypsin (Promega Gold, 20 ng/µL in 50 mM ammonium bicarbonate) were added to the spots for 15 minutes at 4 °C for the gel to absorb the trypsin solution. Bands were then covered with 50 mM ammonium bicarbonate, then incubated for 2h at 37 °C and 400 rpm. A second aliquot of trypsin solution was added after 2h, and incubation continued overnight. The tryptic peptides were extracted sequentially using 1% formic acid in 60% methanol, then 1% formic acid in 50% acetonitrile. The combined fractions were dried in an evaporator and resuspended in 0.1% trifluoroacetic acid. Desalting of peptides was performed using the StageTip protocol (36). After cleaning, samples were dried in an evaporator and resuspended in 0.1% formic acid for LC/MS/MS analysis.

2.5. LC/MS/MS analyses

Samples were injected in an Easy-nLC 1200 UHPLC (Thermo Scientific) and separated with a linear gradient of solvent A (0.1% formic acid) and solvent B (80% acetonitrile in 0.1% formic acid). Each sample was loaded onto a trap column (Acclaim PepMap 100, C18, 3 µm, 75 µm × 2 cm, nanoViper, Thermo Scientific) with 12 µL of solvent A at 500 bar. Then, the trapped peptides were eluted to a C18 column (Acclaim PepMap RSLC, C18, 2 µm, 75 µm × 15 cm, nanoViper, Thermo Scientific) at a flow rate of 300 nL/min. Peptides were separated using a linear gradient of 5−28% B for 80 min followed by a linear gradient of 28−40% B for 10 min. Finally, the percentage of solvent B was increased to 95% in 2 min and the column was washed for 12 min with this solvent proportion. Re-equilibration of the system with 100% A was performed before each injection. Samples were analyzed on a Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Scientific) using a nanospray Flex NG ion source (Thermo Scientific), operating in positive ESI mode, with capillary temperature at 300°C, and S-Lens RF level at 30%. A full MS scan was followed by data dependent MS^2 scans in a 3 s cycle time. Both MS and MS^2 scans were performed in the Orbitrap analyzer. Precursor ions selected for

MS^2 were excluded for subsequent MS^2 scans for 40 s. Precursor ions were fragmented by HCD with a normalized collision energy of 30. The resolution for the full scan mode was set as 120 000 (at *m/z* 200) and the automatic gain control (AGC) target at 5×10^5. The *m/z* 350-1550 was monitored. For accurate mass measurements the lock mass option was enabled in the MS scan and the polydimethylcyclosiloxane ions (*m/z* = 445.1200) were used for internal calibration in real time. Each full scan was followed by a data dependent MS^2 acquisition with a resolution of 30 000 (at *m/z* 200), maximum fill time of 54 ms, and isolation window of 1.2 *m/z*.

2.6. Data analysis

Raw files of all proteomics experiments performed in this study were processed using MaxQuant. (37). The Andromeda algorithm (38) was used for protein identification against the Homo sapiens Uniprot database (downloaded August, 2019; 20416 entries). Error mass tolerance for precursors and fragments were set to 4,5 ppm and 20 ppm, respectively. Cysteine carbamidomethylation was selected as a fixed modification and methionine oxidation and *N*-terminal acetylation were selected as variable modifications. Trypsin was set as digestion enzyme, with a maximum of 2 missed cleavages allowed. A maximum FDR of 1% was allowed both for peptides and proteins identification, and for proteins it was calculated using a decoy database created from the reverse ordination of the protein sequences in the Uniprot database. Protein abundances were quantified by the LFQ algorithm (39) based on the normalized chromatographic peak integrations calculated by MaxQuant. The protein was considered present if at least two peptides (one of them being unique) were detected. Other parameters were kept as default.

All the replicates and different volunteer's samples (n=5 samples, from 3 different individuals) were run in MaxQuant at the same time. The proteins were analyzed using Perseus software (version 1.6.15.0), R (version 1.4.1106) and some graphics were done using GraphPad Prism (version 6.01).

The Perseus software was used to obtain a Principal Component Analysis (PCA) and a heatmap. For PCA of the samples, the LFQ intensities were loaded into the Perseus software, filtered for reverse peptides and potential contaminants, and log 2 transformed. The missing values were filtered using the criteria of at least 4 valid values in at least one group of storage organelle, and the remaining missing values were imputated by the constant 0. For the heatmap of the samples, the mean percentages of the proteins present in at least 3 samples of at least one storage organelle were loaded into the Perseus and normalized using Z-score.

The pRoloc (40) for the R statistical programming language (41) was used for handling of the quantitative proteomics data and the protein-localization prediction. For protein-granules assignment, a supervised classification using a support vector machine (SVM, (42)) was employed. A set of granular markers was curated based on four published manuscripts involving neutrophils granules (**Supplementary table 1**, (15,20–22)). To be considered as a granular marker, the protein had to be cited in two different publications as pertaining to that specific granule. The spreadsheet containing LFQ intensities was filtered to keep only proteins present in at least three samples of one storage organelle. To filter proteins with low prediction scores, a null distribution of prediction scores for the localization assignment was obtained from randomized input data over 1000 iterations. An empirical cumulative function was estimated from the null distribution, and the threshold for each organelle and sample was selected at a cumulative probability of 0.95 (43).

3. Results

In this study, we developed a miniaturized separation of neutrophil granules starting with 40 mL of blood. A subsequent deep proteomic analysis allowed us to characterize neutrophils granules content starting from 9 x 10^6 cells (and less than 1 mL of fractionation volume). Neutrophils were isolated from blood of 3 apparently healthy volunteers, using at least two different technical replicates. After isolation, these cells were disrupted using nitrogen cavitation and neutrophil storage organelles were separated by ultracentrifugation using a 3-layer Percoll density gradient with a total volume of 959 uL. The obtained fractions were collected and analyzed for the presence of specific granular markers using multiple biochemical methods. Fractions containing significant amounts of each specific marker were consolidated and further processed for proteomic analysis. The resulting data were analyzed by multiple bioinformatics and statistic tools. The summarized workflow of the study can be seen in the **Figure 1**.

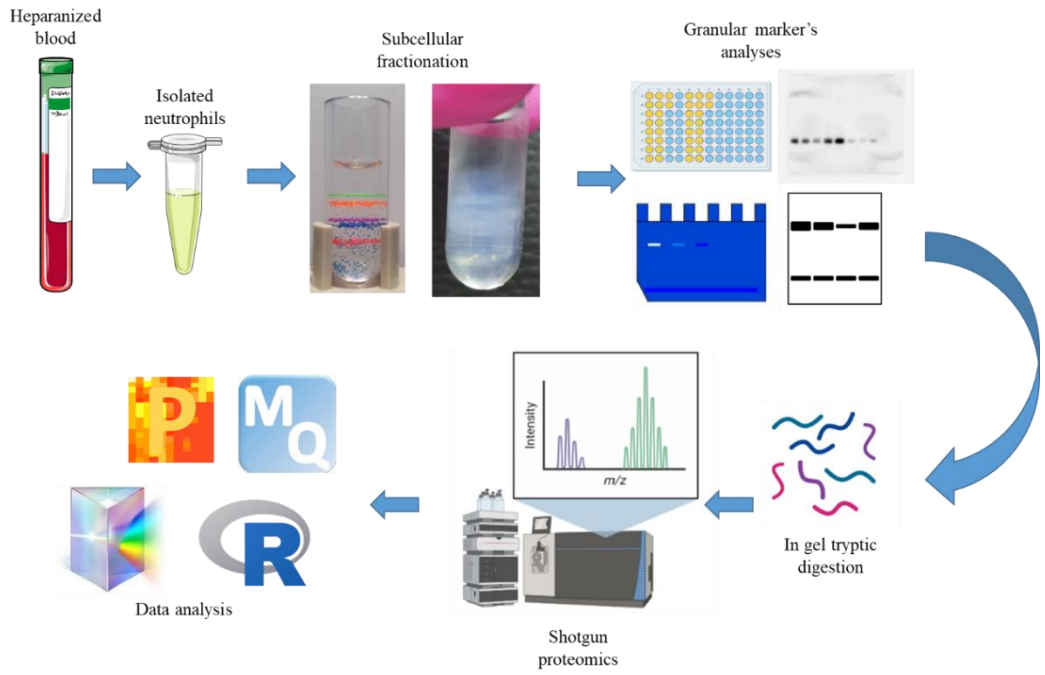


Figure 1. Summarized workflow of the study.

The efficiency of the isolation of each storage organelle by the density gradient was evaluated assessing the presence of the markers in each fraction after its collection. Thus, as described in detail in methods, MPO was analyzed as a marker of azurophilic granule by WB (**Supplemental Figure 1**), while LTF and NGAL were chosen as markers for specific granules and also visualized by WB (**Supplemental Figures 2 and 3**). On the other hand, MMP9 was analyzed as a specific marker for gelatinase granules using gelatin zymography (**Supplemental Figure 4**), and latent AP was taken as a marker for SVs and its activity was measured colorimetrically. The result of the biochemical analyses of the granule markers across collected fractions is shown in **Figures 2A and 2B**. The graphics show a clear change in distribution of the markers throughout the fractions, demonstrating the ability of our miniaturized gradient to separate distinct granules.

A great advantage of the miniaturized method developed in this work is the possibility to perform replicates obtained from the same subject in the same day, allowing to determine the technical variance of the method. The graphic 2A shows the difference in replicates from the same individual in the same day, demonstrating that even with slight differences, our study has a high reproducibility between samples. By combining the results obtained for three different subjects (**Figure 2B**), we also demonstrate that the isolation method is reproducible across different subjects and can therefore be used to study the granules dynamics. The information obtained with the biochemical analyses allowed the identification of the fractions that contain each chosen marker, and consequently, the largest portion of each type of granule. For each subject, fractions were then pooled into four consolidated samples as follows: fractions 2 and 3 were combined and labeled as azurophilic granules; fractions 5 and 6 were combined to represent specific granules; fractions 8 and 9 were pooled and used as markers for gelatinase granules, and fractions 10, 11 and 12 were combined and labeled as secretory vesicles. These consolidated samples representing the four different types of granules were analyzed using

nano-LC/MS/MS. The distribution of the markers across pooled fractions after proteomics analyses shows that the sample pooling was effective (**Figure 2C** for an individual and **Figure 2D** for the 3 subjects). Thus, as measured by MS/MS, around 60 % of all detected MPO was in the first (azurophilic) fraction, while 70 % of NGAL and LTF were found in the specific fraction. Fifty-five % of MMP9 was present in the gelatinase fraction, and around 25 % of this protein was located in the specific fraction. MMP9 has been described as present in both, specific and gelatinase granules (10,24). Lastly, around 50 % of albumin (ALB) occurred in our secretory vesicles fraction. We replaced the AP (monitored by an enzymatic assay) by ALB in the proteomic analysis of fractions because we could not detect AP (a membrane protein) in our MS analyses. Membrane proteins are harder to extract from cells and to digest than soluble proteins (44,45). Nevertheless, ALB has been used as a marker of secretory vesicles before (16,24).

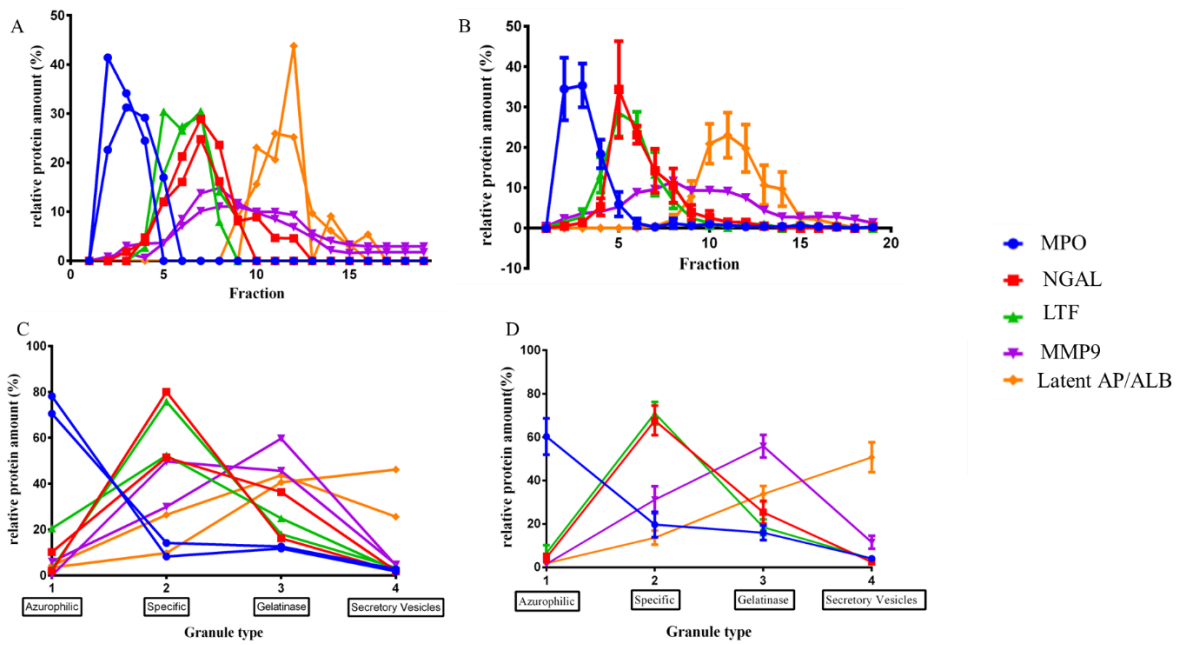


Figure 2. Neutrophil granule's markers distribution profile after 3-layer density gradient using biochemical methods or mass spectrometry. Nineteen fractions of 50µL were collected from the bottom of the tube using a Hamilton syringe and the amount of each marker was evaluated using WB, zymography and enzymatic assays. The markers used were MPO (n=6), NGAL (n=5), LTF (n=6), MMP9 (n=6) and latent AP (n=6). (A) Distribution profile of the granule's markers in two replicates from the same individual. (B) Distribution profile of the granule's markers for all subjects from this study and their replicates (n=5). The results are shown as median ± SEM. The fractions 2 and 3 were selected as representative of azurophilic granules, while fractions 5 and 6 were selected for specific granules, fractions 8 and 9 were selected for gelatinase granules, and fractions 10-12 were selected for the secretory vesicles. (C) Proteomic distribution of the markers in each storage organelle in two replicates from the

same individual. (D) Proteomic distribution of the markers in each storage organelle for all subjects and their replicates (n=5). The results are shown as median ± SEM. The x axis shows the abundance of each marker (as chosen by biochemical methods) across the four different granules, 1 represents azurophilic, 2 for specific, 3 for gelatinase granules and 4 represents the secretory vesicles.

After using the known markers to confirm by proteomics the successful fractionation of each distinct granule, we used Principal Component Analysis (PCA) to inspect the behavior of replicate fractionated samples of 3 different subjects. The results displayed in **Figure 3** show samples cluster together according to the granules they belong. The first two principal components of the analysis account for 49.2% and 14%, respectively, of the total variation in the dataset. Thus, by analyzing the overall variance of our dataset, considering technical and biological replicates, we obtained a significant separation in the PCA, corroborating that the isolation of each granule type was effective.

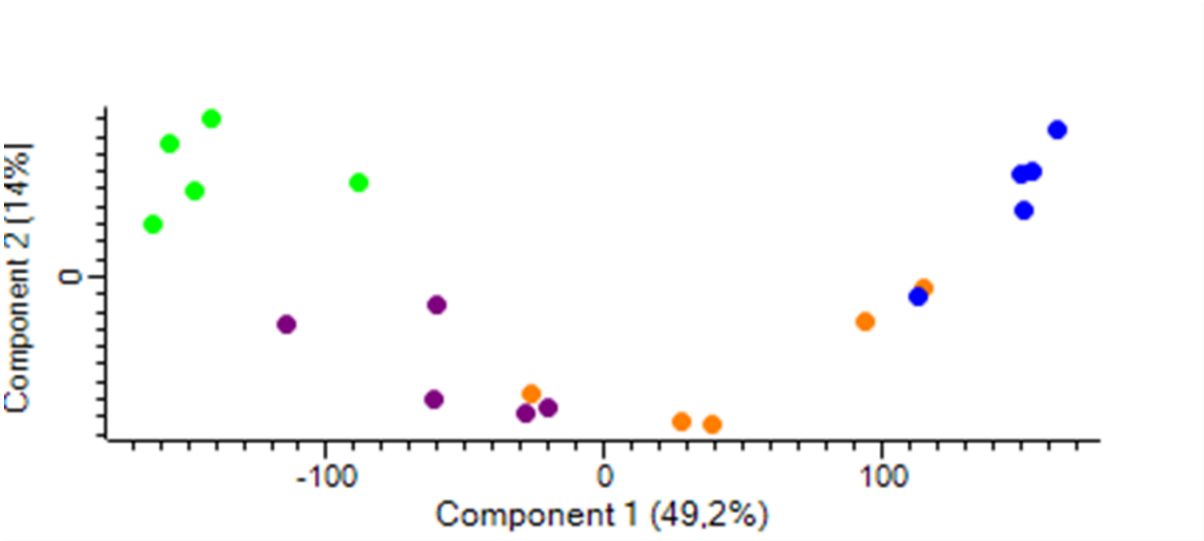


Figure 3. Principal Component Analysis (PCA) of the sample's replicates separated into neutrophil's storage organelles groups. Blue= azurophilic granules, orange = specific granules, purple = gelatinase granules, light green = secretory vesicles.

The results showed above confirmed our miniaturized method was effective to separate neutrophil's granules. The next step was to catalogue the protein granule components. We reasoned that proteins with the same fractionation profile obtained for the known markers

(**Figure 2**) should likely belong to the same granule. Hence, we started by investigating the fractionation profile of the proteins with the highest correlation with the granule's markers shown in **Figure 2**.

The profile plot containing the top 3 proteins of each granule type (based on Pearson's correlation with the marker) is shown in **Figure 4.** **Figure 4A** shows the fractionation profile of the top 3 proteins for all granules. To provide a better visualization of the fractionation patterns, the top three proteins obtained for azurophilic, specific, and gelatinase granules, as well as for secretory vesicles are highlighted respectively in **Figure 4 B, C, D** and **E**. This strategy revealed that neutrophil elastase (ELANE) and myeloblastin (PRTN3), well known as belonging to the azurophilic granules (15,20,22), display the same fractionation profile as MPO, with approximately 60 % of their content concentrated in the first fraction **(Figure 4 B**). Likewise, NGAL and olfactomedin-4 (OLFM4) follow the fractionation pattern seen for LTF, with approximately 75 % of their content concentrated in fraction 2. These proteins are known components of specific granules (**Figure 4C** (22)). Integrin alpha-M (ITGAM) and carbonic anhydrase 4 (CA4) follow the pattern seen for MMP9 (**Figure 4D**), with a slightly shift of ITGAM towards the specific fraction (15,20–22). This shift is expected because this protein is localized in both peroxidase-negative granules. Indeed, the most recent neutrophil granule's proteome study (22) demonstrated that 37% and 27% of the protein ITGAM were localized in specific and gelatinase granules, respectively. The CA4 protein was already described as part of gelatinase granules in Lominadzed *et al* (21). Lastly, discoidin domain-containing receptor 2 or transketolase (TKT) and leukocyte elastase inhibitor (SERPINB1) were seen fractionating closely to ALB (around 45-75 % of these proteins concentrated in the secretory vesicles fraction). The TKT and SERPINB1 proteins were reported in Rørvig *et al* study; the first as belonging to the supposed ficolin granule and the second was considered a plasma protein and thus discarded, even though SV fraction concentrated approximately 30 % of the protein.

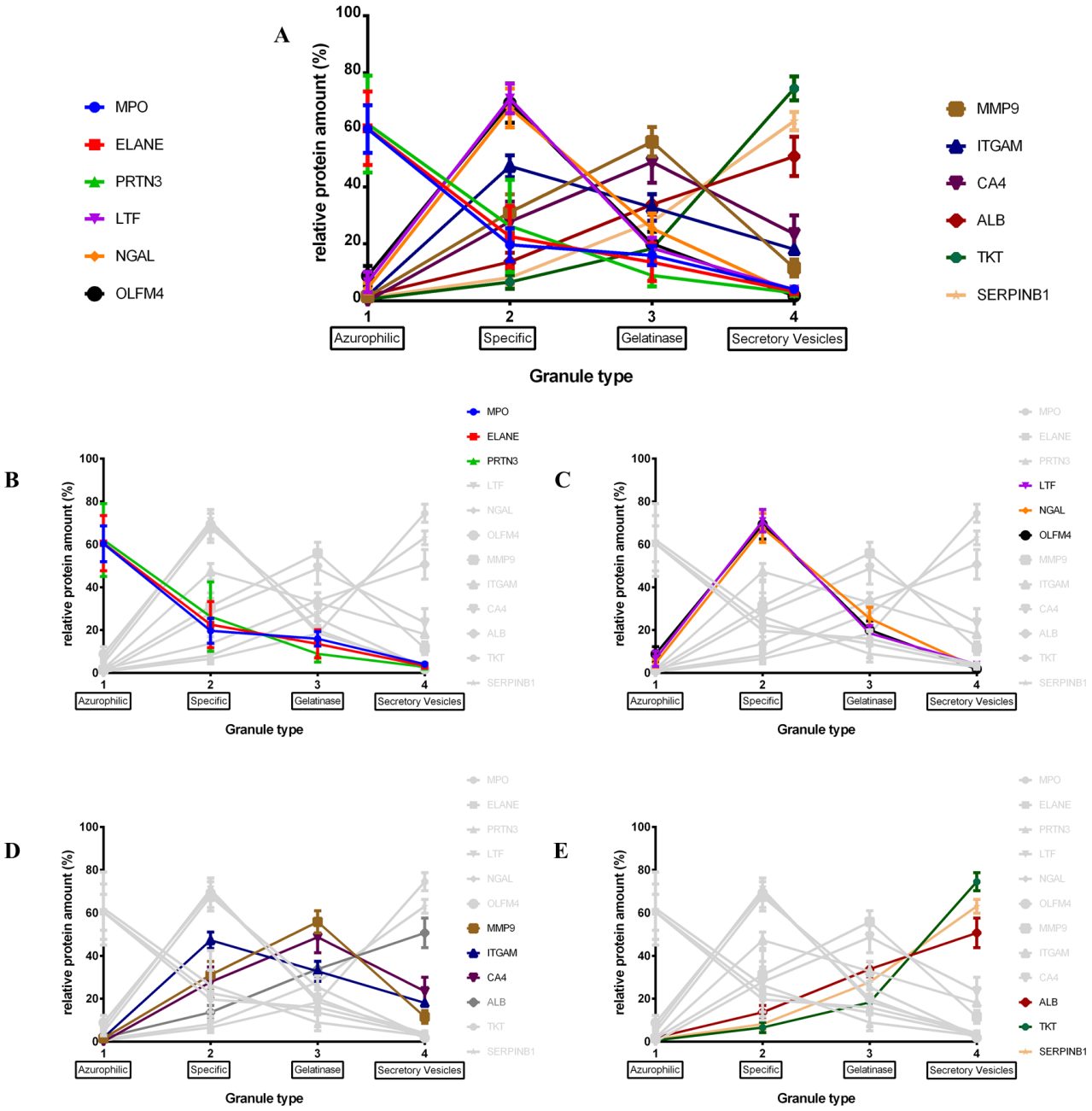


Figure 4. Top 3 proteins present in each storage organelle. The proteins were chosen based on their Pearson correlation to the marker of each granule type and the results are shown as mean ± SEM. (A) Top 3 proteins for each organelle. (B) Top 3 proteins for the azurophil granules are highlighted for better visualization of their distribution profile. The proteins shown are MPO, ELANE and PTNR3. (C) Top 3 proteins for the specific granules are highlighted for better visualization of their distribution profile. The proteins shown are LTF, NGAL and OLFM4. (D) Top 3 proteins for the gelatinase granules are highlighted for better visualization of their distribution profile. The proteins shown are MMP9, ITGAM and CA4. (E) Top 3 proteins for the secretory vesicles are highlighted for better visualization of their distribution profile. The proteins shown are ALB, TKT and SERPINB1.

A wider analysis showed that many more proteins may belong to each specific organelle. In this study, a total of 883 proteins were detected as present in at least one organelle in one sample. After filtering out proteins that were not present in at least 3 samples of at least one storage organelle, 369 proteins remained in the dataset, and their distribution profile across the four organelles was plotted into a heatmap as the mean percentage for each granule. **Figure 5** shows the heatmap of the 369 proteins across the organelles. Azurophilic granules have less protein complexity when compared to the other granules and are represented by the two first clusters that combined show a total of only 12 proteins. The proteins present in these clusters are the usual ones related to azurophilic granules, such as MPO, azurocidin (AZU1), bactericidal permeability-increasing protein (BPI), ELANE, PTRN3 and neutrophil defensin-3 (DEFA3). The overlap between azurophil and specific granules is represented by the cluster 3 that contain only 2 proteins, carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) and CD63. Cluster 4 contains 25 proteins pertaining to gelatinase granules and secretory vesicles. This overlap may be due to the proximity in the density gradient. The cluster 5 represents the secretory vesicles and contains 239 proteins. The secretory vesicles are the most complex in protein content and 6 receptors (APOBR, GRB2, PTPN6, C5AR1, LTB4R and EM3R) localize in this cluster. This result is expected due to the function that these vesicles play in neutrophil physiology of furnishing the cell with receptors in the priming process. In addition, this cluster has proteins related to mobilization, such as tubulins and actins. The abundance of these proteins is related to how readily the vesicles are mobilized (7,8). The cluster number 7 shows 23 proteins with higher abundance in gelatinase granules. A closer inspection to this cluster shows a range of proteins that can be contaminants, such as histones and typical mitochondrial proteins, which may indicate a mitochondrial contamination.

The similarity between specific and gelatinase granules is shown by the presence of 4 clusters that explore the overlap between these granules, the clusters 8, 9, 10 and 11. These

clusters have a combined total of 22 proteins. Interesting to notice that some proteins usually used as markers for gelatinase, such as MMP9 and ficolin-1 (FCN1) are present in these clusters. The cluster that represents the specific granules is the number 12, and it contains 43 proteins and is the largest one between the granular set, demonstrating the proteome complexity of these granules. The cluster 12 contains the Vesicle-associated membrane protein 8 (VAMP8) with a mean percentage of 55.7%. This protein is related to exocytosis and was the only mobilization protein present in a granular set cluster. In addition, the proteins usually related to the specific granules such as NGAL, LTF, OLFM4, ITGAM, resistin (RETN) and haptoglobin (HP) are also present in this cluster. The protein eosinophil peroxidase (EPX) also reported in other granular proteomics studies (21,22), appeared in this cluster in our samples. The mean percentage, and cluster number are displayed in **Supplementary Table 2**.

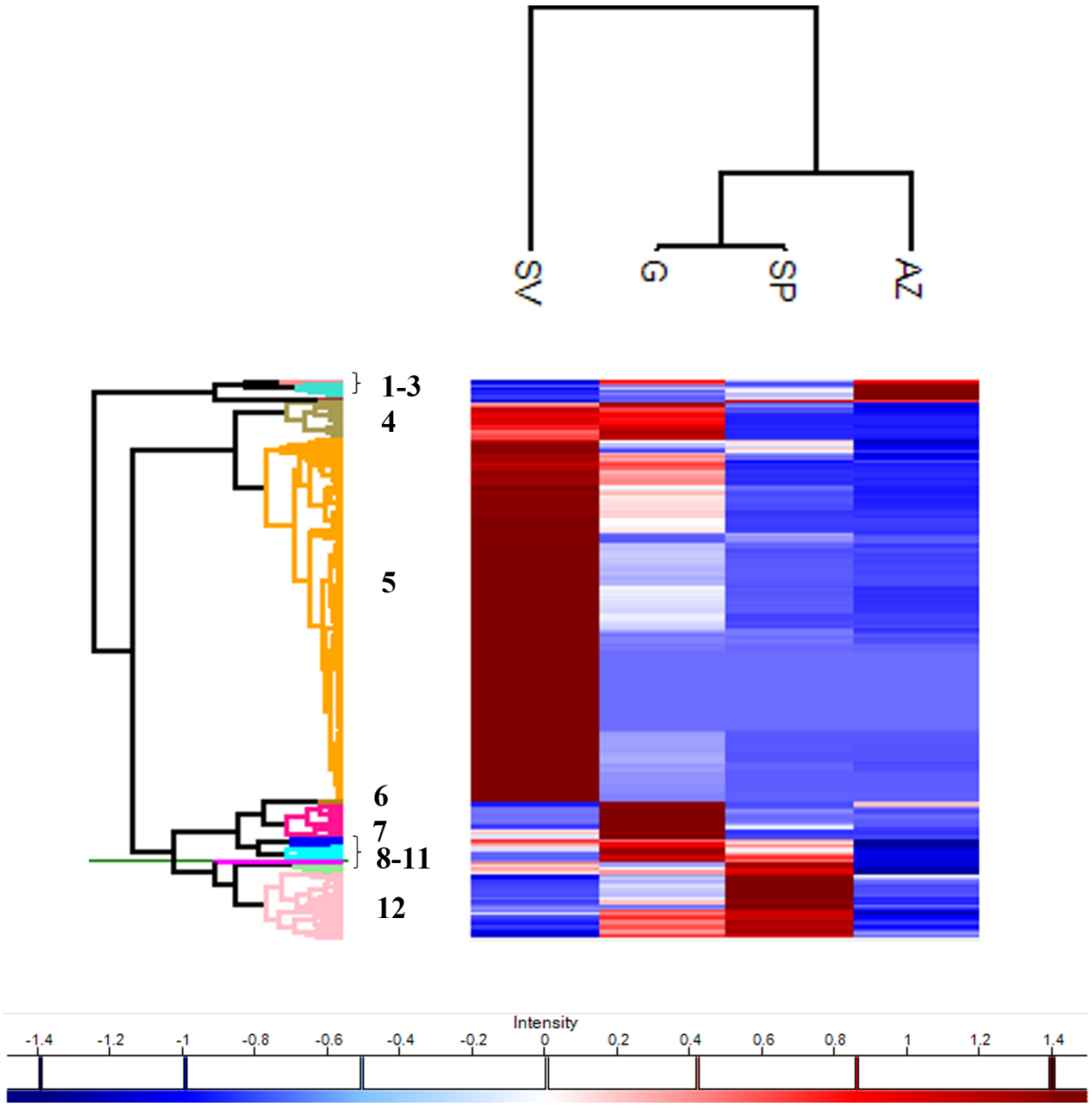


Figure 5. Heatmap of the 369 proteins in at least 3 samples of at least one storage organelle. The heatmap was elaborated using the mean percentage of each protein and normalized with Z-score. The groups were separated by Pearson's correlation and the proteins were grouped in 12 clusters. The scale intensity goes from blue (less abundant) to red (more abundant).

We used a SVM algorithm to assess if protein localization could be attributed accurately by learning the fractionation patterns of organellar markers with well-established localization. Based on four scientific studies (15,21–23), we curated organellar markers for each compartment to investigate if protein localization could be classified based on the fractionation profile. Markers were selected if present in at least two of the studies (**Figure 6A**). A clear

separation between the markers confirms they are reliable to predict the localization of the other proteins. The set of markers were then used by the SVM algorithm to predict the localization of each protein in our dataset of 369 proteins (**Figure 6B**). Using a 95% score cut-off, the localization of 140 proteins were predicted, 9 localized in azurophilic granules, 18 localized in the specific granules, 5 belonging to the gelatinase granules and the remaining 108 were classified as pertaining to the secretory vesicles. Classification probability scores that reflect the reliability of the assignment are provided with the classifications obtained from the SVM algorithm. Low scores are often associated with profiles not directly modeled by the organellar markers used in the algorithm (43). This is the case for proteins localized in more than one granule. The table with the protein localization predicted using SVM with 95% cut-off can be seen in the **Supplementary Table 3**. Thus, using the curated markers, MPO, ELANE, CTSG, CD63, BPI, AZU1, PRTN3 DEFA3 and MAN2B1 were confidently assigned as pertaining to the azurophil granules. Likewise, MMP9, AT6V0D1, B2M, SIRPA and FCN1 were assigned to gelatinase granules. Interestingly, proteins from cluster 7 (**Figure 5**), suspected as contaminants, were not classified by the SVM algorithm as pertaining to gelatinase granules. Proteins such as LTF, OLFM4, ITGAM, RTN and HP were all assigned to the specific granules. The 4 proteins localized in specific granules besides the markers were TCN1, OLR1, PTX3 and CHIT1. Likewise, 94 proteins were predicted to localize in the secretory vesicles beside the predetermined markers. These results clearly show our miniaturized method was effective in isolating neutrophil's granules and identifying their main proteins.

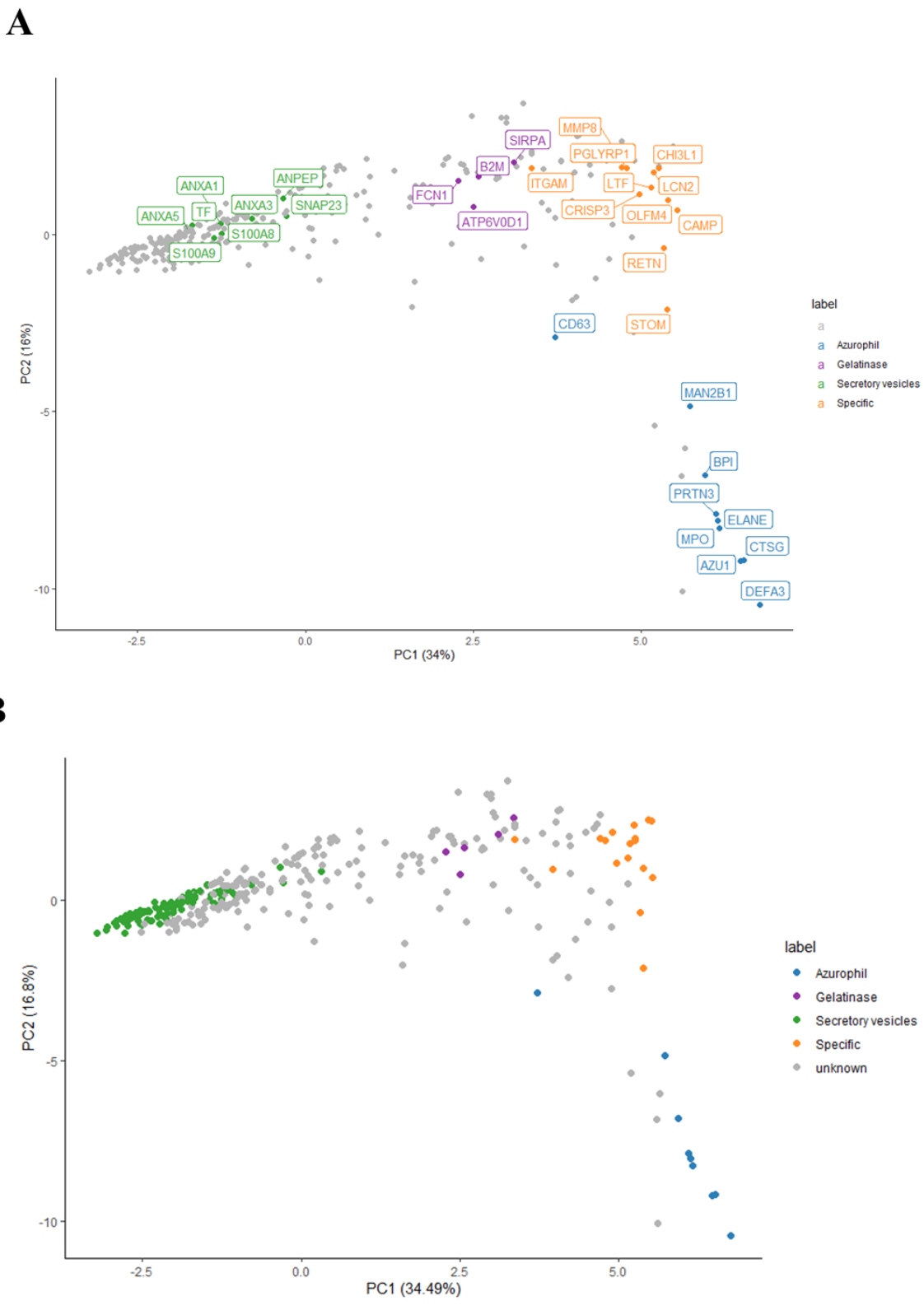


Figure 6. Principal component analysis (PCA) of the proteins present in at least 3 samples of each storage organelle group in our study. (A) PCA analysis of the markers used for prediction of protein localization with the supported vector machine (SVM) approach. The markers were chosen through extensive literature search. (B) PCA analysis of all proteins and their predicted localization with SVM approach. The colored dots represent the proteins with at least 95% confidence in the prediction score and each color is related to a specific storage organelle.

4. Discussion

In this study, we were able to successfully miniaturize the process of neutrophil granules isolation, allowing the assessment of the proteins of multiple biological and technical replicates, using biochemical assays, mass spectrometry-based proteomics and a machine learning approach. There are a few neutrophil granules isolation methods described in the literature, and the most used are the ones based on discontinuous Percoll density gradients (23–25,46), specially the 3-layer Percoll gradient. All these methods, however, have in common the large volume of sample used for one single isolation, about 400 mL of peripheral blood (approximately 3 x 10^8 neutrophils), which doesn't allow for technical replicates. Moreover, the large sample volume also makes difficult the use of biological replicates in a period shorter than 3 months, and complex studies that investigate the proteome of these storage organelles culminates in a more strenuous and expensive process. The miniaturized process developed in this study allows for both biological and technical replicates, and it is reproducible and reliable as shown in the biochemical and proteomic analyses in **Figure 2**. Furthermore, PCA analysis of our five samples have shown that the separation between the granules was effective and clear in all the replicates as shown in **Figure 3**.

The proteome of neutrophil granules is key to understanding neutrophil function. However, due to the challenges involving the isolation process and sample preparation for mass spectrometry, there are few studies that tackle this subject. Two important studies in this field were published in 2005 by Lominadze et al. (21) and in 2013 Rørvig et al. (22), identifying 286 and 1292 proteins, respectively, as pertaining to multiple granules. In the present study, to exclude possible protein variability among individuals, we considered only the proteins present in at least 3 samples in each storage organelle group, and after this stringent filtration, 369 proteins were identified. This is important because the neutrophil population is not homogenous even in healthy subjects (50,51), and therefore the protein content and abundance may vary.

The previous studies, however, did not have biological replicates. Therefore, some of their findings might be tied to one specific sample and might not reflect accurately the majority of proteins belonging to neutrophil's storage organelles. Although the number of proteins identified in our work were lower than in the 2013 study, probably as a reflex of the miniaturization, the number of replicates, both biological and technical, allowed for a more reliable characterization of neutrophil's granule proteome than the ones based in only one sample.

The analyses of granules content (**Figure 5)** showed the azurophil granules were the compartment with the smaller number of proteins. Besides this fact, proteins present in this granule were corroborated by the literature (F**igure 6 and Supplemental Table 3**), proving once again that our isolation was successful. Nevertheless, a deep analysis of the granule's content shows that further studies are necessary to attribute the accurate localization for all proteins investigated. For instance, the Syntenin-1 (SDCBP), present in one of the azurophil clusters in this work, was reported in 2005 as present in the specific and gelatinase granules fractions. However, our study corroborates the reported in the 2013 study, the presence of this protein in the azurophil fraction. In our study, the specific and gelatinase granules, both classified as peroxidase negative, shared 22 proteins. The proteins that usually are used as markers for gelatinase, MMP9 and FCN1 were present in these co-localized clusters, which may indicate that the isolation between specific and gelatinase granules must be improved. Nevertheless, our work was the first to show shared proteins between these granules in a proteomics study. Whether or not the co-localization is an isolation artifact, or if both granules share the proteins, still needs further evaluation. The protein FCN1 has recently been attributed to the ficolin granule, a putative new granular type that would be less dense than the gelatinase granules. In our study, the protein co-localized with specific granules. In fact, the specific granules appear to be the most complex in protein diversity due to the presence of 43 proteins.

Importantly, all of them were reported in previous proteomic studies. However, some discrepancies of localization were noted; the CSC1-like protein 1 (TMEM63A) was reported in 2005 as present in the gelatinase granules, but our study corroborates the localization in the specific granules as reported in the 2013 study. In contrast, the glutaminyl-peptide cyclotransferase (QPCT) was reported in 2013 in the gelatinase granules and our study corroborates the reported in 2005, the localization in the specific granules fraction. The gelatinase granules showed the presence of histones and mitochondrial proteins that may suggest contamination, but all neutrophil granule's studies also reported presence of histones and mitochondrial proteins. These results suggest these proteins are co-isolated during the separation using Percoll gradient. This technique is common to all proteome studies involving neutrophils granules.

All the inconsistencies seen when comparing our work with previous published studies lead us to employ a machine learning approach to compile an accurate list of neutrophil's granules markers. One limitation of our stringent criteria for data curation (present in the same granule in at least two independent studies), and for protein occurrence in this work (present in at least three replicates of the same granule) is the exclusion of many proteins likely belonging to a specific granule. Thus, from our 369 proteins found in at least there samples of at least one organelle, the SVM assigned 140 to pertaining to a specific granule, with a cumulative cut-off score of 95%. Nevertheless, the proteins found are consistent across the available studies, and our results provide a resource for future investigations regarding neutrophil's granules composition and function.

5. Conclusion and Perspectives

The present pioneer study provided a new reproducible miniaturized method for neutrophil granules isolation that allow for multiple technical and biological replicates and is more affordable. The proteins found in this study and their localization can be used in the future as a database for further studies in the neutrophil field. The method described will also allow comparative studies involving neutrophil granules mobilization during priming and activation with different stimuli.

REFERENCES

1. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nature Reviews Immunology. 2013 Mar;13(3):159–75.

2. Hampton MB, Kettle AJ, Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood. 1998 Nov 1;92(9):3007–17.

3. Kettle AJ, Rosen H, Winterbourn CC, Nauseef WM, Klebanoff SJ. Myeloperoxidase: a front-line defender against phagocytosed microorganisms. Journal of Leukocyte Biology. 2013 Feb;93(2):185–98.

4. Sørensen OE, Borregaard N. Neutrophil extracellular traps - The dark side of neutrophils. Journal of Clinical Investigation. 2016 May 2;126(5):1612-20.

5. Ludwig IS, Geijtenbeek TBH, van Kooyk Y. Two way communication between neutrophils and dendritic cells. Current Opinion in Pharmacology. 2006 Aug;6(4)408-13.

6. Condliffe AM, Kitchen E, Chilvers ER. Neutrophil priming: Pathophysiological consequences and underlying mechanisms. Clinical Science. 1998 May;94(5)461-71.

7. Sengeløv H, Kjeldsen L, Borregaard N. Control of exocytosis in early neutrophil. Journal of Immunology. 1993 Feb 15;150(4):1535-43.

8. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. Microbes and Infection. 2003 Nov;5(14):1317-27.

9. Bainton DF, Ullyot JL, Farquhar MG. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. Journal of Experimental Medicine. 1971 Oct 1 134(4):907–34.

10. Kjeldsen L, Bainton DF, Sengeløv H, Borregaard N. Structural and functional heterogeneity among peroxidase-negative granules in human neutrophils: identification of a distinct gelatinase-containing granule subset by combined immunocytochemistry and subcellular fractionation. Blood. 1993 Nov 15;82(10):3183–91.

11. Borregaard N, Miller LJ, Springer TA. Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. Science. 1987 Sep 4;237(4819):1204–6.

12. Le Cabec V, Cowland JB, Calafat J, Borregaard N. Targeting of proteins to granule subsets is determined by timing and not by sorting: The specific granule protein NGAL is localized to azurophil granules when expressed in HL-60 cells. Proceedings of the National Academy of Sciences of the United States of America. 1996 Jun 25;93(13):6454-7.

13. Borregaard N, Sehested M, Nielsen BS, Sengeløv H, Kjeldsen L. Biosynthesis of Granule Proteins in Normal Human Bone Marrow Cells. Gelatinase Is a Marker of Terminal Neutrophil Differentiation. Blood. 1995 Feb 1;85(3):812-7.

14. Lawrence SM, Corriden R, Nizet V. The Ontogeny of a Neutrophil: Mechanisms of Granulopoiesis and Homeostasis. Microbiology and molecular biology reviews. 2018 Feb 7;82(1):e00057-17.

15. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood. 1997 May 15;89(10):3503–21.

16. Borregaard N, Kjeldsen L, Rygaard K, Bastholm L, Nielsen MH, Sengeløv H et al. Stimulus-dependent secretion of plasma proteins from human neutrophils. Journal of Clinical Investigation. 1992 Jul;90(1):86–96.

17. Sengeløv H, Follin P, Kjeldsen L, Lollike K, Dahlgren C, Borregaard N. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. Journal of immunology. 1995 Apr 15;154(8):4157–65.

18. Brumell JH, Volchuk A, Sengeløv H, Borregaard N, Cieutat AM, Bainton DF, et al. Subcellular distribution of docking/fusion proteins in neutrophils, secretory cells with multiple exocytic compartments. Journal of immunology. 1995 Dec 15;155(12):5750–9.

19. Kjeldsen L, Bjerrum OW, Askaa J, Borregaard N. Subcellular localization and release of human neutrophil gelatinase, confirming the existence of separate gelatinase-containing granules. Biochemical Journal. 1992 Oct 15;287(2):603–10.

20. Borregaard N, Sørensen OE, Theilgaard-Mönch K. Neutrophil granules: a library of innate immunity proteins. Trends in Immunology. 2007 Aug;28(8):340–5.

21. Lominadze G, Link AJ, Luerman GC, Powell DW, McLeish KR, Ward RA. Proteomic Analysis of Human Neutrophil Granules. Molecular & Cellular Proteomics. 2005 Oct;4(10):1503–21.

22. Rørvig S, Østergaard O, Heegaard NHH, Borregaard N. Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors. Journal of Leukocyte Biology. 2013 Oct;94(4):711–21.

23. Borregaard N, Heiple JM, Simons ER, Clark RA. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: Translocation during activation. Journal of Cell Biology. 1983 Jul;97(1):52–61.

24. Kjeldsen L, Sengeløv H, Lollike K, Nielsen MH, Borregaard N. Isolation and characterization of gelatinase granules from human neutrophils. Blood. 1994 Mar 15;83(6):1640–9.

25. Clemmensen SN, Jacobsen LC, Rørvig S, Askaa B, Christenson K, Iversen M, et al. Alpha-1-antitrypsin is produced by human neutrophil granulocytes and their precursors and liberated during granule exocytosis. European Journal of Haematology. 2011 Jun;86(6):517–30.

26. Bøyum A. Isolation of Lymphocytes, Granulocytes and Macrophages. Scandinavian Journal of Immunology. 1976 Jun;Suppl 5:9-15.

27. Spitznagel JK, Dalldorf FG, Leffell MS, Folds JD, Welsh ID, Cooney MH et al. Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. Laboratory Investigation; a journal of technical methods and pathology. 1974 Jun;30(6):774–85.

28. Cramer E, Pryzwansky KB, Villeval JL, Testa U, Breton-Gorius J. Ultrastructural Localization of Lactoferrin and Myeloperoxidase in Human Neutrophils by Immunogold. Blood. 1985 Feb 1;65(2):423–32.

29. Borregaard N, Christensen L, Bjerrum OW, Birgens HS, Clemmensen I. Identification of a highly mobilizable subset of human neutrophil intracellular vesicles that contains tetranectin and latent alkaline phosphatase. The Journal of clinical investigation. 1990 Feb;85(2):408–16.

30. DeChatelet LR, Robert Cooper M. A modified procedure for the determination of leukocyte alkaline phosphatase. Biochemical medicine. 1970 Aug;4(1):61–8.

31. Kleiner DE, Stetler-stevenson WG. Quantitative zymography: Detection of picogram quantities of gelatinases. Analytical Biochemistry. 1994 May 1;218(2):325-9.

32. Toth M, Sohail A, Fridman R. Assessment of gelatinases (MMP-2 and MMP-9) by gelatin zymography. Methods in Molecular Biology. 2012;878:121-35.

33. Ren Z, Chen J, Khalil RA. Zymography as a research tool in the study of matrix metalloproteinase inhibitors. Methods in Molecular Biology. 2017;1626:79-102.

34. Karlsson A, Khalfan L, Dahlgren C, Stigbrand T, Follin P. Neutrophil alkaline phosphatase activity increase in bacterial infections is not associated with a general increase in secretory vesicle membrane components. Infection and immunity. 1995 Mar;63(3):911–6.

35. Shevchenko A, Tomas H, Havliš J, Olsen JV., Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nature protocols. 2006 Jan;1(6):2856–60.

36. Rappsilber J, Mann M, Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nature protocols. 2007 Aug;2(8):1896–906.

37. Jürgen Cox, Matthias Mann. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nature Biotechnology. 2008 Dec;26(12):1367–72.

38. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen J V., Mann M. Andromeda: a peptide search engine integrated into the MaxQuant environment. Journal of Proteome Research. 2011 Apr 1;10(4):1794–805.

39. Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, Mann M. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. Molecular & cellular proteomics. 2014 Sep;13(9):2513–26.

40. Gatto L, Breckels LM, Wieczorek S, Burger T, Lilley KS. Mass-spectrometry-based spatial proteomics data analysis using pRoloc and pRolocdata. Bioinformatics (Oxford, England). 2014 May 1;30(9):1322–4.

41. R Core Development Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2013.

42. Christoforou A, Mulvey CM, Breckels LM, Geladaki A, Hurrell T, Hayward PC, et al. A draft map of the mouse pluripotent stem cell spatial proteome. Nature Communications. 2016 Jan 12;7:8992.

43. Jean Beltran PM, Mathias RA, Cristea IM. A Portrait of the Human Organelle Proteome In Space and Time during Cytomegalovirus Infection. Cell systems. 2016 Oct 26;3(4):361-373.e6.

44. Tan S, Hwee TT, Chung MCM. Membrane proteins and membrane proteomics. Proteomics. 2008 Oct;8(19):3924–32.

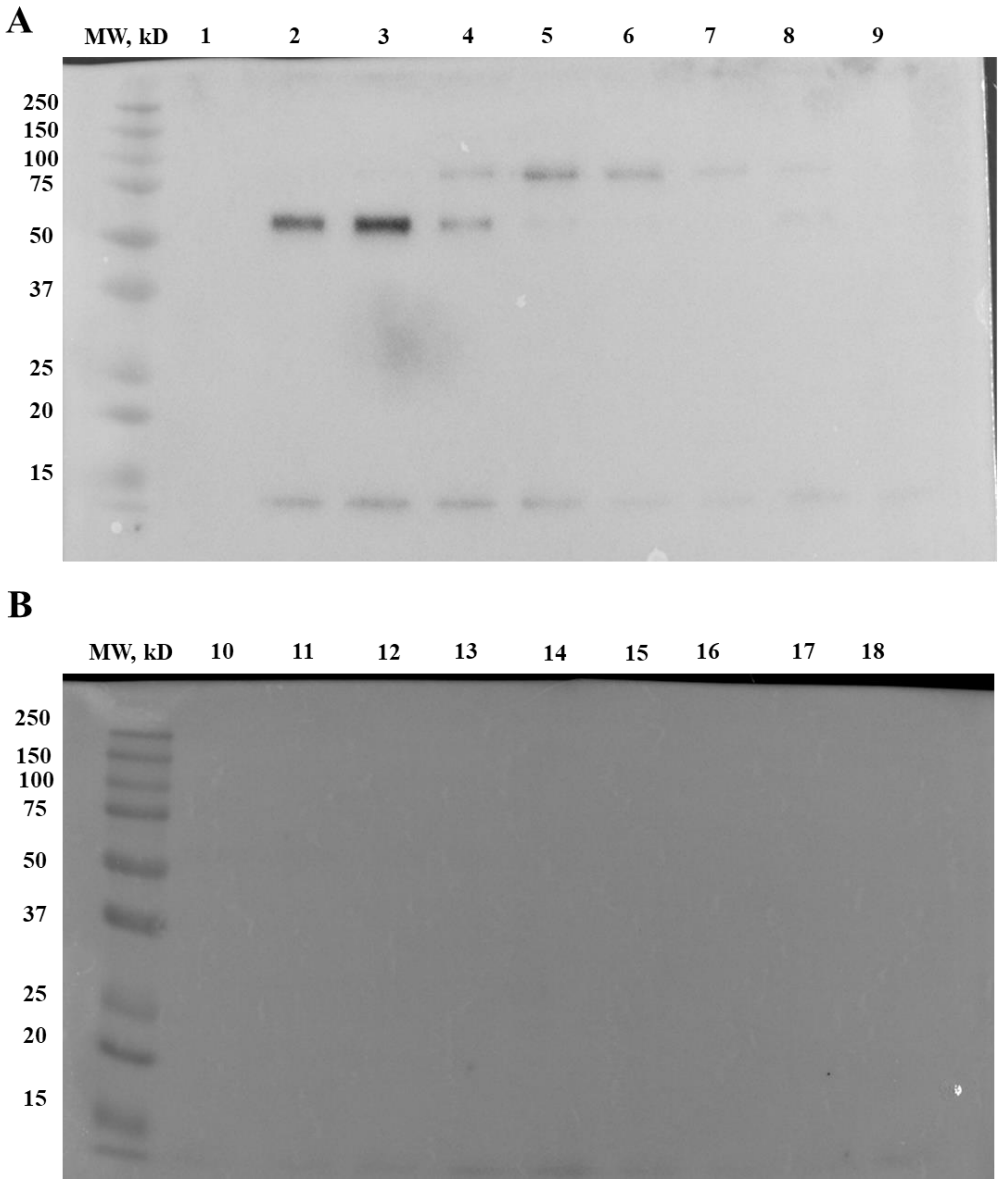
45. Rabilloud T. Membrane proteins and proteomics: love is possible, but so difficult. Electrophoresis. 2009 Jun;30 Suppl 1:S174-80.

46. Kjeldsen L, Sengeløv H, Borregaard N. Subcellular fractionation of human neutrophils on Percoll density gradients. Journal of Immunological Methods. 1999 Dec 17;232(1-2):131-42.

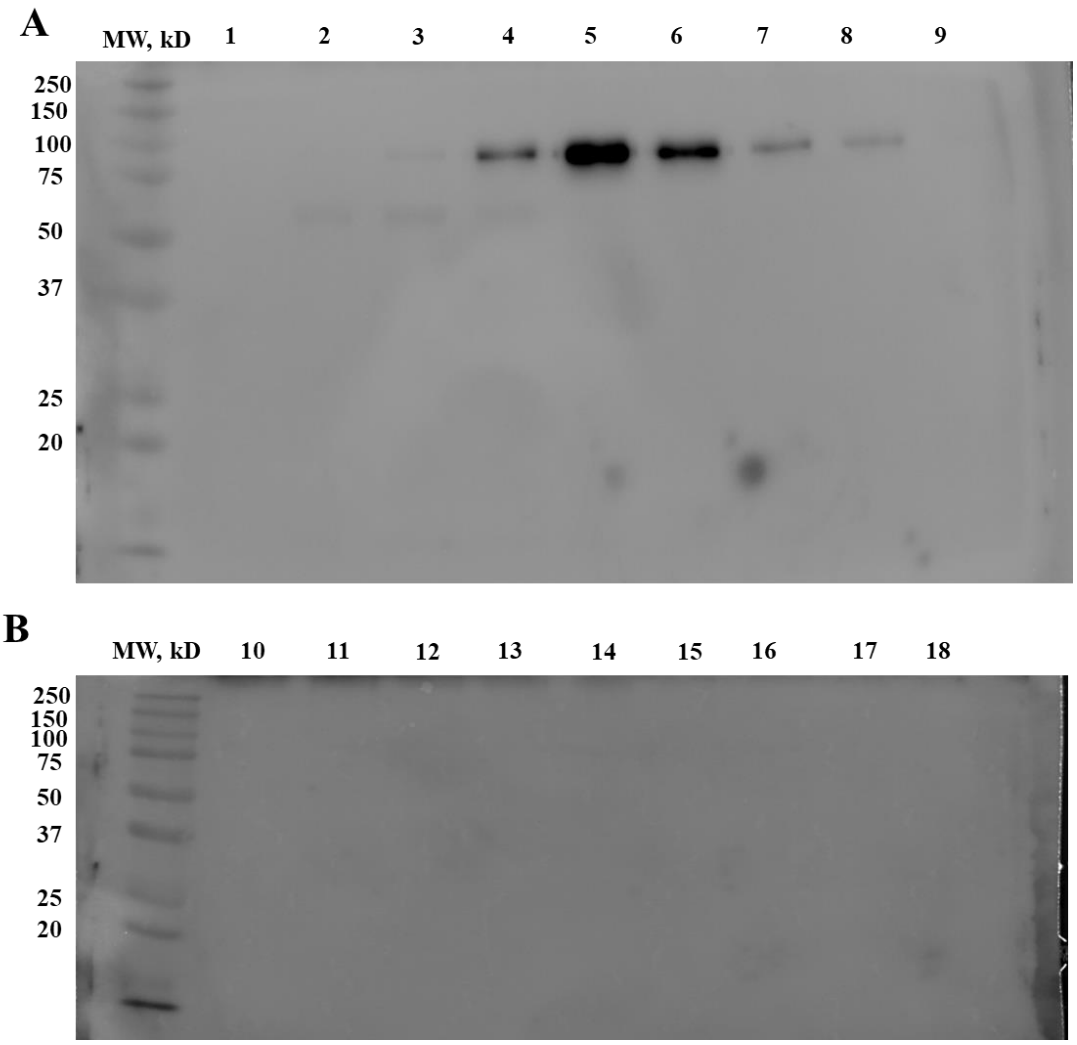
47. Silvestre-Roig C, Fridlender ZG, Glogauer M, Scapini P. Neutrophil Diversity in Health and Disease. Trends in immunology. 2019 Jul;40(7):565–83.

48. Bonaventura A, Montecucco F, Dallegri F, Carbone F, Lüscher TF, Camici GG, et al. Novel findings in neutrophil biology and their impact on cardiovascular disease. Cardiovascular research. 2019 Jul 1;115(8):1266–85.

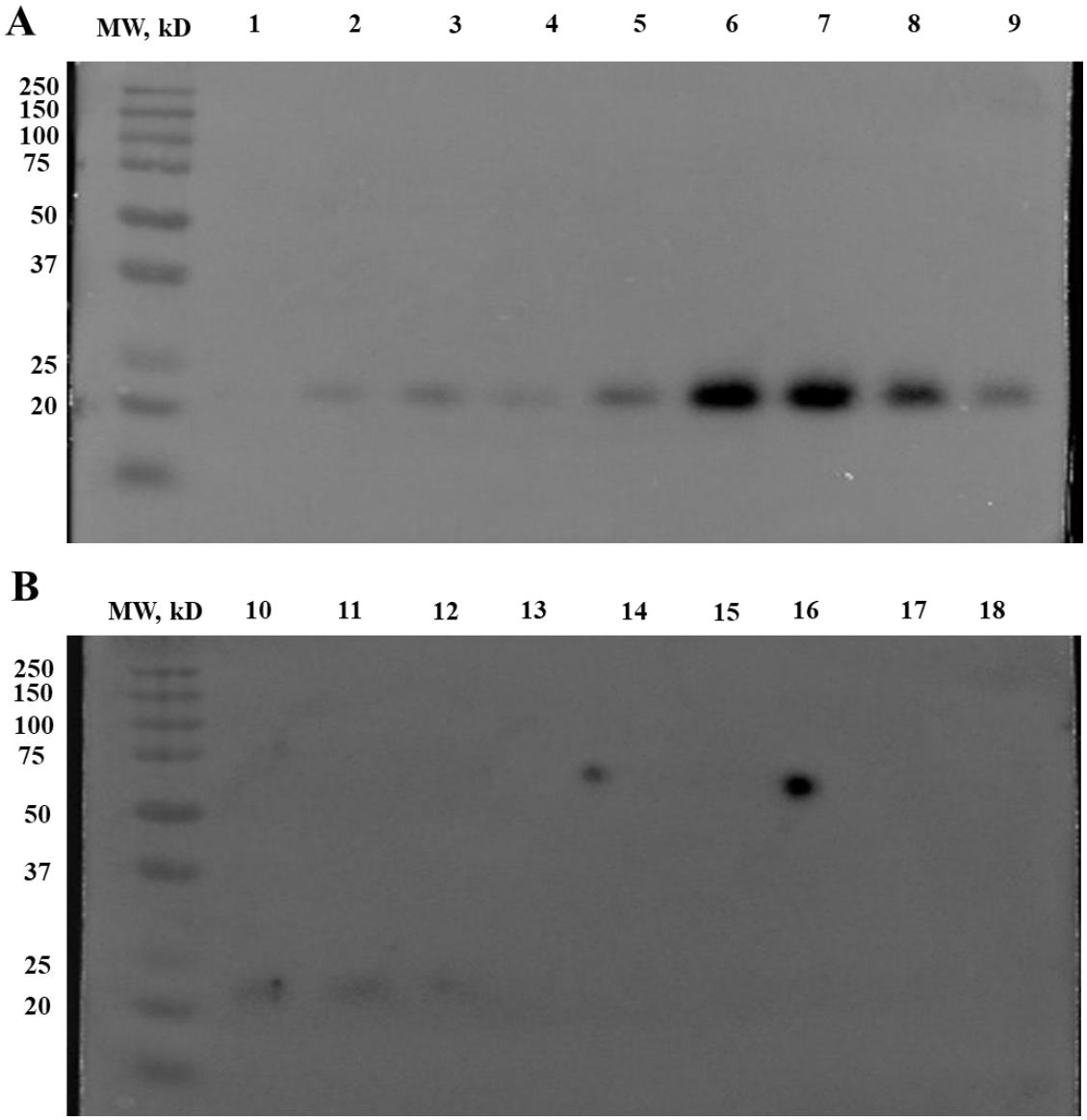
Supplementary information



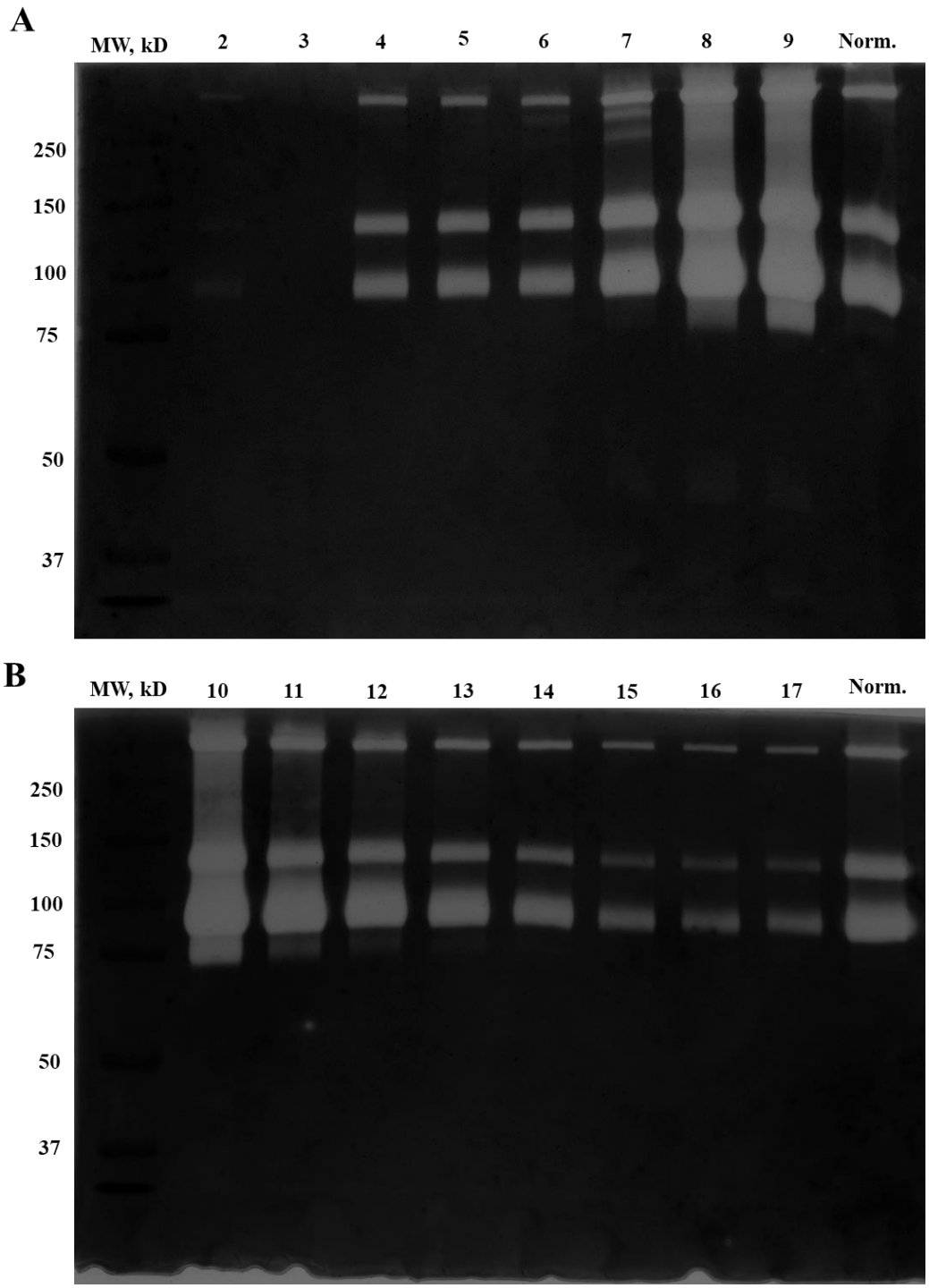
Supplementary figure 1. Representative WB membranes marked with rabbit polyclonal primary antibody anti-MPO for the fractions collected from the miniaturized 3-layer Percoll density gradient. The heavy chain of MPO (60 kDa) band was analyzed in ImageJ software.



Supplementary Figure 2. Representative WB membranes marked with mouse monoclonal primary antibody anti-LTF conjugated with HRP for the fractions collected from the miniaturized 3-layer Percoll density gradient. The LTF bands (90 kDa) were analyzed in ImageJ software.



Supplementary Figure 3. Representative WB membranes marked with rabbit polyclonal primary antibody anti-NGAL for the fractions collected from the miniaturized 3-layer Percoll density gradient. The NGAL bands (25 kDa) were analyzed in ImageJ software.



Supplementary Figure 4. Representative zimography gels for the fractions collected from the miniaturized 3-layer Percoll density gradient. The MMP9 band at 92kDa was analyzed in ImageJ. The normalizer (0,125ug of protein from the cavitate) was used to compare the bands from different gels. The other bands present in the gels are complex of MMP9-NGAL, and dimers.

ATTACHMENT LIST

1- Supplementary table 1

2- Supplementary table 2

3- Supplementary table 3

Visualize the attachment tables at: https://github.com/GabyAMF/A-MAP-OF-NEUTROPHIL-GRANULES-BY-PROTEOMICS

CURRICULUM VITAE

1. PERSONAL DATA

Name: Gabrielly Alexandria de Moura Freitas
Place and date of birth: Niterói-RJ, 15/03/1996

2. EDUCATION

Colégio Salesianos Santa Rosa, Niterói/RJ (2011-2013)

High school degree

Universidade Federal Fluminense, Niterói/RJ (2015-2018)

Bachelor's degree in Biomedicine with clinical analysis

3. COMPLEMENTARY EDUCATION

Instituto Nikola Tesla (2021)

Fisiologia do Esporte - 60h

Universidade de São Paulo, São Paulo/SP (2020)

2º curso EaD de Armazenamento e Compatibilidade de Reagentes – 10h

Johns Hopkins University, EUA (2020)

R programming – 58h

Duke University, EUA (2020)

Introduction to probability and data with R – 15h

4. OCUPATION

Undergraduate fellowship, FAPERJ, 01/2016-10/2017

Undergraduate fellowship, PIBIC, 11/2017-05/2018

Master's fellowship, CAPES, 03/2019 – 08/2021

Post-graduation student representative, IQ-USP, 27/09/2020 – 26/09/2021

Biomedical analyst, ISA Lab, 02/2022 – current

5. PUBLICATIONS

SOUZA JUNIOR, D. R. ; SILVA, AMANDA RIBEIRO MARTINS ; ROSA-FERNANDES, LIVIA ; REIS, LORENNA ROCHA ; **ALEXANDRIA,G.** ; BHOSALE, SANTOSH D. ; GHILARDI, FABIO DE ROSE ; DALÇÓQUIO, TALIA FALCÃO ; BERTOLIN, ADRIADNE JUSTI ; NICOLAU, JOSÉ CARLOS ; MARINHO, CLAUDIO R.F. ; WRENGER, CARSTEN ; LARSEN, MARTIN R. ; SICILIANO, RINALDO FOCACCIA ; DI MASCIO, PAOLO ; PALMISANO, GIUSEPPE ; RONSEIN, GRAZIELLA ELIZA . HDL proteome remodeling associates with COVID-19 severity. Journal of Clinical Lipidology, v. 15, p. 796-804, 2021.

6. PARTICIPATION IN EVENTS

I Simpósio dos Pós-Graduandos da Bioquímica: a academia e além – 2022

Organization of the event

20th International Union for Pure and Applied Biophysics (IUPAB) - 2021

Neutrophil granules isolation: a new miniaturized method (poster).

3rd RIDC Redoxoma Meeting with the Advisory Committee - 2020

Mapping Neutrophil Granules (poster).

I Simpósio de Fisiologia e Farmacologia Experimental - 2020