RESEARCH ARTICLE

Characterising the transcriptome of hypersegmented human neutrophils [version 1; peer review: 2 approved with reservations]

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Abstract

Background: Mature human neutrophils are characterised by their multilobed nuclear morphology. Neutrophil hypersegmentation, a pathologic nuclear phenotype, has been described in the alveolar compartment of patients with acute respiratory distress syndrome and in several other contexts. This study aimed to characterise the transcriptional changes associated with neutrophil hypersegmentation.

Methods: A model of hypersegmentation was established by exposing healthy peripheral blood neutrophils to the angiotensin converting enzyme inhibitor (ACEi) captopril. Laser capture microdissection (LCM) was then adapted to isolate a population of hypersegmented neutrophils. Transcriptomic analysis of microdissected hypersegmented neutrophils was undertaken using ribonucleic acid (RNA) sequencing. Differential gene expression (DEG) and enrichment pathway analysis were conducted to investigate the mechanisms underlying hypersegmentation.

Results: RNA-Seq analysis revealed the transcriptomic signature of hypersegmented neutrophils, with five genes differentially expressed. *VCAN*, *PADI4* and *DUSP4* were downregulated, while *LTF* and *PSMC4* were upregulated. Modulated pathways included histone modification, protein-DNA complex assembly and antimicrobial humoral response. The role of *PADI4* was further validated using the small molecule inhibitor, Cl-amidine.

Conclusions: Hypersegmented neutrophils display a marked transcriptomic signature, characterised by the differential expression of five genes. This study provides insights into the mechanisms underlying neutrophil hypersegmentation and describes a novel method to isolate and sequence neutrophils based on their morphologic subtype.

Keywords

Neutrophil, nuclear morphology, hypersegmentation, RNAseq

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Introduction

Neutrophils are the most numerous white blood cells in humans (Rosales 2018) and the principal effectors of the innate immune system. To fulfil this role, neutrophils are able to readily migrate to sites of inflammation where they recognise, phagocytose, and kill invading microorganisms¹.

Human neutrophils have a dynamic nuclear architecture. Immature (banded) neutrophils are often released into the peripheral blood during severe sepsis and have a single dumb-bell shaped nucleus. Once mature, the chromatin of human neutrophils is clustered in lobes linked to one another by dense heterochromatin filaments^{2,3}, with on average two to four lobes. In 1904, Arneth quantified the proportion of neutrophils with each number of lobes under homeostatic conditions; he established that 5% of human neutrophils had horseshoe-shaped or indented nuclei, 35% had two lobes; 41% had three, 17% four and $\leq 2\%$ had five lobes⁴. Neutrophil hypersegmentation is considered pathological, and is defined by the presence of more than 2% of cells with five lobes, or the presence of cells with six or more lobes². The presence of hypersegmentation was previously associated with the maturation state of a neutrophil⁵, but, more recently, hypersegmentation has been observed in other contexts⁶.

The capacity of neutrophils to alter their nuclear architecture is poorly understood, and the advantages of having a multi-lobed nuclear morphology are unclear⁷. It has been hypothesised that the multilobed phenotype increases the deformability of neutrophils and facilitates their migration through thin tissue spaces⁸. Alternatively, it may enhance phagocytosis by offering a wider cytoplasmic space to engulf pathogens⁹. However, during acute inflammation, immature banded neutrophils released into the circulation have been shown to have similar migratory properties to those of mature, multilobed neutrophils¹⁰. Moreover, the nuclear morphology of neutrophils varies widely across mammalian species, yet they achieve similar anti-microbial tasks, suggesting this hypothesis may be incorrect¹¹.

Juss *et al.* reported an increased number of hypersegmented neutrophils in the bronchoalveolar lavage of patients with acute respiratory distress syndrome (ARDS), when compared to peripheral blood neutrophils sampled from the same patients, and to peripheral blood from age/sex-matched healthy controls⁶. More than 25% of the alveolar neutrophils of patients with ARDS had five or more lobes, compared to around 10% in blood neutrophils from the same patients, and around 2% in blood obtained from healthy volunteers. Neutrophil hypersegmentation has also been observed in other contexts including bacterial infection, in association with tumours, and in the lungs of patients with chronic obstructive pulmonary disease^{12–14}. Very little is known about the mechanisms of hypersegmentation, or the functional consequences of the chromatin rearrangement occurring during this alteration in neutrophil morphology.

Transcriptomic analysis of human neutrophils has been largely neglected. For many years it was held that the short lifespan

of neutrophils, their paucity of mRNA, and their high content of endonucleases made such analyses too challenging^{15,16}. Recent studies have demonstrated that neutrophils are multifaceted cells with an active transcriptional program amenable to whole genome analysis^{17,18}. To permit the characterisation of the transcriptome of hypersegmented neutrophils, we combined a robust model of human neutrophil hypersegmentation with the use of laser capture microscopy (LCM) to identify and collect cells for RNA sequencing based on their morphology. Here we describe for the first time, the transcriptome of microdissected human neutrophils, identifying genes and pathways associated with hypersegmentation.

Methods

Ethical considerations

Ethical permission was granted by the Cambridge Research Ethics Committee (06/Q0108/281). All participants gave written informed consent.

Isolation of human neutrophils

Peripheral venous blood was sampled from age-matched healthy adult volunteers (no significant chronic illness or need for long-term medication) who had provided written informed consent. Participants were recruited from a bank of healthy volunteers, who volunteered to join the research blood donation programme after responding to posters placed at Addenbrooke's Hospital and the University of Cambridge. A research nurse obtained informed consent from participants between June and August 2019. Sex-disaggregated data is not available as the terms of our ethical permissions do not allow us to record this data.

Using a 19-gauge butterfly needle, two aliquots of 40 mL of peripheral venous blood were anticoagulated with 3.8% sodium citrate (Martindale Pharmaceuticals) and circulating neutrophils were immediately purified using discontinuous plasma-Percoll density gradient centrifugation under sterile conditions as previously described¹⁹. Neutrophil purity was routinely $>98\%$ with eosinophils as the major contaminant. Neutrophils were immediately processed for subsequent experiments as outlined below.

Neutrophil morphology analysis

Freshly isolated neutrophils were centrifuged for 3 minutes at 300 rpm onto polyethylene naphthalate (PEN)-membrane microscope slides (Leica Biosystems) or PolysineTM microscope slides (VWR International) using a Shandon Cytospin 3 (Shandon). Neutrophil morphology was assessed by a researcher blinded to the experimental conditions. Neutrophils were considered apoptotic if they showed dense condensation of chromatin forming a single nucleus, hypersegmented if they possessed five lobes or more, and normally lobulated if they had between two and four lobes⁴.

Induction of hypersegmentation in primary human neutrophils

Human neutrophils were centrifuged (Hettich Rotina, model 420r) for 5 min at 256 g and resuspended in 10% autologous serum

(AS) in Hank's Balanced Salt Solution (HBSS) (Thermo Fisher Scientific) (2.5×10^6 cells/mL) before stimulation with the following increasing captopril concentrations: 0 μ M, 10 μ M, 30 μ M, 100 μ M, 300 μ M and 1000 μ M. Plates were placed in a shaker (Eppendorf Thermomixer) at 300 rpm at 37 °C for 3 hours. Once the optimal concentration was identified, neutrophils were incubated in HBSS supplemented with 10% AS and 300 μ M captopril in a shaking plate at 37 °C for 0 h, 2 h, 4 h or 6 h.

Laser capture microdissection

We adapted the laser capture microdissection (LCM) technique to identify and isolate a population of hypersegmented neutrophils for transcriptomic analysis. LCM has the capacity to separate a defined area from a heterogeneous cell population and is applicable for use on histologically stained paraffin-embedded tissues, cell smears and cytopins²⁰. RNA sequencing was then undertaken on microdissected cells to characterise the differential gene expression profile of hypersegmented neutrophils.

The fixing and staining of neutrophil cytopins were optimised for RNA Integrity Number (RIN) preservation. Neutrophils on PEN-membrane glass slides were fixed with ice-cold 95% ethanol for 30 sec, then air-dried and stored at -80 °C for a maximum of three days. Slides were then stained for 1 min with hematoxylin and dehydrated in increasing ethanol concentrations to remove any remaining moisture: 50% for 10 sec, 70% for 20 sec, 95% for 15 sec, 100% for 25 sec and 100% for 30 sec. Laser capture microdissection was undertaken using 63x magnification on a Leica LMD6 system (Leica, Biosystems) using the Leica LMD software V7.6 (Leica, Biosystems), which allowed for the identification and selection of 1500 neutrophils per condition (750 in duplicate), which were then collected into a 0.5 mL collection tube (Greiner Bio-One).

RNA extraction and next generation sequencing

Microdissected neutrophils were lysed in Buffer RLT (Qiagen) supplemented with 1% of β -mercaptoethanol (β -ME), and this was followed by a brief vortex to homogenise the cells before storage at -80 °C for a maximum of three days. RNA extraction was performed according to Qiagen's RNeasy UCP Micro Kit protocol (Qiagen) including an on-column DNase digest under clean laboratory conditions. The resultant RNA was divided into two equal technical replicates for the cDNA libraries preparation. Prior to cDNA library preparation, an additional DNase incubation step was performed according to manufacturer's instructions (Heat&Run gDNA removal kit, ArcticZymes). The SMART Seq Stranded Micro kit (Takara Bio) was optimised for cDNA library preparation, and the following workflow was undertaken. RNA was reverse-transcribed and converted to cDNA after 4 min of fragmentation. Illumina adapters and barcodes were added through ten cycles of polymerase chain reaction (PCR) (following the ultra-low input protocol option). The PCR products were then purified by immobilisation onto AMPure beads (Beckman Coulter Life Science), ribosomal cDNA was depleted using mammalian-specific probes, and the remaining fragments were amplified with 10 additional PCR cycles before a final

purification. The resulting cDNA fragments were assessed using the DNA HS kit according to manufacturer's instructions on the Bioanalyser 2100 (Agilent). Paired-end 150 base pair (bp) sequencing was undertaken using an Illumina NovaSeq 6000 System S1 Flow Cell (Illumina) after normalisation of concentrations to the sample with the lowest concentration; 12.5 fM per sample were submitted for sequencing.

Bioinformatic analysis

The sequencing results were analysed in collaboration with biostatisticians from the Institute of Metabolic Science, University of Cambridge. Reads were mapped to the human genome using TopHat (v2.1.1)²¹ and Bowtie (v2.2.3)²². Transcripts were assembled, and the expression levels of genes were estimated using Cufflinks. Reads were normalised to counts per million (cpm) for all conditions, and differential gene expression was calculated using edgeR (3.26.8)²³. Significant differential gene expression was defined as false discovery rate (FDR) < 0.05. Differentially expressed genes were selected for pathway analysis using Metascape²⁴.

Real-time quantitative PCR (qPCR)

RNA from healthy volunteer neutrophils was isolated using the RNeasy Mini kit (Qiagen, Germany) in accordance with the manufacturer's instructions and included an on-column DNase digestion step. cDNA libraries for qPCR analysis were generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. qPCR reactions were prepared using SYBR green PCR Jumpstart Taq ReadyMix (Sigma-Aldrich) and ROX Reference Dye (Thermo Fisher Scientific), and these were added to the cDNA input in a total volume of 10 μ L. Validated oligonucleotides to genes of interest were ordered from Quantitect (Qiagen) (Table 1) and added 1:10. The gene expression was quantified using a QuantStudio 6 Flex (Applied Biosystems). For each sample, triplicate amplifications were undertaken, and mean measurements were used for data analysis. Gene expression was normalised to the geometric mean of three reference genes Beta-2-Microglobulin (B2M), ribosomal protein L32 (RPL32) and receptor of activated protein

Table 1. Quantitect codes of optimised oligonucleotides used for the quantitative polymerase chain reaction (qPCR) validation of the differential gene expression of hypersegmented neutrophils identified by ribonucleic acid (RNA)-seq.

Gene	GeneGlobe Id	Catalog No
VCAN	QT00064064	249900
LTF	QT00096677	249900
PSMC4	QT00035511	249900
PADI4	QT00094689	249900
DUSP4	QT00067018	249900

C kinase 1 (RACK1), and the N-fold differential expression was determined with the $2^{-\Delta\Delta CT}$ method²⁵.

Pharmacological inhibition of PADI4

Neutrophils (2.5×10^6 cells /mL) from healthy volunteers were isolated using dextran sedimentation and discontinuous plasma-Percoll gradients and resuspended in HBSS supplemented with 10% AS. Neutrophils were then incubated with 200 μ M of the PADI4-inhibitor Cl-amidine (Cayman Chemical) for 15 min alongside an untreated age-matched control (in triplicate) at 37 °C while shaking at 300 rpm. Following this incubation, three cytopspins per triplicate condition were made. The slides were then fixed with methanol (4 min) and stained with Diffquick Red (1 min) then Blue (3 min) and three pictures per slide were taken using light microscopy (Olympus CX31RBSF) at 40x magnification.

Statistical analysis

Statistical analysis was undertaken using [GraphPad Prism 7.0a](#) software (GraphPad Software Inc.), but similar analyses could be undertaken using the open source platform [R](#). Data were analysed with the non-parametric Friedman test and values of $p \leq 0.05$ were considered to indicate statistical significance. Graphs present the median and range unless otherwise specified.

Results

Captopril induces neutrophil hypersegmentation in primary human neutrophils

To further our understanding of neutrophil hypersegmentation, we first had to overcome the lack of availability of hypersegmented neutrophils from patients. To do so, we established a robust model of neutrophil hypersegmentation *in vitro* by inducing hypersegmentation in healthy human neutrophils using the angiotensin converting enzyme inhibitor (ACEi), captopril. Captopril induced hypersegmentation in a study reporting the presence of hypersegmented neutrophils in the circulation of children treated with this ACEi²⁶. This effect was further characterised by Shrestha *et al.* who described the concentration and time effect of captopril on neutrophil lobularity along with the induction of an antitumor phenotype^{13,27}. We replicated these experimental findings and identified the optimal concentrations of captopril needed to induce hypersegmentation.

Human neutrophils from three healthy volunteers were stimulated with increasing concentrations of the ACEi captopril ranging from 0 to 1000 μ M for 3 hours. We observed a dose-dependent increase in hypersegmentation with captopril ([Figure 1A](#)). Having determined that 300 μ M was the optimum concentration of captopril, we then explored the time-dependent effects of ACEi on hypersegmentation. Neutrophils were incubated with 300 μ M captopril for 0 to 6 hours, with hypersegmentation peaking at 2 hours, reaching 24.4% ([Figure 1D–F](#)).

After establishing this model of neutrophil hypersegmentation, we sought to identify genes and pathways involved in this dynamic chromatin rearrangement.

Laser capture microdissection (LCM) allows the isolation of neutrophils according to their morphology

Until now, the inability to separate an enriched population of hypersegmented neutrophils has limited our understanding of this nuclear phenotype. We adapted the laser capture microdissection (LCM) technique to observe and isolate a population of captopril-induced hypersegmented neutrophils from a morphologically heterogeneous neutrophil sample in order to characterise the genomic context of hypersegmentation.

[Figure 2](#) illustrates our final optimised LCM workflow. [Figure 3](#) shows the isolation of a normally segmented control neutrophil using LCM: a gate is manually drawn around the selected neutrophil ([Figure 3A](#)), the laser cuts following the defined borders ([Figure 3B, C](#)), and the PEN-membrane with the neutrophil falls in the lid of a collector Eppendorf ([Figure 3D](#)).

Next generation sequencing of total RNA from laser capture microdissected neutrophils

Following the optimisation of each step of the sample preparation workflow, we conducted RNA-seq to assess whether hypersegmentation in neutrophils was associated with transcriptomic changes. Four populations of neutrophils were manually isolated using LCM: normally segmented baseline neutrophils, normally segmented 2 h age-matched neutrophils, normally segmented 2 h captopril-treated neutrophils, and captopril-treated hypersegmented neutrophils.

The average library length of the submitted samples was 354 bp and ranged from 296 to 438 bp, with an average molarity of 4690 pM/L. Bioinformatic quality control analysis did not report any consequent bacterial contamination or presence of forward strands. A minimum mappability threshold was established in order to select only high-quality samples in the analysis and to avoid introducing biases. Four samples with a mappability below 40% were not included in the analysis. Baseline samples had an average of 36 million reads, and their percentage of uniquely mapped reads to the human genome ranged between 64% and 80%. The age-matched neutrophils had an average of 28 million reads, with uniquely mapped reads ranging between 63% and 83%; the normally segmented captopril treated condition also had an average of 28 million reads with a 59–79% mappability. Hypersegmented neutrophils had 31 million reads on average with 72–82% uniquely mapped reads.

Transcriptomic signature of hypersegmented neutrophils reveals significantly differentially expressed genes as well as novel modulated pathways

Differential gene expression and pathway enrichment analysis revealed that neutrophil hypersegmentation was associated with marked transcriptomic changes (Accession number GSE173553; <https://identifiers.org/geo:GSE173553>). In total, five genes significantly regulated in hypersegmented neutrophils, with several pathways differentially modulated; this provided further insights into the mechanisms associated with this chromatin rearrangement ([Figure 4A](#); [Table 2](#)).

Figure 1. The angiotensin converting enzyme (ACE)-inhibitor captopril induces a concentration-dependent increase in neutrophil nuclear hypersegmentation. (A–C) Healthy peripheral blood neutrophils (2.5×10^6 cells/mL) were cultured in Hank's Balanced Salt Solution (HBSS) with 10% autologous serum. Neutrophils were treated with increasing concentrations of captopril (0 μM to 1000 μM) in triplicate, for 3 hours at 37 $^{\circ}\text{C}$ shaking at 300 rpm. Cytospins were prepared for each condition, slides were air-dried then fixed with methanol for 4 min, stained with Diff Quick Red for 1 min and Diff Quick Blue for 3 min. Neutrophil morphology was assessed in a blinded manner by light microscopy, and each neutrophil was categorised as apoptotic, normally lobulated (2 to 4 lobes) or hypersegmented (5 lobes or more). There is an increase in the percentage of hypersegmented neutrophils (A, black arrows) with increasing captopril concentrations (B, C) ($p \leq 0.05$ when analysed with the non-parametric Friedman test ($n=3$)). (D–F) Neutrophils were treated with 300 μM captopril for 0 to 6 hours, in triplicate, at 37 $^{\circ}\text{C}$, shaking at 300 rpm. Morphologic analysis was undertaken on cytopsin preparations in a similar blinded manner to above. Hypersegmentation was quantified (A, black arrows) and peaked following a 2-hour incubation reaching 24.4% (B, C) ($n=3$). No modifications were made to the microscopy images presented.

Figure 2. Workflow of laser capture microdissection transcriptomic analysis of neutrophils. Cytospins of freshly isolated healthy peripheral blood neutrophils were either made immediately using polyethylene naphthalate (PEN)-membrane microscope slides (baseline) or cells were treated for two hours at 37°C in the presence and absence of captopril prior to cytospins being made. Slides were then fixed, stained and dehydrated following a protocol optimised to preserve ribonucleic acid (RNA) integrity, before laser capture microdissection was used to select 1500 neutrophils for each condition, according to their morphology. RNA was extracted using spin column, and an additional DNase incubation step was undertaken prior to cDNA library preparation. Paired-end 150 bp sequencing was undertaken using an Illumina NovaSeq 6000 S1 Flow Cell. The transcriptome of hypersegmented neutrophils was compared to that of captopril-treated non-hypersegmented neutrophils.

Figure 3. Laser capture microdissection allows the isolation of neutrophils according to their morphology. (A) A gate is drawn around a selected neutrophil. (B–C) The laser cuts the polyethylene naphthalate (PEN)-membrane allowing the dissected area to fall into a collection tube. (D) The micro-dissected neutrophils are visible in the collection tube. No modifications were made to the microscopy images presented.

Figure 4. Hypersegmented neutrophils have differentially expressed genes and pathways compared to captopril-treated non-hypersegmented neutrophils. (A) The ribonucleic acid (RNA)-seq analysis revealed that five transcripts were differentially expressed in hypersegmented neutrophils (false discovery rate (FDR) ≤ 0.05). The volcano plot shows the differentially expressed genes with the significant genes labelled in blue, and the ones with a fold change ≥ 1.5 in orange for scale. (B) Pathway enrichment analysis was undertaken using Metascape based on differentially expressed genes, highlighting the pathways modulated in hypersegmentation like signalling by interleukins, protein-DNA complex assembly and histone modification.

The protein coding gene *versican* (*VCAN*) was downregulated in hypersegmented neutrophils with a log fold change of -1.4 and an FDR of 8.7×10^{-21} . *VCAN* is known to be associated with cellular processes such as protein-DNA complex assembly, cell proliferation and adhesion, and post-translational protein phosphorylation^{24,28,29}. It has been extensively studied for its role in inflammation and in lungs disorders like chronic obstructive pulmonary disease (COPD) or asthma^{30,31}.

Another significantly downregulated gene, *PADI4*, has also been extensively linked to post-translational modification in neutrophils, and more precisely to histone citrullination. *PADI4* is known for its role in chromatin decondensation in neutrophils

and its role in neutrophil extracellular trap (NET) formation^{32,33}. Our RNA-seq dataset revealed a small but significant down-regulation of *PADI4* in hypersegmented neutrophils, suggesting the potential involvement of histone citrullination and chromatin condensation in the hypersegmentation process.

Lactotransferrin (*LTF*) and proteasome 26S Subunit, ATPase 4 (*PSMC4*) genes were significantly upregulated with log fold changes of 2.1 and 1.8 respectively, and dual specificity phosphatase 4 (*DUSP4*) was downregulated.

Other potentially relevant genes appeared to be differentially expressed in our dataset without reaching statistical significance,

Table 2. 50 genes most differentially expressed in hypersegmented neutrophils, ordered by false discovery rate (FDR).

Gene ID	log FC	log CPM	P Value	FDR
VCAN	-1.4151	7.3287	0.0000	0.0000
LTF	2.0905	4.5718	0.0000	0.0000
PSMC4	1.7925	3.8879	0.0000	0.0033
PADI4	-0.5583	7.3147	0.0000	0.0077
DUSP4	-2.5397	3.1804	0.0000	0.0078
TNS1	-1.7744	3.6529	0.0000	0.0659
HIVEP2	0.3217	9.1743	0.0000	0.0659
CD300E	-1.1306	5.7591	0.0001	0.0672
DDX42	-0.9023	4.9935	0.0001	0.0672
PAXBP1	-0.7003	7.1133	0.0001	0.0963
PGAP1	2.2096	2.9492	0.0001	0.1419
SUMF1	2.1503	2.9332	0.0002	0.1550
CXCL2	-1.1536	4.3920	0.0002	0.1626
LOC102723897	2.7223	2.4229	0.0002	0.1986
CDK7	1.6503	3.3478	0.0003	0.2540
CDC34	-0.8803	4.9062	0.0003	0.2647
SLC12A9	-0.6326	5.8967	0.0004	0.2772
C19orf53	2.0897	2.8261	0.0004	0.2772
METRNL	-0.4251	7.0392	0.0004	0.2772
AIP	-1.2074	3.8774	0.0004	0.2772
TUBB1	-1.5626	3.5285	0.0005	0.3136
LCN2	1.6680	3.5509	0.0005	0.3136
AUTS2	3.1728	2.0110	0.0005	0.3136
PKD2	-1.7118	2.9855	0.0006	0.3142
STOM	0.9988	4.4799	0.0006	0.3142
TGFB1	-0.7666	5.8627	0.0007	0.3631
FAM213B	2.9241	2.0674	0.0007	0.3631
LLGL2	-2.3218	2.4330	0.0008	0.3631
COMMD10	-3.0367	1.9960	0.0008	0.3631
IL21R	2.9734	2.0758	0.0009	0.3631
RNGTT	-1.0281	4.1108	0.0009	0.3631
OSMR	1.4713	3.4561	0.0010	0.3631
WNT4	-1.3698	3.5114	0.0010	0.3631
OXR1	1.0551	4.0716	0.0010	0.3631
RP11-692N5.1	-0.8644	4.8713	0.0010	0.3631
FYN	1.5121	3.3973	0.0010	0.3631

Gene ID	log FC	log CPM	P Value	FDR
DDB1	-1.4732	3.3701	0.0011	0.3631
INTS6-AS1	-1.7352	2.8438	0.0011	0.3631
TALDO1	0.2873	8.9019	0.0011	0.3631
ADA	-2.9625	1.9112	0.0011	0.3631
HIST1H2BE	-3.3716	1.7402	0.0012	0.3631
ANXA4	2.8339	2.1095	0.0012	0.3631
VAPA	0.3719	7.2958	0.0012	0.3631
MIER2	3.5781	1.7505	0.0012	0.3631
CCDC43	3.5252	1.7614	0.0012	0.3631
ING5	-1.3851	3.3167	0.0012	0.3631
AP2A1	-0.6175	5.5657	0.0012	0.3631
WDR5B	-1.8002	2.7340	0.0012	0.3631
CELF1	0.4404	6.7763	0.0012	0.3631
TTN	-0.3440	7.4947	0.0012	0.3631

and it is possible an enlarged experiment would have increased statistical power (Table 2). For example, the WAS protein family homolog 9 was found to be upregulated by a log fold change of 2.7 and a p-value of 2×10^{-4} but an FDR of 0.19. This gene is involved in the process of Arp2/3 complex-mediated actin nucleation, the process by which actin monomers combine to form new branches on existing actin filaments³⁴.

To further understand this subset of neutrophils, we conducted a pathway enrichment analysis on the differentially expressed genes. Unsurprisingly, histone modification was one of the main enriched pathways, alongside protein-DNA complex assembly, signalling by interleukins and antimicrobial humoral response (Figure 4B).

Validation of expression of genes involved in hypersegmentation

We confirmed the changes in gene expression observed in the RNA-seq dataset using qPCR. A bulk pool of captopril-treated neutrophils from four additional healthy volunteers was compared to age-matched neutrophils, instead of morphology-selected cells. *VCAN*, *LTF*, *PSMC4*, *PADI4* and *DUSP4* expressions were quantified, and all but one (*DUSP4*) were found to be differentially expressed in the same direction as in the RNA-seq dataset, thereby confirming our findings (Figure 5). Interestingly, *DUSP4* was also the only gene for which the primers had a 67% efficiency; this is far below the established accepted threshold of 90%, and this may explain the discrepancy.

Validation of the transcriptomic dataset in primary human neutrophils by pharmacological inhibition of PADI4

We sought to further explore the role of PADI4 in the process of hypersegmentation due to its well-documented roles in

Figure 5. Validation of RNA-seq differential gene expression analysis by q-PCR in hypersegmented neutrophils normalised to age-matched neutrophils. The ribonucleic acid (RNA) of captopril-treated neutrophils and age-matched neutrophils from four additional healthy volunteers was isolated and the levels of the five significantly differentially expressed genes were quantified by quantitative polymerase chain reaction (q-PCR). Four out of the five genes were found to be up- or down-regulated in the same direction as in the RNA-seq dataset when normalised to the age-matched controls. *DUSP4* was found to be upregulated instead of downregulated in the RNA-seq but the primers efficiency was also found to be low for this particular gene. No modifications were made to the microscopy images presented.

chromatin packing and NET formation^{32,33,35,36}. Primary human neutrophils were treated either with the PADI4 pharmacological inhibitor Cl-amidine, or with captopril alongside baseline and age-matched untreated control. Morphologic analysis revealed that Cl-amidine treatment led to neutrophil hypersegmentation. The proportion of cells with five or more lobes was similar to those obtained with captopril (around 20%), validating the RNA-seq findings (Figure 6A, B). Additionally, the lobe distributions highlighted the striking similarities in the right shifts caused by captopril and Cl-amidine thereby validating

the involvement of PADI4 downregulation in captopril-induced neutrophil hypersegmentation (Figure 6C).

Discussion

This study successfully established the first transcriptomic analysis of neutrophil hypersegmentation. Although neutrophil nuclear hypersegmentation, present in various disease states and in response to diverse stimuli, has frequently been described^{12–14,27,37}, very little is known about the mechanism or pathways involved. We established a model of hypersegmentation

Figure 6. Treating neutrophils with Cl-amidine confirms the role of PADI4 on hypersegmentation. (A, B) Healthy peripheral blood neutrophils (2.5×10^6 cells/mL) were cultured in Hank's Balanced Salt Solution (HBSS) with 10% autologous serum. Neutrophils were either centrifuged directly on cytopins for the baseline condition or incubated in a thermomixer shaking gently at 300 rpm, at 37 °C, in triplicate, with Cl-amidine (15 min, 200 μ M) or captopril (2 h, 300 μ M). Cytopins were prepared for each condition, slides were air-dried then fixed with methanol for 4 min, stained with Diff Quick Red for 1 min and Diff Quick Blue for 3 min. Neutrophil morphology was assessed in a blinded manner by light microscopy, and each neutrophil was categorised as apoptotic, normally lobulated (2 to 4 lobes) or hypersegmented (5 lobes or more). Neutrophils treated with Cl-amidine or captopril were hypersegmented (A, black arrows) and exhibited a higher percentage of cells with five lobes compared to an untreated control (B) ($p \leq 0.05$ when analysed with the non-parametric Friedman test ($n=6$)). (C) The lobe distributions obtained in response to each of the treatments, with each circle representing the median of the three biological replicates, highlight the similarities in the right shift caused by captopril and Cl-amidine.

in primary human neutrophils using the ACEi captopril and undertook RNA sequencing of an enriched population of hypersegmented neutrophils, selected according to their nuclear morphology using LCM.

We identified five differentially expressed transcripts in microdissected hypersegmented neutrophils. Our dataset highlights that neutrophil hypersegmentation is seemingly associated with the differential expression of multiple genes – *PADI4*, *VCAN*, *LTF*, *PSMC4*, and *DUSP4*.

The involvement of *PADI4* in the hypersegmentation process, validated by both qPCR and the use of the small molecule inhibitor Cl-amidine, provides a novel mechanism for this chromatin rearrangement. *PADI4* is one of the five isozymes of the peptidyl-arginine deiminase enzymes involved in various physiological processes from myelin sheath maintenance to gene expression regulation³⁸. *PADI4* catalyses post-translational protein citrullination, a process by which the amino acid arginine is converted into citrulline³⁹. Histone citrullination is crucial in neutrophil NET formation and extrusion as it leads to chromatin decondensation and the formation of DNA structures that characterise these extracellular traps⁴⁰.

Due to *PADI4*'s role in chromatin decondensation in NET formation and its downregulation in our transcriptomic analysis, we hypothesised that this protein may play an important role in the chromatin rearrangement observed in hypersegmentation. *PADI4* downregulation may be associated with increased chromatin condensation and potentially more lobe formation in an opposite fashion to that in NET formation. In agreement with this idea, a study by Wang *et al.*, demonstrated that *PADI4* activation in the neutrophil-like cells, human promyelocytic leukaemia cells (HL-60s), was associated with the loss of their multilobar morphology⁴⁰. This finding along with our observations further supports the association between a downregulation of *PADI4* and increased lobularity.

The differential expression of *VCAN* in our dataset adds another layer of complexity to this already poorly characterised gene. From its contribution to extracellular matrix accumulation and leukocyte infiltration in inflammatory respiratory diseases such as asthma³⁰ to its upregulation in skin tumorigenesis⁴¹, cutaneous melanoma³¹, and prostate cancer⁴², the role of *VCAN* is broad. Initially believed to be mostly involved in extracellular signalling such as cell adhesion, proliferation, or migration, it is now thought to be additionally involved in intracellular processes such as cell mobility, growth and differentiation⁴³. The observed downregulation of *VCAN* mRNA in our transcriptomic analysis is hard to interpret without further validation as its role in neutrophils has yet to be characterised.

Although lactotransferrin is one of the most abundant proteins in mature neutrophils and is a major constituent of the neutrophil antimicrobial response, literature has shown that its mRNA levels decrease after granulocyte differentiation and are low in mature circulating blood neutrophils^{44,45}. The upregulation

observed in our dataset may therefore be representative of a differential maturation status. Alternatively, this increase could be a marker of an increase in lactotransferrin and antimicrobial response initiated at the transcriptome level and hence a sign of a functional difference since lactotransferrin stimulates the TLR4 signalling pathway leading to NK-kappa-B activation and subsequent pro-inflammatory cytokine production⁴³.

PSMC4 gene, traditionally known to be part of the 26S proteasome and involved in the maintenance of protein homeostasis by removing misfolded or damaged proteins, has more recently proven itself to be involved in many different modulated pathways, including cell extension formation in fibroblast enabling matrix remodelling⁴⁶ and cell proliferation and tumorigenesis⁴⁷. Assuming that hypersegmentation is associated with a shift in the cellular machinery of neutrophils leading to certain proteins becoming redundant or damaged, and based on *PSMC4*'s function, we can speculate that its upregulation may be involved in the removal of misfolded, damaged, or no longer used proteins, but this has not yet been demonstrated.

Finally, the importance of *DUSP4* in neutrophil hypersegmentation is still to be determined. Having not been validated by qPCR analysis but with the confounding factor of low primer efficiency, it is unclear whether *DUSP4* downregulation plays a role in this process or if it is an experimental artefact. The dual-specificity phosphatase (DUSP) gene family is well characterised for its involvement in immunity and infection due to its role in reversible phosphorylation and signal transduction⁴⁸. Despite many members of the DUSP family having been shown to be present in neutrophils⁴⁹, the presence of *DUSP4* and its role in neutrophil function remain poorly characterised.

Laser capture microscopy (LCM) is an ideal tool for the collection of cells or tissue based on their morphology and may easily be coupled with increasingly sensitive transcriptomic analyses that require only small amounts of input. Despite its many advantages, LCM remains an expensive and time-constraining technique where every step must be rapidly undertaken in order to preserve RNA integrity. Additionally, the isolation of single cells according to their morphology is a laborious process. Ideally, future advances will lead to automatic image analysis based on user-defined selection criteria for more rapid selection whilst retaining the precision of the overall method.

The adaptation of LCM to the isolation of neutrophils according to their morphology will allow further research on neutrophil morphologic phenotype. Our study represents, the first transcriptomic dataset of microdissected neutrophils. Analyses of this dataset revealed that transcriptomic analysis using RNA-seq can be achieved in neutrophils despite the challenges associated with their low levels of RNA. Additionally, our data also constitutes the first RNA-seq analysis of both hypersegmented and *ex vivo* aged human neutrophils, providing insights into both the mechanisms of hypersegmentation and highlighting that neutrophils are transcriptionally active

cells undergoing marked transcriptional changes in as little as two hours.

Data availability

NCBI Gene Expression Omnibus: RNAseq data. Accession number [GSE173553](https://identifiers.org/geo/GSE173553); <https://identifiers.org/geo/GSE173553>.

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Ravi Lokwani

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The Fox et al, paper on transcriptome of hypersegmented neutrophils provides great insight into the mechanism involves in hypersegmentation of neutrophils and how hypersegmentation is achieved in the laboratory.

In the discussion section and also in the introduction, authors have mentioned previous studies that have reported the presence of hypersegmented neutrophils in a different disease.

I have a few comments that I would like the author to address in the revision of this paper:

1. Are the hypersegmented neutrophils formed after captopril treatment in this study featuring some of the common hypersegmented neutrophil traits that was reported in previous studies i.e. Reduced CD62L expression, High oxidative burst, Increased CD11b expression, etc? I would like the author to carry out a small study to demonstrate these captopril treated hypersegmented neutrophils to validate that these neutrophils feature similar characteristics as hypersegmented neutrophils reported in previous studies. Because if these hypersegmented neutrophils feature similar characteristics as previously reported then it can significantly assist future studies in using this model to study disease associated hypersegmented neutrophils in more detail.
2. The author had shown that VCAN was downregulated in hypersegmented neutrophils. Since authors have suggested that VCAN have a role in cellular adhesion, it will be interesting to see if the reduced CD62L expression (one of well known feature of hypersegmented neutrophils) have any association with VCAN. I would like the author to comment on this.

Overall, the study is very promising and provides very detailed insight into hypersegmentation of neutrophils.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Characterizing neutrophil subsets in obstructive airway disease using microscopy and flow-cytometry, Characterizing immune response against biomaterial in mice using flow cytometry.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 07 January 2022

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Sarah Walmsley

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Fox and colleagues use captopril to induce neutrophil hyper-segmentation and investigate the transcriptional signature of hyper-segmented neutrophils. They identify 5 genes of interest and subsequently explore whether the treatment of neutrophils with the PAD14 inhibitor Cl-amidine can promote neutrophil hyper-segmentation. The manuscript would be considerably strengthened by the authors addressing a number of experimental limitations by providing further clarification of the experimental groups being compared and the provision of additional control data.

1. In general, the number of replicates is extremely limited. Where laser capture microdissection and RNAseq data sets are generated this would be understandable,

however, the morphological data presented in figure 1 is an $n=3$, and whilst there is limited data variation, this should be highlighted in the results text. With respect to the RNAseq data sets, information regarding n number and biological replicates is currently missing so it is difficult to understand how many replicates were generated for each experimental condition studied.

2. Apologies, but I am unclear as to what data sets are being compared in figure 4. It must be 2hr hyper-segmented captopril PMN vs another group but I am unsure what that group is, is it baseline PMN, 2 hr normal segmented PMN untreated, or 2hr normal segmented neutrophils captopril treated. This is important information as it impacts upon the conclusions drawn.
3. How specific is Cl-amidine for PAD14 inhibition, at a minimum, the authors should provide evidence of PAD14 inhibition by Cl-amidine at the concentration studied.
4. Validation of RNAseq data sets is not undertaken in the hyper-segmented PMN population making it difficult to interpret. If the transcriptional signature is specific to the hyper-segmented PMN, given they form only a small percentage of the total PMN population in culture even following exposure to captopril, then it would be surprising to see any difference at all in the "whole" PMN population.

5. How does the pathway enrichment analysis relate to the 5 genes of interest.

Conceptually, there are a number of important points that warrant further discussion,

How does a hyper-segmented neutrophil induced by culture with captopril relate to a hyper-segmented neutrophil observed in a pathophysiological context?

Why does a transcriptional change in PAD14 implicate NETosis in neutrophil hyper-segmentation. Are the authors proposing that hyper-segmented PMN are cells that fail to NET, or that by inhibiting NETosis cells develop a hyper-segmented phenotype; why is this a small percentage of the total population, is there any evidence that speaks to either of these potential pathways?

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: neutrophil biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.