Comparing Effects of Polypharmacy on Inflammatory Profiles in Older Adults and Mice: Implications for Translational Ageing Research

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Abstract

Background Ageing and multimorbidity are associated with inflammation. Polypharmacy is common in older people with multimorbidity. Given the potential for interactions between polypharmacy and inflammation, the relationship between inflammation and polypharmacy were studied in older adults with multimorbidity and in healthy ageing mice.

Methods A cross-sectional analysis of data from the five-year wave of the Concord Health and Ageing in Men Project, a population-based study of community-dwelling men aged ≥ 70 years. Serum concentrations of 27 cytokines were measured using a multiplex immunoassay. Associations between polypharmacy (≥5 medications) and cytokines were evaluated using multivariable linear regression adjusting for age, frailty, comorbidities and individual drug classes. Interaction between polypharmacy and Drug Burden Index (DBI - drugs with anticholinergic and sedative effects) was analysed. Effects of polypharmacy and DBI on serum levels of 23 cytokines were determined in ageing male mice treated with chronic polypharmacy or control.

Results Compared to the non-polypharmacy group (n=495), CHAMP participants with polypharmacy (n=409) had significantly higher concentrations of IL-8, IL-6, CCL3, Eotaxin, IL-1ra, IL-1β, IP-10 and lower concentrations of anti-inflammatory cytokine IL-4. In fully-adjusted multivariable models, polypharmacy was positively associated with concentrations of IL-8 and CCL3. There were no significant differences in inflammatory profiles between control and polypharmacy-treated mice. The relationship was not influenced by DBI in men or in mice.
Conclusions Inflammatory markers associated with polypharmacy in older adults were not seen in healthy aged mice administered polypharmacy, and may be related to underlying diseases. The polypharmacy mouse model provides opportunities for mechanistic investigations in translational research.

Key words: Ageing, Drug Burden Index, Geroscience, Inflammation, Polypharmacy, Translational Research.
Introduction

Polypharmacy has emerged as a major public health challenge (1), affecting approximately 1 million people in Australia (2) and 40 million people in the United States (3). It is associated with significant mortality and morbidity, such as delirium, falls and functional impairment (4-6).

Recent population-based studies have highlighted the growing burden of geriatric syndromes related to polypharmacy, such as dementia, delirium and falls (7-9). To date, translational studies investigating the pathophysiological mechanisms underlying the deleterious effects of polypharmacy are lacking. To reduce the burden of geriatric syndromes and medication-related problems, successful translation of mechanistic studies and therapeutic innovations through translational ageing research is fundamental.

Much attention has been devoted in recent years to assess the role of inflammation in ageing biology (10). The elucidation that inflammatory cytokines such as IL-6 and IL-8 may be contributors to the development of chronic diseases provides further insights into the complex interaction between inflammatory pathways and the disease processes (11). Inflammation is gaining recognition as critical in initiating and maintaining the senescence-associated secretory phenotype (SASP) of the senescence response (12). Polypharmacy is common in older adults with multimorbidity (13). Furthermore, the inflammatory processes might be modulated by the use of drugs with pro- and anti-inflammatory actions. Studies of circulating inflammatory cytokines may provide a biological basis to better understand the complex pathophysiological processes of polypharmacy-related adverse health outcomes in older adults. Studies in humans and animals have demonstrated that increasing anticholinergic and sedative exposure, which can be measured with the Drug Burden Index, is associated with frailty and functional impairment (14). It is possible that the effects may be mediated by both
direct pharmacological actions and inflammatory responses. In recent years, our research group has developed a unique animal model of polypharmacy to investigate the relationship between polypharmacy and clinical outcomes in old age (6, 15, 16).

No previous studies have compared the relationship between polypharmacy and inflammatory profiles in humans and mice. We hypothesized that polypharmacy might be associated with a pro-inflammatory state, which may be modulated by drug effects or underlying comorbidity. The aims of this study are (a) to examine the association between polypharmacy and inflammation in humans and mice, and (b) to determine whether our interventional mouse model is useful to investigate potential mechanisms of polypharmacy related adverse health outcomes independent of diseases, an interaction difficult to study without confounding among older adults.

Methods

Human study design and population

The Concord Health and Ageing in Men Project (CHAMP) is a prospective observational cohort study of community-dwelling older men in Australia. The study was approved by the Sydney Southwest Area Health Service Human Research Ethics Committee, Concord Hospital, Sydney, Australia. The rationale, design and selection criteria of the study have been described previously (17). In brief, a total of 1705 community-dwelling older men were enrolled from 2005 to 2006. To be eligible for the study, participants had to be men aged ≥ 70 years and live in the local government areas surrounding Concord Hospital in Sydney, Australia. Those who resided in a nursing home at the time of enrolment were excluded. The sampling frame was the New South Wales Electoral Roll, on which all Australian residents must register. All participants underwent detailed baseline assessments. Four follow-ups have
been conducted since 2006. Data in the present study originated from the 5-year follow-up and comprised 915 participants in whom serum inflammatory profiles were measured at the follow-up clinic visit. Participants with missing medication data (n=11) were excluded, leaving a total of 904 individuals available for analyses in this study. Follow-up assessment involved a structured medication history, biopsychosocial history, physical performance measures and laboratory assessment. The study followed the principles outlined in the Declaration of Helsinki. All participants provided written informed consent.

**Definition of polypharmacy**

Polypharmacy was defined as the concurrent use of ≥ 5 regular prescription medications. All participants were classified according to polypharmacy status. Medication data were collected during the follow-up visit. Participants were encouraged to bring all their medications (taken within the last month) to the study clinic, allowing verification by trained research staff, who also conducted a structured medication history and recorded the name, dose, frequency, duration, and prescription pattern (regular or as required) for all medications the participant had taken. Medication data were coded using the Iowa Drug Information Service (IDIS) drug code numbers. Analysis of drug type was performed at the drug class level.

As a supplemental analysis, we tested whether the Drug Burden Index (DBI) modified the relationship between polypharmacy and inflammation using the interaction term polypharmacy x DBI (a continuous variable) in linear regression analysis. The DBI for each participant represented the sum of exposure to medications with anticholinergic or sedative effects. It is calculated using the equation, Drug Burden = \(\sum[D/(\delta + D)]\), where the sigma sign (\(\sum\)) is the sum score of the prescribed drugs, \(D\) is the daily dose taken, and \(\delta\) is the
minimum licensed daily dose according to the Therapeutic Goods Administration Australia. \( \delta \) is used as an estimate of the \( DR_{50} \) (daily dose required to achieve 50% of the maximal anticholinergic or sedative effect at steady state). Complementary medications or medications taken as required were not used in the DBI calculation.

**Baseline covariates definitions**

Frailty was defined according to Fried frailty phenotype criteria in the Cardiovascular Health Study, which comprised of weight loss, exhaustion, low activity, slowness and weakness, with a few modifications as described previously (18). Participants were classified as frail, prefrail or robust if they met \( \geq 3, 1 \) or 2, and none of the criteria respectively. Comorbidities data were obtained from a standardized questionnaire. Comorbidities burden was defined by a composite score assigning one point to each of the following conditions: diabetes, hypertension, stroke, myocardial infarction, heart failure, peripheral vascular disease, angina, cancer, chronic kidney disease, chronic liver disease, chronic obstructive pulmonary disease, depression, dementia, epilepsy, Parkinson’s disease, thyroid dysfunction, osteoporosis, Paget’s disease and osteoarthritis. Smoking status was classified as never smoker, ex-smoker or current smoker. Alcohol intake was based on the self-reported amount of alcoholic drinks consumed per week. Height and weight were measured using standard protocols. Body mass index (BMI) in kg/m\(^2\) was calculated.

**Measurement of serum cytokine concentration**

At the follow-up clinic visit, venous blood samples were collected in a fasting state from participants. The blood samples were then centrifuged, serum was aliquoted and frozen at -80°C until analysis. Circulating serum cytokine concentrations were determined by a
multiplex immunoassay at the Australian Proteome Analysis Facility, using the Bio-Plex Pro Human Cytokine 27-plex Assay kit (Bio-Rad). When the concentrations of any cytokine fell below the limit of detection in >25% of the study population, the cytokine was excluded from further analysis (IL-2, IL-15 and GM-CSF). For the present study, a panel of 24 cytokines were analysed (FGF basic, Eotaxin, G-CSF, IFN-γ, IL-1β, IL-1ra, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-17, IP-10 (CXCL10), MCP-1 (MCAF), CCL3, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF).

Animal study methods

Animal Details

The study was performed within a longitudinal study of healthy ageing male mice described previously (15). All experiments were performed in accordance with the guidelines of the National Health and Medical Research Council of Australia, and approved by the Animal Ethics Committee of the Northern Sydney Local Health District, Sydney, Australia. Healthy male C57BL/6J (B6) mice (Animal Resources Centre, Perth, WA, Australia) were housed in cohorts of up to five animals per cage at the Kearns facility (Kolling Institute of Medical Research, Sydney, Australia). They had ad libitum access to water and food (Rat and Mouse Premium Breeder Diet containing 23% protein, Gordon Specialty Feed, NSW, Australia), and a 12-hour light-dark cycle was maintained (lights on at 07:00, off at 19:00). At 12 months, mice were randomly assigned to a non-medicated control feed or one of the three polypharmacy feeds, which included zero DBI (simvastatin 20 mg/kg/day, metoprolol 350 mg/kg/day, omeprazole 10mg/kg/day, paracetamol 100mg/kg/day and irbesartan 5mg/kg/day), low DBI (simvastatin 20 mg/kg/day, metoprolol 350 mg/kg/day, omeprazole 10mg/kg/day, paracetamol 100mg/kg/day and citalopram 10 mg/kg/day) and high DBI (simvastatin 20 mg/kg/day, metoprolol 350 mg/kg/day, oxycodone 5 mg/kg/day, oxybutynin...
27.2 mg/kg/day and citalopram 15 mg/kg/day). These medications are selected because they are commonly prescribed in older people (19). They have similar pharmacokinetics and pharmacodynamics in humans and mice, and are not known to be toxic when given to healthy mice. Medication was administered in the chow and water. Body weight, food and water intake were assessed weekly. After 14 months of treatment (old age = 26 months), venous blood samples were collected from the inferior vena cava, centrifuged and frozen at -80°C until analysis.

**Measurement of serum cytokine concentration**

Circulating serum cytokine concentrations were determined by a multiplex immunoassay at the Australian Proteome Analysis Facility. Since there were significantly more serum samples available in the control group (n=15) and low DBI polypharmacy group (n=10), compared with the high DBI polypharmacy group (n=7) and zero DBI polypharmacy group (n=8), a random number generator in Microsoft Excel 2019 (Microsoft Inc., Redmond) was used to randomly select one serum sample from each animal cohort of the control group and the low DBI polypharmacy group for inclusion in this study. As a result, the total numbers of animals included in this study for the control, zero DBI, low DBI and high DBI groups were 9, 8, 8 and 7 respectively. Serum samples from both groups were thawed, vortexed, and filtered through a 0.22μm centrifugal filter for 15mins at 14,000 x g, at 4°C. Sample aliquots were diluted 1:4 with sample diluent from a multiplex kit (Bio-Plex Pro Mouse Cytokine 23-plex Assay, Bio-Rad, Australia). The assay was conducted according to the manufacturer’s instructions. All samples were analysed on a 96-well plate, standards and samples were assayed on a robotic liquid handling workstation epMotion 5075 (Eppendorf), the plate was washed on Bio-Plex Pro II magnetic plate washer (Bio-Rad), and read with the Bio-Plex Systems 200. During incubation, the assay plates were shaken at 850 rpm at 25°C and
protected from light. Samples were analysed and standard curves were generated using the Bio-Plex Manager v6.0 software. For the animal study, cytokines with >40% of the values below the lower limit of detection (LLOD) were excluded from further analysis. For those cytokines with <40% of the values below LLOD, concentrations below LLOD were replaced with the respective LLOD divided by the square root of 2 (20).

Statistical analysis
Continuous variables are presented as means ± standard deviations, or median with interquartile range (Q1 – Q3) as appropriate. Categorical variables are presented as frequencies and percentages (%). Comparisons between groups were performed using the Student’s t-test (parametric) or the Mann-Whitney U test (non-parametric) for continuous variables. Categorical variables were compared using the chi-square or the Fisher exact test as appropriate. The Kolmogorov-Smirnov test was performed to confirm the normal distribution of data. Because cytokine concentrations were not normally distributed, values were logarithmically transformed to reduce skewness before linear regression analyses. A series of sequential multivariable linear regression models were performed to assess the association of polypharmacy status with the cytokines that were significant after bivariable analyses (IL-8, CCL3, Eotaxin, IL-6, IL-1ra and IL-1β): model 1 adjusting for age; model 2 adjusting for age and frailty status; model 3 adjusting for model 2 variables plus comorbidity burden; model 4 adjusting for model 3 variables plus additional adjustment for the following medication classes that were used significantly more in the polypharmacy group than in those without polypharmacy: statin, antiplatelet, angiotensin receptor blocker (ARB), angiotensin-converting enzyme inhibitor (ACEI), proton pump inhibitor (PPI), beta-blocker, bisphosphonate, antidepressant, corticosteroid, nonsteroidal anti-inflammatory drug (NSAID), antineoplastic and opioid. These covariates were selected as potential confounders
Based on their clinical and biological plausibility (21). Each cytokine was entered separately in each model. Standardized beta-coefficients for polypharmacy are reported for each cytokine in each model, with positive values indicating higher relative concentrations in participants in the polypharmacy group compared with those in the non-polypharmacy group, and negative standardized beta-coefficients indicating lower relative concentrations in the polypharmacy group.

For the animal study, continuous variables are presented as median with interquartile range (Q1 – Q3). Normality was assessed using the Kolmogorov-Smirnov test. Student’s t-test (parametric) and the Mann-Whitney U test (non-parametric) were used to compare variables between the control (no medications) and polypharmacy groups (5 different medications) as appropriate. Comparisons between three groups were performed using the Kruskal Wallis test (non-parametric) or one-way analysis of variance test (ANOVA) (parametric) as appropriate. Data analysis was performed using Statistical Package for the Social Sciences (SPSS) version 27 for Windows (IBM Corp, Armonk, New York, USA). A two-sided P-value < 0.05 was considered to be statistically significant. Adjustment for multiple comparisons was made using the False Discovery Rate Method by Benjamini and Hochberg at the 0.10 level (22).

Results

Demographic and clinical characteristics

In total, 904 CHAMP participants were included in the study, comprising 409 men in the polypharmacy group (≥ 5 medications) and 495 men in the non-polypharmacy group (< 5 medications). The demographic and clinical characteristics of the study population are shown in Table 1. There was no significant difference in body mass index, alcohol intake and number of current smokers between polypharmacy and non-polypharmacy groups. Compared
with the non-polypharmacy group, those in the polypharmacy group were significantly older, frailer, had a greater number of comorbidities and were more likely to be ex-smokers. They were more likely to be taking a statin, antiplatelet, ACEI, ARB, PPI, beta-blocker, bisphosphonate, antidepressant, corticosteroid, NSAID, antineoplastic and opioid.

Serum cytokines according to polypharmacy status

Concentrations of each cytokine by polypharmacy status are provided in Table 2. Compared with the non-polypharmacy group, those in the polypharmacy group had significantly higher concentrations of IL-8 (median, 9.3 pg/ml versus median, 8.3 pg/ml, \( P < 0.001 \)), IL-6 (2.8 pg/ml versus 2.1 pg/ml, \( P = 0.004 \)), CCL3 (0.8 pg/ml versus 0.7 pg/ml, \( P = 0.006 \)), Eotaxin (77.7 pg/ml versus 72.2 pg/ml, \( P = 0.008 \)), IL-1ra (22.3 pg/ml versus 20.5 pg/ml, \( P = 0.017 \)), IL-1β (0.7 pg/ml versus 0.6 pg/ml, \( P = 0.020 \)), IP-10 (CXCL-10) (314.9 pg/ml versus 292.1 pg/ml, \( P = 0.044 \)), and lower concentrations of anti-inflammatory cytokine IL-4 (median, 1.46 pg/ml versus median, 1.52 pg/ml, \( P = 0.040 \)). The differences in serum concentrations of IL-8, IL-6, Eotaxin, CCL3, IL-1β and IL-1ra remained significant between polypharmacy and non-polypharmacy groups after adjustment for multiple comparisons by using the false discovery rate method, whereas the differences in serum concentrations of IP-10 (CXCL-10) and IL-4 between the two groups were attenuated and failed to retain statistical significance upon adjustment for multiple comparisons.

Multivariable linear regression was undertaken with the concentrations of the following six cytokines (IL-8, IL-6, Eotaxin, CCL3, IL-1β and IL-1ra) as the dependent variables, which are summarized in Table 3. The positive association of polypharmacy status with concentrations of IL-8 (\( P < 0.001 \)), CCL3 (\( P = < 0.001 \)) and Eotaxin (\( P = 0.038 \)) remained significant when adjusted for age (model 1). Further adjustment for potential confounders
such as age and frailty (model 2) did not significantly change these associations, except Eotaxin, which was no longer significant when adjusted for age and frailty. The associations between polypharmacy status and higher concentrations of IL-8 \( (P=0.016) \) and CCL3 \( (P=0.006) \) persisted in more extensive models that included additional adjustment for comorbidity burden (model 3) and the following medications that were used significantly more in the polypharmacy group than in those without polypharmacy (model 4: statin, antiplatelet, ACEI, ARB, PPI, beta-blocker, bisphosphonate, antidepressant, corticosteroid, NSAID, antineoplastic and opioid).

**Association between inflammatory cytokines and polypharmacy x DBI interaction**

In a supplementary analysis, we examined the effects of polypharmacy x DBI interaction on serum cytokine concentrations, the results did not reach statistical significance after adjusting for multiple comparisons for any of the 24 cytokines analysed (Supplementary Table 1).

**Preclinical study results**

To further understand the impact of polypharmacy on inflammation, we compared the inflammatory profiles between the control and polypharmacy-treated animals (Table 4, Supplementary Table 2-4). There were no statistically significant differences in the concentrations of cytokines between the control and polypharmacy groups (combined groups administered zero DBI, low DBI and high DBI polypharmacy). Comparisons between zero DBI, low DBI and high DBI polypharmacy regimens also showed no significant differences in inflammatory profiles (Supplementary Table 5).
Discussion

To the best of our knowledge, this is the first study to comprehensively compare the effects of polypharmacy on inflammatory profiles in both older adults and mice. Our current analyses provided important and preliminary insights by demonstrating that older men with polypharmacy had significantly higher concentrations of cytokines, such as IL-8, CCL3, Eotaxin, IL-6, IL-1ra and IL-1β compared with those in the non-polypharmacy group. In fully-adjusted multivariable models, there was a significant association between polypharmacy and increasing concentrations of IL-8 and CCL3. The relationship was not modified by DBI. In our preclinical study, no association was observed between polypharmacy or DBI and serum levels of inflammatory markers. These findings demonstrate that the association between polypharmacy and inflammation observed in the CHAMP population may be related to residual confounding from underlying diseases, or other cross-species differences between humans and mice. Our findings have the potential to drive research innovations in the field of translational ageing research.

There are several potential explanations for the contrasting results observed between the human and animal studies. These include different study designs, study populations and interventions. The CHAMP study was observational, with the possibility for residual confounding due to unmeasured factors, while the preclinical study used a randomized interventional design. Our murine polypharmacy model is unique and significant with the following features: 1) healthy aged mice without comorbidities, such as hypertension and dyslipidaemia, treated with polypharmacy. Therefore, our polypharmacy model enables us to evaluate the independent effects of polypharmacy on inflammatory markers. This may be more difficult to achieve in clinical studies, where factors such as multimorbidity and drug-disease interactions may play a role in the underlying pathophysiological pathways. 2) From
middle age, the mice are administered with different combinations of medications that are commonly prescribed in older people. As such, our polypharmacy model mimics the human clinical course, and represents a useful model to investigate the effects of polypharmacy on different clinical outcomes.

The exact mechanisms responsible for elevated inflammation in people with polypharmacy remain to be fully elucidated. Many chronic diseases such as cancer and heart diseases are associated with chronic low-grade inflammation and increased levels of pro-inflammatory cytokines (23). We postulate that the association between polypharmacy and inflammatory markers observed in the CHAMP study may be related to underlying diseases. Moreover, increasing evidence suggests that inflammation plays a prominent role in the senescence-associated secretory phenotype (SASP) of the senescence response and the underlying ageing process (10, 12). Previous studies have demonstrated that inflammatory cytokines IL-6 and IL-8 are associated with the SASP response (12, 24) and are related to frailty in older adults (21). Inflamm-ageing refers to a state of chronic sterile low-grade inflammation and increased circulating levels of pro-inflammatory mediators in older adults (25). Chronic inflammation has a negative influence on the health outcomes in older people and is a key driver of accelerated ageing, disability and frailty (10, 26). The effect size of the differences in cytokine levels (in particular for IL-1β, IL-6 and IL-8) between the polypharmacy and non-polypharmacy groups is comparable to previous ageing studies of community-dwelling old adults with dementia or frailty (27, 28) and similar to studies in old mice treated with ACEI (with respect to Eotaxin) (29), although we acknowledge that it is often difficult to compare the cytokine levels across different studies due to high variability in study populations, modes of cytokine measurements (different immunoassay methods) and physiological factors.
Several drugs in our mouse polypharmacy regimens have been shown to affect inflammatory markers, such as statins, angiotensin-converting-enzyme inhibitors, angiotensin receptor blocker, beta-blockers and proton pump inhibitors (30-33). The anti-inflammatory properties of statins may be mediated in part by reduction of STAT3 phosphorylation in hepatocytes (30) and reduced inflammatory gene expression in vascular cells, such as a decrease in the levels of IL-1, IL-6, IL-8, IL-12, TNF-α and IFN-γ (34). Angiotensin II is a pro-inflammatory mediator that regulates the expression of cytokines (IL-6, TNF-α), chemokine (MCP-1) and adhesion molecules (35). By inhibiting the formation of angiotensin II, angiotensin-converting-enzyme inhibitors have been shown to reduce vascular inflammation in addition to their blood-pressure lowering effects (31). Observational studies have demonstrated that in patients with dilated cardiomyopathy, beta-blocker therapy is associated with reduced levels of anti-inflammatory IL-10 and pro-inflammatory TNF-α (32). Experimental preclinical and human studies have sought to determine the impact of anti-inflammatory drugs on serum cytokine levels. Handa et al. performed an in vitro experiment using human gastric epithelial cells and umbilical vein endothelial cells (33). They found that omeprazole and lansoprazole significantly inhibited IL-8 production by gastric epithelial cells and vascular endothelial cells in the presence of IL-1β and H. pylori-induced gastric inflammation (33). The potential mechanism of anti-inflammatory effects of proton pump inhibitors may be related to the inhibition of Nuclear Factor Kappa B (NF-κB) nuclear translocation and activation (33). Furthermore, some men in the CHAMP analysis took other anti-inflammatory drugs, such as corticosteroids and NSAID.

Drug therapy may also have pro-inflammatory effects. There is emergent evidence that anticholinergic drugs can impair cognitive function by suppressing the acetylcholine-
dependent anti-inflammatory pathway, resulting in increased IL-1β expression in the brain (36). In addition, polypharmacy has been previously shown to be associated with increased risks of drug-drug interactions, drug-disease interactions and decreased adherence (37), which may lead to suboptimal control of the underlying medical conditions, and may exacerbate the chronic pro-inflammatory state in older people. Further studies of the underlying mechanisms of the pro-inflammatory state in people with polypharmacy are warranted to gain further insight into these findings.

Our finding that a systemic pro-inflammatory state exists in people with polypharmacy may have important clinical implications. Inflammation plays a prominent role in drug metabolism (38). Experimental and clinical studies demonstrate that inflammation is a major regulator of drug-metabolising enzymes and transporters, which influence pharmacokinetics and drug clearance (39). Several pro-inflammatory cytokines, such as IL-1α, IL-1β, IL-6, IL-8 and TNF-α can reduce the hepatic expression and activity of major cytochrome P450 enzymes, such as CYP3A, CYP4A and CYP2C (40, 41). Inflammation-mediated changes in the expression of drug transporters, such as p-glycoproteins can result in altered plasma drug concentrations and variability in drug efficacy and toxicity (38, 42). Other pharmacologic effects of inflammation include changes in fluid volume, plasma protein level and increased susceptibility to drug-induced hepatotoxicity (43). Further studies are needed to evaluate whether polypharmacy-associated inflammation alters drug metabolism.

**Strengths and limitations**

This study has a number of strengths, including its translational study design and robust methodology. Examination of both clinical and preclinical data provides mechanistic insights and opportunities for translation. Strengths of the CHAMP study include a large study
population from a representative sample of community-based older men with standardized measurements of baseline covariates. However, the results of the CHAMP study are based on post hoc analyses, which are subjected to selection bias. Our clinical study population consisted of community-dwelling older men living in a defined region in Australia. Therefore, our results may not be generalisable to women, younger populations and those in other geographic regions or in non-community settings (e.g. hospitalized patients with acute inflammation). Future studies should confirm these findings across other populations using longitudinal repeated sampling. Although we adjusted for several potential confounders in the model examining the association between polypharmacy and inflammation, residual confounding due to unmeasured factors remains a potential explanation for the observed findings. Furthermore, because of the cross-sectional and post hoc nature of the analysis, our results should be considered as hypothesis-generating, and cannot prove causality. Our preclinical study is limited by the small sample size, male mice and absence of diseases. The relatively small sample size may be underpowered to detect changes in inflammatory markers, increasing the chance of a type 2 error. Future studies should test larger sample sizes to confirm these findings. In addition, the concentrations of several cytokines were below the detection range and were not analysed. This has limited our ability to compare the findings between the human and animal studies for those cytokines. Further validation of our results in other human and animal cohorts is needed. The relative duration of polypharmacy treatments differs between our mouse and human studies. Fourteen months of treatment in mice is equivalent to 46 years of treatment in humans (44). The duration of treatment in the CHAMP population is likely to vary between drugs and participants.
Conclusion

In a large community-based population cohort of older men, we have provided the first evidence of the association between polypharmacy and higher concentrations of cytokines, such as IL-8, CCL3, Eotaxin, IL-6, IL-1ra and IL-1β. The same relationship was not observed in the animal study. These findings demonstrate that the pro-inflammatory state in people with polypharmacy may be related to residual confounding from factors such as underlying diseases, rather than being a direct drug effect. Our polypharmacy mouse model represents a useful model for translational ageing research on mechanistic investigations of polypharmacy.
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Author Contributions

S.N.H. and D.G.L.C. conceptualized and designed the study, supervised acquisition, analysis and interpretation of the data, and assisted with drafting and revising the manuscript. H.W. was involved in the acquisition of data, statistical analysis, interpretation of data, drafting and revising the manuscript. J.M contributed to study design, data acquisition and manuscript revision. D.G., V.N., F.M.B., L.M.W., and D.J.H. contributed to study design, participant recruitment, data collection, and revised the manuscript critically for important intellectual content. All authors reviewed the manuscript and approved the final version of the manuscript for submission.
Conflict of interest

The authors declare that they have no conflict of interest.
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Table 1. Human study: Demographic and clinical characteristics.

| Characteristics                  | Non-Polypharmacy Group (n=495) | Polypharmacy Group (n=409) | P value |
|----------------------------------|--------------------------------|----------------------------|---------|
| **Age, years**                   | 81.0 ± 4.5                     | 81.8 ± 4.7                 | 0.012   |
| **Frailty status**               |                                |                            |         |
| Robust, n (%)                    | 260 (52.5)                     | 133 (32.5)                 | <0.00   |
| Prefrail, n (%)                  | 207 (41.8)                     | 214 (52.3)                 | 0.002   |
| Frail, n (%)                     | 21 (4.2)                       | 57 (13.9)                  | <0.00   |
| **Number of Comorbidities**      | 1.8 ± 1.3                      | 3.2 ± 1.6                  | <0.00   |
| **Body Mass Index, kg/m²**       | 27.1 ± 4.4                     | 27.6 ± 4.7                 | 0.084   |
| **Alcohol intake (drinks/week)** | 7.6 ± 9.5                      | 7.1 ± 8.7                  | 0.340   |
| **Smoking status**               |                                |                            |         |
| Never smoked, n (%)              | 213 (43.0)                     | 147 (35.9)                 | 0.022   |
| Ex-smoker, n (%)                 | 256 (51.7)                     | 246 (60.1)                 | 0.017   |
| Current smoker, n (%)            | 19 (3.8)                       | 14 (3.4)                   | 0.720   |
| **Medications**                  |                                |                            |         |
| Statin, n (%)                    | 189 (38.2)                     | 277 (67.7)                 | <0.00   |
| Antiplatelet, n (%)              | 141 (28.5)                     | 239 (58.4)                 | <0.00   |
| Angiotensin receptor blocker, n (%) | 122 (24.6)                  | 153 (37.4)                 | <0.00   |
| Angiotensin-converting enzyme inhibitor, n (%) | 89 (18.0)              | 126 (30.8)                 | <0.00   |
| Proton pump inhibitor, n (%)     | 88 (17.8)                      | 173 (42.3)                 | <0.00   |
| Beta-blocker, n (%)              | 76 (15.4)                      | 177 (43.3)                 | <0.00   |
| Bisphosphonate, n (%)            | 22 (4.4)                       | 50 (12.2)                  | <0.00   |
| Antidepressant, n (%)            | 22 (4.4)                       | 43 (10.5)                  | <0.00   |
| Corticosteroid, n (%)            | 20 (4.0)                       | 57 (13.9)                  | <0.00   |
| NSAID, n (%)                     | 17 (3.4)                       | 39 (9.5)                   | <0.00   |
| Antineoplastic, n (%)            | 11 (2.2)                       | 20 (4.9)                   | 0.028   |
| Opioid, n (%)                    | 7 (1.4)                        | 19 (4.6)                   | 0.004   |
| Antihistamine, n (%) | 3 (0.6) | 6 (1.5) | 0.313 |
|---------------------|---------|---------|-------|
| Antipsychotic, n (%)| 2 (0.4) | 6 (1.5) | 0.150 |

Abbreviation: NSAID, Nonsteroidal anti-inflammatory drug.
Table 2. Human study: Serum cytokine concentrations by polypharmacy status.

| Cytokines                  | Non-Polypharmacy Group (n=495) | Polypharmacy Group (n=409) | P-value | Adjusted P-value |
|----------------------------|-------------------------------|----------------------------|---------|------------------|
| Eotaxin, pg/ml             | 72.2 (45.2 - 99.5)            | 77.7 (53.0 - 107.4)        | 0.008*  | 0.049†           |
| FGF basic, pg/ml           | 5.3 (0 - 9.1)                 | 4.8 (0 - 9.1)              | 0.622   | 0.711            |
| G- CSF, pg/ml              | 22.2 (15.7 - 29.1)            | 21.0 (15.5 - 29.1)         | 0.618   | 0.742            |
| IFN- γ, pg/ml              | 31.1 (22.5 - 41.8)            | 29.7 (22.3 - 41.6)         | 0.479   | 0.639            |
| IL- 1ra, pg/ml             | 20.5 (14.8 - 28.4)            | 22.3 (16.2 - 31.2)         | 0.017*  | 0.081†           |
| IL- 1β, pg/ml              | 0.6 (0.5 - 0.9)               | 0.7 (0.5 - 1.0)            | 0.020*  | 0.079†           |
| IL- 4, pg/ml               | 1.52 (1.3 - 1.8)              | 1.46 (1.3 - 1.7)           | 0.040*  | 0.138            |
| IL- 5, pg/ml               | 1.9 (0.1 - 3.4)               | 1.9 (0.4 - 3.9)            | 0.299   | 0.478            |
| IL-6, pg/ml                | 2.1 (1.0 - 4.2)               | 2.8 (1.4 - 5.0)            | 0.004*  | 0.046†           |
| IL-7, pg/ml                | 4.1 (2.6 - 5.9)               | 4.1 (2.6 - 6.1)            | 0.933   | 0.974            |
| IL-8, pg/ml                | 8.3 (6.7 - 10.4)              | 9.3 (7.3 - 12.1)           | <0.001* | <0.001†          |
| IL-9, pg/ml                | 7.0 (5.1 - 8.8)               | 7.2 (5.3 - 9.3)            | 0.277   | 0.475            |
| IL-10, pg/ml               | 3.1 (1.7 - 5.7)               | 3.4 (1.7 - 6.5)            | 0.416   | 0.588            |
| IL-12 (p70), pg/ml         | 2.8 (1.0 - 6.4)               | 3.5 (1.4 - 7.3)            | 0.100   | 0.240            |
| IL-13, pg/ml               | 0.8 (0.2 - 1.8)               | 0.9 (0.2 - 1.8)            | 0.884   | 0.964            |
| IL-17, pg/ml               | 11.4 (6.1 - 17.3)             | 12.3 (6.6 - 18.4)          | 0.394   | 0.591            |
| IP- 10 (CXCL-10), pg/ml    | 292.1 (195.3 - 410.9)         | 314.9 (220.1 - 437.4)      | 0.044*  | 0.131            |
| MCP- 1 (MCAF), pg/ml       | 12.3 (2.2 - 22.4)             | 13.2 (2.3 - 22.8)          | 0.585   | 0.740            |
| CCL3, pg/ml                | 0.7 (0.4 - 1.0)               | 0.8 (0.5 - 1.1 )           | 0.006*  | 0.051†           |
| MIP- 1β, pg/ml             | 4.7 (2.1 - 8.3)               | 5.1 (2.6 - 9.1)            | 0.119   | 0.237            |
| PDGF- BB, pg/ml            | 223.4 (103.6 - 385.8)         | 212.2 (103.3 - 388.4)      | 0.933   | 0.933            |
| RANTES, pg/ml              | 45.0 (33.8 - 57.8)            | 45.4 (33.8 - 61.1)         | 0.194   | 0.357            |
| TNF- α, pg/ml              | 9.0 (3.7 - 13.2)              | 9.9 (4.9 - 14.3 )          | 0.094   | 0.252            |
| VEGF, pg/ml                | 14.3 (5.5 - 27.4)             | 15.7 (6.8 - 32.0)          | 0.105   | 0.229            |

Table 2 Notes:

Data are presented as median (interquartile range). Comparisons between groups were performed using the Mann-Whitney U test.

Statistically significant P values are highlighted in bold.

* Unadjusted P-value < 0.05 prior correction for false discovery rate.

† Adjusted P-value <0.10 after correction for false discovery rate.
The adjusted $P$-value corrects for multiple comparisons by using the false discovery rate method by Benjamini and Hochberg at the 0.10 level.

IL-2, IL-15 and GM-CST were excluded because most values were below the lower limit of detection.
Table 3. Human study: Multivariable regression models showing association between polypharmacy and log IL-8, log CCL3, log Eotaxin, log IL-6, log IL-1ra and log IL-1β.

| Cytokines   | Model 1 | Model 2 | Model 3 | Model 4 |
|-------------|---------|---------|---------|---------|
|             | β- Coefficient | P-value | β- Coefficient | P-value | β- Coefficient | P-value | β- Coefficient | P-value |
| Log IL-8    | 0.127   | <0.001  | 0.115   | <0.001  | 0.093   | **0.012** | 0.104   | **0.016** |
| Log CCL3    | 0.116   | <0.001  | 0.117   | <0.001  | 0.111   | **0.003** | 0.119   | **0.006** |
| Log Eotaxin | 0.069   | **0.038** | 0.054 | 0.115 | 0.047 | 0.212 | 0.017 | 0.701 |
| Log IL-6    | 0.049   | 0.138   | 0.039   | 0.258   | 0.047   | 0.209   | 0.056   | 0.197   |
| Log IL-1ra  | 0.046   | 0.173   | 0.033   | 0.343   | 0.050   | 0.182   | 0.025   | 0.559   |
| Log IL-1β   | 0.035   | 0.295   | 0.033   | 0.333   | 0.04    | 0.287   | 0.031   | 0.481   |

Table 3 Notes:

β-Coefficient = Standardized β-Coefficient.

Positive β-Coefficient indicates higher concentration of cytokines in the polypharmacy group compared with the non-polypharmacy group.

Model 1 adjusting for age; model 2 adjusting for age and frailty status; model 3 adjusting for model 2 variables plus comorbidity burden; model 4 adjusting for model 3 variables plus additional adjustment for the following medication classes that were used significantly more in the polypharmacy group than in those without polypharmacy: statin, antiplatelet, angiotensin receptor blocker (ARB), angiotensin-converting enzyme inhibitor (ACEI), proton pump inhibitor (PPI), beta-blocker, bisphosphonate, antidepressant, corticosteroid, nonsteroidal anti-inflammatory drug (NSAID), antineoplastic and opioid.

Statistically significant P-values are highlighted in bold.
Table 4. Animal study: Serum cytokine concentrations in the control and polypharmacy groups.

| Cytokines            | Control (n=9)       | Polypharmacy (n=23) | P-value |
|----------------------|---------------------|---------------------|---------|
| Eotaxin, pg/ml       | 1197.2 (852.6 – 1510.8) | 1177.5 (795.0 - 1967.7) | 0.95   |
| G- CSF, pg/ml        | 268.1 (244.2 – 580.3)  | 303.5 (229.0 - 352.1)  | 0.54   |
| IFN- γ, pg/ml        | 17.8 (8.0 – 53.5)    | 23.0 (17.5 - 31.1)    | 0.45   |
| IL-1α, pg/ml         | 5.7 (4.4 – 10.9)     | 6.3 (2.4 - 10.7)      | 0.88   |
| IL-9, pg/ml          | 17.2 (10.8 – 54.0)   | 22.4 (10.8 - 28.7)    | 0.80   |
| IL-12 (p40), pg/ml   | 1232.0 (951.2 – 2532.2) | 1363.6 (967.6 - 1985.4) | 0.99   |
| IL-12 (p70), pg/ml   | 116.5 (7.0 – 190.5)  | 122.7 (76.6 - 212.6)  | 0.29   |
| IL-17A, pg/ml        | 140.9 (5.0 – 177.4)  | 53.6 (4.7 - 175.4)    | 0.59   |
| KC, pg/ml            | 40.8 (22.2 – 56.0)   | 42.9 (23.1 - 59.7)    | 0.95   |
| MIP- 1β, pg/ml       | 210.9 (159.1 – 599.0) | 155.3 (125.3 - 220.7) | 0.09   |
| RANTES, pg/ml        | 207.6 (105.7 – 634.6) | 126.7 (78.5 - 202.2)  | 0.14   |

Table 4 Notes:

Data are presented as median (interquartile range).

Comparisons between groups were performed using the Mann-Whitney U test or Student’s t-test as appropriate.

Statistically significant P-values are highlighted in bold.

IL- 1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, GM-CSF, CCL3, MCP-1 (MCAF) and TNF-α were excluded because over 40% of values were below the lower limit of detection.