Frailty is associated with neutrophil dysfunction which is correctable with phosphoinositol-3-kinase inhibitors

Daisy Wilson PhD¹, William Drew MBBS ², Alice Jasper BSc ², Helena Crisford BSc², Peter Nightingale PhD³, Paul Newby BSc ², Thomas Jackson PhD¹, Janet M Lord PhD ¹,4, Elizabeth Sapey PhD²,5

1. MRC-Versus Arthritis Centre for Musculoskeletal Ageing Research, Institute of Inflammation and Ageing, University of Birmingham, Queen Elizabeth Hospital Birmingham, Edgbaston, Birmingham, B15 2WD, UK.

2. Birmingham Acute Care Research, Institute of Inflammation and Ageing, University of Birmingham, Queen Elizabeth Hospital Birmingham, Edgbaston, Birmingham, B15 2WD, UK.

3. NIHR Clinical Research Facility, University Hospitals Birmingham NHS Foundation Trust, Edgbaston, Birmingham, UK.

4. NIHR Birmingham Biomedical Research Centre, University Hospitals Birmingham NHS Foundation Trust and University of Birmingham, Birmingham, UK.

5. Corresponding author: Dr E Sapey, Birmingham Acute Care Research Group, Institute of Inflammation and Ageing, University of Birmingham, Queen Elizabeth Hospital Birmingham, Edgbaston, Birmingham, B15 2WD, UK.  E.sapey@bham.ac.uk. Tel: 44 121 246 2000 Twitter @e_sapey

© The Author(s) 2020. Published by Oxford University Press on behalf of The Gerontological Society of America.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
Abstract

Neutrophil dysfunction has been described with age, appears exaggerated in infection, with altered phosphoinositol signalling a potential mechanism. However, functional ageing is heterogeneous. Frailty is a negative health status and is more common in older adults. We hypothesised that neutrophil migration may be compromised in frailty, associated with the degree of frailty experienced by the older person. We compared measures of frailty, neutrophil function and systemic inflammation in forty young and seventy-seven older community dwelling adults in the United Kingdom. Systemic neutrophils exhibited an age-associated reduction in the accuracy of migration (chemotaxis) which was further blunted with frailty. The degree of migratory inaccuracy correlated with physical (adjusted hand grip strength) and cognitive (Stroop test) markers of frailty. Regression analysis demonstrated that age, Charlson Co-morbidity Index and Frailty Index were able to predict neutrophil chemotaxis. Reduced chemotaxis of neutrophils from frail adults could be reversed using selective PI3K inhibitors. Exposure of neutrophils from young adults to plasma from chronically inflamed frail older adults could not recapitulate the migratory deficit in vitro and there were no relationships with systemic inflammation and neutrophil dysfunction. Frailty exaggerated the neutrophil deficits seen with advanced age but aspects of the frailty-associated deficit in neutrophil function are rescuable and thus potentially form a therapeutic target to improve outcomes from infection in older adults.

Key words: Innate immunity, proteinases, inflammation, co-morbidity
Introduction

There is increasing interest in therapeutic strategies to maintain health with advancing age. Frailty is a manifestation of unhealthy ageing[1] and an independent predictor of poor outcomes[2]. Frailty is distinct from physiological ageing, disability or co-morbidity, although more common in older people with disability and co-morbidity[3]. Clinically, frailty is identified using composite scores, including Rockwood’s Frailty Index[4].

Inflammation and immune cell function have been implicated in the pathophysiology of frailty[5]. Frailty appears associated with an increase in systemic inflammation[6], although this has not been consistently replicated[7]. Adaptive immune cell subtypes have been shown to be altered in frailty[8], with less known about innate immune cells such as neutrophils.

The susceptibility to infections increases with both age and frailty[9, 10]. Neutrophils are of primary importance during infective challenges and previous work by our group and others have described neutrophil dysfunction with an individual’s increasing age. This includes reduced phagocytosis to some but not all pathogens[11], a failure to prevent apoptosis in the presence of inflammatory stimuli, reduced neutrophil extracellular trap formation[12] and inaccurate migration[13, 14]. These altered functions have been postulated to represent a marker of biological age[15].

Two interwoven factors may contribute to the mechanism of reduced neutrophil migratory accuracy. Increased intracellular class 1 delta(δ) and gamma(γ) Phosphoinositol-3-Kinase (PI3K) signalling have been implicated in inaccurate neutrophil migration with age, which in vitro selective PI3K inhibitors can restore[16]. The systemic inflammation associated with ageing and
It is hypothesized that frailty might alter neutrophil behaviours, making the cells less responsive to chemotactic cues seen during inflammation and infection.

We hypothesised that frailty would be associated with inaccurate neutrophil migration and an increased inflammatory burden. We further hypothesised that PI3K signalling would be implicated in altered neutrophil migration.

This study had two aims. First, to determine if neutrophil migration was altered in cells isolated from frail older adults compared with healthy older and younger subjects. Second, to assess the mechanism of effect including whether PI3K signaling was implicated in migratory function and thus could form a therapeutic target and/or whether an inflammatory systemic environment could induce compromised migratory behaviour.

Materials and methods

Study subjects

117 participants were recruited to three groups: healthy younger adults (HY), healthy older adults (HO) and frail older adults (FO). See online supplement for details. All subjects were recruited between 2015 and 2019.

HY were aged >18 and <35 years and HO were aged >65 years. FO were aged >65 years, had a Frailty Index (FI) >0.2[4]. Table S1 in the online supplement provides a full list of exclusion and inclusion criteria. All participants were clinically characterised by a single assessor who was clinically qualified and an expert in geriatric medicine. These assessments were used to form the Frailty Index, as described in the online supplement (Table S2).
Isolation of blood neutrophils and neutrophil migration

For full methods see online supplement. In brief, neutrophils were isolated from whole blood and were (>95% pure, >97% viable, by exclusion of trypan blue) and migration was assessed using an Insall chamber (Weber Scientific International Ltd, UK), as described previously[17]. 10 nM N-Formylmethionine-leucyl-phenylalanine(fMLP) 10nM and 100 nM Interleukin 8(CXCL8) were used as chemoattractants or appropriate vehicle control. PI3K isoform selective inhibitors (IC50 concentrations) included: class 1δ (Cal-101 75nM; Selleck), γ (AS-252424 33nM; Selleck) or vehicle control and were incubated with neutrophils for 45 minutes prior to migration. Neutrophils from HY were studied following 45-minute incubation with pooled plasma (PP) from 18 HO (mean FI – 0.04) or 18 FO (mean FI – 0.41) following washing and resuspending in RPMI-1640 (Sigma Aldrich, UK). A 250uL aliquot of plasma was combined from each individual sample to form the pool which was used for all experiments. Donors of pooled plasma samples were representative of the cohorts as a whole with no significant differences in demographics. All analysis was carried out by a single analyst utilising a randomisation method for initial cell selection. Images were captured by a Leica DMI6000B with DFC360FX camera as described previously(19). The images were analysed using Image J software (Wayne Rasband, NIH, USA).

Two migratory parameters were assessed: mean cell speed of movement defined as the distance travelled between frames in any direction over time (termed chemokinesis) and mean cell velocity, defined as the speed in a consistent direction toward the chemoattractant (termed chemotaxis)[17], both measured in micrometres per minute.
Neutrophil elastase activity

\( \alpha \)-Val360, a neutrophil elastase (NE) specific fibrinogen degradation product and a surrogate marker of NE activity in vivo, was measured in plasma as described previously[18].

Cytokine and high sensitivity (hs)CRP plasma concentration

Inflammatory mediators were measured in plasma using commercially available kits, as per manufacturer’s instructions (Bio-Plex Pro Human Cytokine Standard 27-Plex and Bio-Rad; hsCRP kit, IBL International).

Statistics

Statistical analyses were performed using SPSS (Version 22.0, IBM Corp, USA). Data were tested for normality using the Shapiro-Wilk or Kolmogorov Smirnov test and the appropriate parametric (ANOVA, post-hoc Tukey’s, Pearson correlation (shown as ‘r’), Kruskal-Wallis with Dunn’s multiple comparison test, Wilcoxon signed ranks test or Spearman’s rank correlation (shown as ‘rho’)). Linear regression modelled if chemotaxis predicted frailty index. A p value < 0.05 was considered to be statistically significant. All p values are reported and all tests are described in the text or table/figure legends with adjustment for multiple comparisons where stated.

Data Sharing Statement

For original data, please contact d.v.wilson@bham.ac.uk.
Results.

All subjects gave their informed written consent following approval from the Health Research Authority and Research Ethics Committee (15/WM/0002). Table 1 describes participant demographics. For all results, frail older adults are referred to as FO, healthy old adults as HO and healthy young as HY.

Neutrophil chemokinesis is preserved with frailty but chemotaxis is reduced.

There were no significant differences in the speed of migration in any direction (chemokinesis) of isolated neutrophils to fMLP or CXCL8 between the three groups, FO, HO, or HY (Figure S1A and S1C of the online supplement). There was a reduction in chemotaxis towards fMLP across the groups (Independent Kruskal-Wallis p < 0.0001) where chemotaxis was reduced in HO (Dunn’s multiple comparison test, p < 0.0002) and FO (Dunn’s multiple comparison test, p < 0.0001) compared with HY. Neutrophils from HO and FO were less accurate in their migratory pathways towards fMLP and CXCL8 than those isolated from young adults. FO neutrophils showed a reduction in chemotaxis to CXCL8 compared to HO but not fMLP (Figure S1B and S1D of the online supplement).

Neutrophil chemotaxis relates to physical and cognitive parameters of frailty.

When HY, HO, FO participants were included together, chemotaxis correlated positively with grip strength (adjusted for gender and BMI[19]) and Stroop score and negatively with overall Frailty Index. For migration to fMLP: adjusted hand grip, r=0.5239, p<0.0001 (Figure 1A); Stroop score, r=0.359, p=0.0025 (Figure 1B) and Frailty Index, rho= -0.4012, p=0.002 (Figure 1C): For migration to CXCL8, adjusted hand grip r = 0.215, p = 0.08, Stroop score, r = 0.364, p = 0.0026 (Figure 1D) and Frailty Index rho = -0.312, p = 0.04. There were no significant
relationships between other frailty parameters including the short physical performance battery and Addenbrookes cognitive examination and neutrophil chemotaxis.

**Neutrophil chemotaxis relates to Charlson Co-morbidity Index (CCI) and predicts frailty.**

When HY, HO, FO participants were included together, there was a negative relationship between CCI and chemotaxis towards fMLP (Spearman’s rho -0.423, p < 0.0001) and CXCL8 (rho -0.414, p=0.0003) but not chemokinesis (fMLP: Spearman’s rho = -0.1992, p = 0.08; CXCL8 rho -0.21, p = 0.06).

Linear regression modelling demonstrated that chemotaxis towards CXCL8 and fMLP could be predicted by age, frailty and Charlson Co-morbidity index (CCI) (standardised β: Age = -0.301, p=0.013; Frailty Index = -0.294, p = 0.015; CCI = -0.253, p = 0.037).

**Selective PI3K inhibitors improve migratory accuracy in neutrophils from FO adults**

FO neutrophil chemotaxis towards CXCL8 increased following incubation with Class 1 δ and γ PI3K inhibitors. Median and (IQR) for all: Vehicle Control: 0.44 µm/min (0.19 – 0.68) vs δ 0.81 µm/min (0.43 – 1.31), Wilcoxon test, p = 0.005 and γ 0.88 µm/min (0.67 – 1.31), Wilcoxon test p = 0.0068. See Figure 2.

The PI3K-inhibitor-associated improvement in chemotaxis related to both Frailty Index (with individuals with greater frailty showing greater improvements from baseline) and adjusted hand grip strength (with individuals with low grip strength (adjusted for gender and BMI) having greater improvements from baseline) when expressed as a fold rather than an absolute change. Spearman’s correlation between the: fold change in chemotaxis, vehicle control to PI3K δ and Frailty Index, rho =0.452. p=0.001; fold change in chemotaxis, vehicle control to PI3K δ and...
adjusted hand grip, \( \rho = -0.509, p<0.001 \) and fold change in chemotaxis, vehicle control to PI3K \( \gamma \) and Frailty Index, \( \rho = 0.435, p=0.001; \) fold change in chemotaxis, vehicle control to PI3K \( \gamma \) and adjusted hand grip, \( \rho = -0.388, p=0.004. \)

**Systemic neutrophil elastase activity footprint is reduced in frailty.**

Neutrophil migration through tissue is achieved through release of the protease neutrophil elastase. \( \text{AαVal360} \) measures the activity of \( \text{NE} \) *in vivo*, a biomarker of chemotactic accuracy and neutrophil activity. Systemic neutrophil elastase activity differed across groups. \( \text{AαVal360}, \) median (IQR) for all: HY 8.75nM (6.35 – 9.93) vs. HO 16.50 nM (14.00 – 19.00) vs FO 9.50 nM (7.00 – 13.00), Kruskal Wallis \( p < 0.0001 \). \( \text{AαVal360} \) was higher in HO compared to FO subjects (\( p = 0.0003 \)), and HO compared to HY (\( p < 0.0001 \)) but there were no differences between HY and FO (\( p = 0.783 \)), Dunn’s Multiple Comparison test.

**Systemic inflammation in frailty**

The concentration of hsCRP was increased in FO adults compared to both HY and HO adults (see Figure S2 online supplement). There was no correlation between neutrophil chemotaxis and hsCRP concentration (Pearson’s correlation \( r = -0.239, p = 0.07 \)).

For cytokine measurement, only results where more than 50% of values were above the minimum level of detection are reported (16 of 27 inflammatory mediators). In these mediators, 7 had comparable concentrations between the 3 groups, 7 were higher in both older adult groups compared with young adults (IL-ra, IL-4, CXCL8, IL-17, Eotaxin, IP10, MIP1a). MCP1 and IL-17 were lower in FO than HO groups and MIP1a was higher in FO adults compared to HO adults. See Table S3 of the online supplement.
The frail neutrophil functional phenotype is not inducible by plasma from frail adults.

Neutrophils from 11 HY adults were incubated with pooled plasma (PP) made up from 18 FO (FO-PP), 18 HO (HO-PP) or 18 HY (HY-PP) and migration experiments repeated.

Incubation with FO-PP or HO-PP did not change the HY neutrophils migratory speed or accuracy towards CXCL8 compared to HY neutrophils being incubated with HY-PP. Chemokinesis: median (IQR); HY-PP 2.51 μm/min (2.18–3.49) vs HO-PP 3.63 μm/min (2.83 – 4.17) vs FO-PP 3.29 μm/min (2.43 – 4.93), Friedman test p=0.420. Chemotaxis: median (IQR); HY-PP 1.68 μm/min (0.45 – 2.81) vs HO-PP 1.13 μm/min (0.96 – 2.01) vs FO-PP 0.66 μm/min (0.31 – 2.87), Friedman test, p=0.317.

Discussion

This study presents novel data on the differential effects of age and frailty on neutrophil function, linking migratory accuracy with global markers of function (grip strength and cognitive ability). Frailty was also associated with reduced migratory accuracy towards CXCL8 compared with healthy young and old adults. This phenotype could not be induced by exposing neutrophils from young adults to frail older plasma, perhaps supporting an intrinsic deficit, but was rescuable using selective PI3K γ and PI3K δ inhibitors.

This study is of importance. It highlights potential cellular mechanisms for reduced neutrophil migration which are targetable therapeutically. Inhibition of PI3K δ and γ restored the chemotactic ability of neutrophils from frail older adults, offering the possibility of modifying the innate immune system during infections in frail elders to improve responses. The PI3K pathway could be targeted at differing entry points. For example, simvastatin inhibits GTPases[20] which represent a downstream effector of PI3K signalling and adjuvant statins were associated with both
improvements in neutrophil migratory accuracy and survival benefit in hospitalised patients with community acquired pneumonia in a cohort of older, frail adults [14]. The negative relationship between grip strength and fold change improvement in chemotaxis following PI3K inhibition further highlights the close relationship between frailty and immune ageing (immunosenescence) and may help clinically identify a suitable population for this therapeutic intervention.

The reduction in AαVal360 in frail old adults compared to healthy older adults is of interest. Previous studies from our group report AαVal360 levels of approximately 7.5nM in young adults and approximately 17nM in healthy older adults [16], supporting increased neutrophil activity with age, mirroring the levels described in the current study. The reduced systemic neutrophil proteinase activity in frailty alongside reduced migratory accuracy might suggest a double insult to cell function, impeding bacterial killing further. Although previous investigation of frailty-associated immunosenescence is limited, systemic neutrophils isolated from frail older adults were shown to produce less chemokines than neutrophils from healthy older adults [21]. This requires further study at baseline and following inflammatory challenges in vitro and in vivo to confirm this interpretation.

A second key finding is the heterogeneity of systemic inflammation in frailty. In a systematic review of inflammation in over 3000 frail adults, the variance in inflammatory mediators was noted, including differences between the findings of longitudinal and cross sectional studies [7]. It is likely that there will be different cohorts of frail adults, some with or without co-morbidities, with only a proportion demonstrating a pro-inflammatory phenotype. However, as the FO neutrophil migration phenotype was not inducible by acute exposure to plasma from FO adults, it is likely that the change in neutrophil function is not merely a response to systemic inflammation alone. This suggests anti-inflammatory strategies may not be sufficient to improve cell function.
Third, it highlights the need for careful characterisation of patients when studying the effects of age or immunosenesence. Here frailty, age and co-morbid disease burden all related to the accuracy of neutrophil migration. Another study investigating neutrophil chemotaxis in healthy older adults grouped by level of daily physical activity reported similar heterogeneous results, with activity positively correlating with migratory accuracy[22]. Previous studies[16, 23, 24] have demonstrated a reduction in migratory accuracy with age but only considered the impact of specific diseases and did not consider the potential effect of frailty or co-morbidity.

There are a number of important limitations to this work. The frail older adults were older than the healthy older adults. These data are cross-sectional, and therefore cannot comment on the time course between the onset of frailty and the reduced migratory phenotype. As physical activity appears to affect neutrophil migratory accuracy[22], it is possible that reduced physical activity in the frail older adults was a contributor, but we did not assess daily physical activity objectively in our cohorts, instead using self-reported levels of activity as a part of the frailty assessment. We did not look specifically at each component of the frailty index to see what characteristic might most predict neutrophil dysfunction. Isolated neutrophils were used for these studies. The process of isolation can impact on the cell, however the current study finds clear differences between frail old, healthy old and young adults’ neutrophils under the same conditions. The Insall chamber, while allowing single cell migration to be assessed, does not provide the same migratory platform as is found in vivo and more physiological models should now be assessed. Not all aspects of migration were assessed, including responses to different chemoattractant concentrations over different time courses. Not all neutrophil functions were studied due to experimental constraints using these short-lived cells. Although mechanisms were suggested by association, these are not confirmed and in-depth studies are required. Although PI3K inhibitors have been associated with
improved neutrophil migration in vitro, the effects of PI3K inhibition on other cellular functions should be assessed. This study utilised systemic neutrophils, and neutrophil functions may alter during transmigration. Studying transmigrated cells during particular infections or inflammatory events would be of interest.

In summary, neutrophil chemotaxis is compromised with age and is further challenged with frailty. Neutrophils from frail adults appear to represent an exaggerated version of neutrophils from healthy older adults, but with some distinct characteristics (such as reduced evidence of degranulation) which are not inducible by inflammatory cues but are correctable by PI3K inhibition. Frail older adults are known to suffer with more infections and experience worse outcomes from these infections. Targeting neutrophil functions may provide a new therapeutic strategy for this vulnerable group.
Funding

Daisy Wilson was funded by a studentship from the MRC-Versus Arthritis Centre for Musculoskeletal Ageing Research. Janet Lord is supported by the NIHR Birmingham Biomedical Research Centre at the University Hospitals Birmingham NHS Foundation Trust and the University of Birmingham. Elizabeth Sapey is supported by the HDR-UK Health Data research Hub in acute care: PIONEER.

Acknowledgements

We are grateful for the support of the participants who took part in this study. The Neutrophil elastase footprint assay is patented and was used with the kind permission of Professor Robert A. Stockley, Birmingham, UK. This work was completed with the support of the NIHR Clinical Research Facility in Birmingham, UK.

Authorship contributions

D.W. recruited subjects, performed experimental assays, completed the statistical analysis and prepared the manuscript for publication; W.D., A.J., H.C. and P.N. assisted with experimental assays; P.Nightingale assisted with statistical analysis; TJ provided input into study design and interpretation. JML and ES conceived and oversaw the project and contributed to manuscript preparation. ES oversaw study governance and the clinical care of the participants included in the study. All authors approved the final version of the manuscript.

Disclosure of conflicts of interests

The authors declare no financial conflicts of interest.
References

1. Morley JE, Vellas B, van Kan GA, Anker SD, Bauer JM, Bernabei R, Cesari M, Chumlea WC, Doehner W, Evans J et al: Frailty consensus: a call to action. J Am Med Dir Assoc 2013, 14(6):392-397. doi: 10.1016/j.jamda.2013.03.022

2. Millett ERC, De Stavola BL, Quint JK, Smeeth L, Thomas SL: Risk factors for hospital admission in the 28 days following a community-acquired pneumonia diagnosis in older adults, and their contribution to increasing hospitalisation rates over time: a cohort study. BMJ Open 2015, 5(12):e008737. DOI: 10.1136/bmjopen-2015-008737

3. Collard RM, Boter H, Schoevers RA, Oude Voshaar RC: Prevalence of frailty in community-dwelling older persons: a systematic review. J Am Geriatr Soc 2012, 60(8):1487-1492. DOI: 10.1111/j.1532-5415.2012.04054.x

4. Mitnitski AB, Mogilner AJ, Rockwood K: Accumulation of Deficits as a Proxy Measure of Aging. TheScientificWorldJOURNAL 2001. DOI: 10.1100/tsw.2001.58

5. Wilson D, Jackson T, Sapey E, Lord JM: Frailty and sarcopenia: The potential role of an aged immune system. Ageing Res Rev 2017, 36:1-10. DOI: 10.1016/j.arr.2017.01.006

6. Marzetti E, Picca A, Marini F, Biancolillo A, Coelho-Junior HJ, Gervasoni J, Bossola M, Cesari M, Onder G, Landi F et al: Inflammatory signatures in older persons with physical frailty and sarcopenia: The frailty “cytokinome” at its core. Experimental Gerontology 2019, 122:129-138. DOI: 10.1016/j.exger.2019.04.019

7. Soysal P, Stubbs B, Lucato P, Luchini C, Solmi M, Peluso R, Sergi G, Isik AT, Manzato E, Maggi S et al: Inflammation and frailty in the elderly: A systematic review and meta-analysis. Ageing Research Reviews 2016, 31:1-8. DOI: 10.1016/j.arr.2016.08.006

8. Ng TP, Camous X, Nyunt MSZ, Vasudev A, Tan CTY, Fulop T, Yap KB, Larbi A: Markers of T-cell senescence and physical frailty: insights from Singapore Longitudinal Ageing Studies. NPJ Aging Mech Dis 2015, 1:15005-15005. DOI: 10.1038/npjamd.2015.5

9. Drew W, Wilson DV, Sapey E: Inflammation and neutrophil immunosenescence in health and disease: Targeted treatments to improve clinical outcomes in the elderly. Exp Gerontol 2018, 105:70-77. DOI: 10.1016/j.exger.2017.12.020

10. Drew W, Wilson DV, Sapey E: Frailty and the Immune System. Journal of Aging Research And Healthcare 2017, 2(1):1-14. DOI:10.14302/issn.2474-7785.jarh-17-1578

11. Butcher SK, Chahal H, Nayak L, Sinclair A, Henriquez NV, Sapey E, O’Mahony D, Lord JM: Senescence in innate immune responses: reduced neutrophil phagocytic capacity and CD16 expression in elderly humans. J Leukoc Biol 2001, 70(6):881-886. https://doi.org/10.1189/jlb.70.6.881

12. Fortin CF, Larbi A, Dupuis G, Lesor U, Fulop T: GM-CSF activates the Jak/STAT pathway to rescue polymorphonuclear neutrophils from spontaneous apoptosis in young but not elderly individuals. Biogerontology 2007, 8(2):173-187. DOI: 10.1007/s10522-006-9067-1

13. Sapey E, Patel JM, Greenwood HL, Walton GM, Hazeldine J, Sadhra C, Parekh D, Dancer RCA, Nightingale P, Lord JM et al: Pulmonary Infections in the Elderly Lead to Impaired Neutrophil Targeting, Which Is Improved by Simvastatin. Am J Respir Crit Care Med 2017, 196(10):1325-1336. DOI: 10.1164/rccm.201704-0814OC

14. Sapey E, Patel JM, Greenwood H, Walton GM, Grudzinska F, Parekh D, Mahida RY, Dancer RCA, Lugg ST, Howells PA et al: Simvastatin Improves Neutrophil Function and Clinical Outcomes in Pneumonia: a Pilot Randomised Controlled Trial. American Journal of Respiratory and Critical Care Medicine 2019, 200:1282 - 1293. DOI: 10.1164/rccm.201812-2328OC

15. Martínez de Toda I, Maté I, Vida C, Cruces J, De la Fuente M: Immune function parameters as markers of biological age and predictors of longevity. Aging (Albany NY) 2016, 8(11):3110-3119. DOI: 10.18632/aging.101116

16. Sapey E, Greenwood H, Walton G, Mann E, Love A, Aaronson N, Insall RH, Stockley RA, Lord JM: Phosphoinositide 3-kinase inhibition restores neutrophil accuracy in the
elderly: toward targeted treatments for immunosenescence. Blood 2014, 123(2):239-248. DOI: 10.1182/blood-2013-08-519520

17. Sapey E, Stockley JA, Greenwood H, Ahmad A, Bayley D, Lord JM, Insall RH, Stockley RA: Behavioral and structural differences in migrating peripheral neutrophils from patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2011, 183(9):1176-1186. DOI: 10.1164/rccm.201008-1285OC

18. Carter RI, Mumford RA, Treonze KM, Finke PE, Davies P, Si Q, Humes JL, Dirksen A, Piitulainen E, Ahmad A et al: The fibrinogen cleavage product Aalpha-Val360, a specific marker of neutrophil elastase activity in vivo. Thorax 2011, 66(8):686-691. DOI: 10.1136/thx.2010.154690

19. Fried LP, Tangen CM, Walston J, Newman AB, Hirsch C, Gottdiener J, Seeman T, Tracy R, Kop WJ, Burke G: Frailty in older adults evidence for a phenotype. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 2001, 56(3):M146-M157. DOI: 10.1093/gerona/56.3.m146

20. Rikitake Y, Liao JK: Rho GTPases, statins, and nitric oxide. Circulation research 2005, 97(12):1232-1235. DOI: 10.1161/01.RES.0000196564.18314.23

21. Juthani-Mehta M, Guo X, Shaw AC, Towle V, Ning Y, Wang X, Allore HG, Fikrig E, Montgomery RR: Innate Immune Responses in the Neutrophils of Community Dwelling and Nursing Home Elders. J Aging Sci 2014, 2. DOI: 10.4172/2329-8847.1000115

22. Bartlett DB, Fox O, McNulty CL, Greenwood HL, Murphy L, Sapey E, Goodman M, Crabtree N, Thogersen-Ntoumani C, Fisher JP et al: Habitual physical activity is associated with the maintenance of neutrophil migratory dynamics in healthy older adults. Brain Behav Immun 2016, 56:12-20. DOI: 10.1016/j.bbi.2016.02.024

23. Wenisch C, Patruta S, Daxbock F, Krause R, Hror W: Effect of age on human neutrophil function. J Leukoc Biol 2000, 67(1):40-45. DOI: 10.1002/jlb.67.1.40

24. Niwa Y, Kasama T, Miyachi Y, Kanoh T: Neutrophil chemotaxis, phagocytosis and parameters of reactive oxygen species in human aging: cross-sectional and longitudinal studies. Life Sci 1989, 44(22):1655-1664. DOI: 10.1016/0024-3205(89)90482-7
**Figure Legends**

**Figure 1. Migration of peripherally isolated neutrophils and relationship with frailty parameters**

Neutrophils isolated from healthy young adults (HY), healthy older adults (HO) and frail older adults (FO) were migrated towards fMLP or CXCL8. Each dot represents neutrophil migration for one person. There were relationships between migration towards fMLP and 1A. adjusted hand grip, $r = 0.5239$, $p < 0.0001$; 1B. Stroop score result, $r = 0.359$, $p = 0.0025$: 1C. Frailty index rho = -0.401, $p = 0.002$. There were also relationships between migration towards CXCL8 and 1D. Stroop score result, $r = 0.364$, $p = 0.0026$. Pearson’s correlation coefficient for A, B, D. Spearman’s correlation for C.

**Figure 2. PI3K inhibition restores neutrophil migratory accuracy in frail older adults**

Neutrophils isolated from frail older adults were migrated towards CXCL8 100nM following incubation with VC (RPMI and DMSO) or the PI3K δ (Figure 2A) or PI3K γ inhibitors (Figure 2B). Each dot represents neutrophil migration for one person. Chemotaxis (migratory accuracy) is shown and expressed as $\mu$m/min. The frailty associated reduction in chemotaxis was improved following incubation with PI3K inhibitors δ and γ (p values as given, Wilcoxon signed rank for both).
Table 1. Demographics of healthy young adult, healthy older adults and frail older adults.

|                          | HY              | HO              | FO              | Group comparison | Pairwise comparison |
|--------------------------|-----------------|-----------------|-----------------|------------------|--------------------|
| Number                   | 40              | 40              | 37              |                  |                    |
| Gender % (F:M)           | 50:50           | 67:33           | 54:46           |                  | p=0.330            |
| Age                      | 26.0 (21.3-31.0)| 71.0 (70.0-79.0)| 84.0 (73.8-88.5)| **p<0.001**      | HY-HO p<0.001      |
|                          |                 |                 |                 | HY-FO p<0.001    | HO-FO p<0.019      |
| Co-morbid conditions (%) |                 |                 |                 |                  |                    |
| 0                        | 100             | 77.5            | 34.3            | **p<0.001**      | HY-HO p=0.124      |
| 1                        | 20              | 20              | 40              | HY-FO p<0.001    | HO-FO p<0.001      |
| 2                        | 2.5             | 14.3            |                 |                  |                    |
| 3                        | 11.4            |                 |                 |                  |                    |
| Frailty Index            | 0.02 (0.00-0.02)| 0.04 (0.02-0.08)| 0.30 (0.25-0.36)| **p<0.001**      | HY-HO p=0.005      |
|                          |                 |                 |                 | HY-FO p<0.001    | HO-FO p<0.001      |
| Adjusted grip strength   | 1.71 (1.61-1.81)| 1.31 (1.23-1.39)| 0.73 (0.64-0.82)| **p<0.001**      | HY-HO p<0.001      |
|                          |                 |                 |                 | HY-FO p<0.001    | HO-FO p<0.001      |
| Walk speed (m/sec)       | 1.62 (1.34-1.79)| 1.26 (1.17-1.43)| 0.33 (0.19-0.50)| **p<0.001**      | HY-HO p=0.007      |
|                          |                 |                 |                 | HY-FO p<0.001    | HO-FO p<0.001      |
| SPPB                     | 12.0 (12.0-12.0)| 12.0 (10.0-13.0)| 2.0 (1.0-4.0)   | **p<0.001**      | HY-HO p=0.051      |
|                          |                 |                 |                 | HY-FO p<0.001    | HO-FO p<0.001      |

Legend. The demographics of the three different groups are described. HY = healthy young adults (n= 40), HO = healthy older adults (n = 40) and FO = frail older adults (n=37). Categorical data are presented as proportions of total. Age, frailty index, walk speed and short physical performance battery (SPPB) are presented as the median and inter quartile range in parentheses. Adjusted grip strength is presented as mean with 95% confidence intervals and compared between groups using an ANOVA with post-hoc Tukey’s for pairwise comparison Gender was assessed using a Chi-squared test and all other statistical tests are Independent Kruskal-Wallis (I K-W) with Dunn’s pairwise comparison adjusted with Bonferroni correction. All statistically significant values appear in bold text.
Figure 1

**A**
Adjusted grip strength vs. Chemotaxis (um/min)

**B**
Stroop Score Result vs. Chemotaxis (um/min)

**C**
Frailty Index vs. Chemotaxis (um/min)

**D**
Stroop Score Result vs. Chemotaxis (um/min)
Figure 2

A. p = 0.005

B. p = 0.0068