Dust, Cadmium and Rheumatoid Arthritis

Volume 1 of 2

Submitted by Daniel Martin Murphy to the University of Exeter
as a thesis for the degree of Doctor of Philosophy in Medical Studies, August 2018

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

Abstract

Background

Rheumatoid arthritis (RA) is a systemic, inflammatory disease with an estimated global prevalence of 0.3–1.0%. Evidence suggests that RA is initiated in the lungs. Cigarette smoking and various occupations associated with vapour, gas, dust, and fume (VGDF) inhalation can increase the risk of RA development. The association of VGDF, smoking, development of rheumatoid autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) and their relationship to RA disease development is poorly understood.

Structure

There are seven chapters in the dissertation. Chapter 1 introduces the dissertation reasoning and hypothesis. Chapter 2 is a published review of literature on RA and inhaled occupational exposures. Chapters 3 and 4 are published empirical studies analysing the clustering pattern of RF and ACPA, suggesting a potential common autoantigen in RA. Chapter 5 is a published empirical study analysing the pattern of autoantibody development with inhalational exposures to smoking and VGDF in male RA. Chapter 6 analyses the role of cadmium (as a common factor in smoking and VGDF), in relation to autoantibody development in nodular and non-nodular RA. Chapter 7 discusses further the strengths, limitations, unanswered questions and future direction of research.

Conclusions

Overall, this research provides evidence that RA, particularly in males, is precipitated by inhaled environmental exposures and RA patients with multiple inhalational insults are likely to have higher RF and ACPA levels. Empirical and laboratory evidence suggests a common autoantigen in RA to explain autoantibody clustering. Nodular RA patients demonstrate higher rheumatoid autoantibody levels, and significantly higher cadmium levels were found in female nodular RA patients. A model of heavy metal adsorption onto VGDF particles *in vitro* is proposed, stimulating pulmonary nodule formation and generating autoantibodies in response to a common autoantigen: post-translationally modified heavy chain fragments of immunoglobulin G.

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Author declaration

My contribution to this research

I declare that all the research reported in this dissertation is my own work. The work contained within has been planned, written and conducted by me. I was solely responsible for collating into a dissertation, writing additional chapters with the approval of my supervisory team.

Further first author peer-reviewed publications related to this dissertation are detailed in Appendix 4, written to establish my hypotheses in the literature.

Chapters 1-6 consist of studies that have been written as manuscripts for publication in peer-reviewed journals. Chapters 1-5 have been published, Chapter 6 has been submitted awaiting editorial decision at the time of thesis submission. All manuscripts are co-authored but are the result of this project. All manuscripts on which I am first author (Chapters 1-3, 5-6) have been written by me. Chapter 4 was written by me in collaboration with my research team and may also be submitted to the University of Exeter by joint first author Mr. Alex Clarke as part of his PhD thesis. I am not the first author on the manuscript for this particular chapter.

A detailed breakdown of author contributions to each manuscript is given below.

Author contributions to publications included as dissertation chapters

Chapter 1 (clinical case report)

Dr. D. Murphy: Conception and design of the work, data collection, data analysis and interpretation, drafting the article, critical revision of the article, final approval of the version to be published. I was responsible for writing up as a case report, obtaining written patient consent, ascertaining detailed clinical information from the patient, researching patients detailed medical history from old notes, integrating with the hypothesis through reflection with supervisor Dr. D. Hutchinson, taking clinical samples and sending for analysis, interpreting results, submitting for publication and responding to peer review as the corresponding author.

Prof. R. Marshall: critical revision of the article, final approval of the version to be published. Prof Marshall added his considerable experience and expertise in histological analysis of lung granuloma formation amongst kaolin workers to provide historical context and provided images of kaolinosis lung from his personal archive.

Dr. C. Harrington: Data collection, revision of the article, final approval of the version to be published. Dr. Harrington conducted urinary cadmium analysis along with Dr. Taylor from provided urinary samples to approved methodology.

Dr. A. Taylor: Data collection, revision of the article, final approval of the version to be published. Dr. Taylor conducted urinary cadmium analysis along with Dr. Harrington from provided urinary samples to approved methodology.

Dr. D. Hutchinson: Data collection, critical revision of the article, final approval of the version to be published. Dr. Hutchinson identified the patient in the clinical case as a typical representation of the hypothesis discussed.

Chapter 2

Dr. D. Murphy: Conception and design of the work, literature review, data collection, data analysis and interpretation, drafting the article, critical revision of the article, final approval of the version to be published. I identified the areas to be researched, conducted the literature review alongside Dr. Hutchinson as supervisor, identified gaps in knowledge and on seeking advice from supervisory team extended the literature search in appropriate directions as identified. I constructed the model for literature retrieval and analysis, structured the paper, submitted for publications and revised in response to peer review as corresponding author.

Dr. D. Hutchinson: Data collection, literature review supervision, critical revision of the article, final approval of the version to be published. Dr. Hutchinson gave guidance on key papers identified, assisted in addressing gaps in knowledge by highlighting key areas of pathophysiology in which to deepen understanding.

Chapter 3

Dr. D. Murphy: Conception and design of the work, literature review, data collection, data analysis and interpretation, drafting the article, critical revision of the article, final approval of the version to be published. I conducted this piece of work as an interim analysis on data collected. Hypothesis generation was completed in discussion with Dr. Hutchinson as supervisor. I drew attention to the striking subtleties in autoantibody pattern association, challenging accepted dogma. I suggested corroboration of findings with a different RA cohort and a need to overcome the fact that we did not have shared epitope data. I structured the paper, conducted data analysis and interpretation for cohort 1, submitted for publication and revised in response to peer review as corresponding author.

Dr. D. Mattey: Data collection, data analysis and interpretation, critical revision of the article, final approval of the version to be published. Dr. Mattey provided data on a pre-existing cohort in which shared epitope data already existed. He provided independent statistical analysis on cohort 1 findings and conducted statistical analysis on cohort 2.

Dr. D. Hutchinson: Conception and design of the work, data analysis and interpretation, critical revision of the article, final approval of the version to be published. Dr. Hutchinson discussed interim data analysis with me, suggesting the direction of analysis from the shape of data generated in the role of supervisor. He was responsible for the identification of a second cohort from previous collaboration with Dr. Mattey.

Chapter 4

Dr. D. Murphy: Conception and design of the work, data analysis and interpretation, critical revision of the article, final approval of the version to be published. I was responsible for hypothesis generation in discussion with Dr. Hutchinson, Dr. Eggleton and Mr. Clarke as a direct result of the findings detailed in Chapter 3. I redrafted the manuscript for publication and had input on the interpretation of data and figure design. I was not responsible for data acquisition.

Mr. A. Clarke: Conception and design of the work, data collection, data analysis and interpretation, drafting the article, final approval of the version to be published. Mr Clarke was primarily responsible for conducting the laboratory

analysis detailed in the paper under the supervision of Dr. Eggleton. He conducted data acquisition on peptide sequencing.

Dr. D. Hutchinson: Conception and design of the work, literature review, data collection, data analysis and interpretation, drafting the article, critical revision of the article, final approval of the version to be published, responsibility for data accuracy and integrity. Dr. Hutchinson was responsible for hypothesis generation and identification of laboratory specialist work to be conducted. He was responsible for the integrity of the data and accuracy of data analysis alongside Dr. Eggleton.

Dr. K. Heesom: Data analysis and interpretation, critical revision of the article, final approval of the version to be published. Dr. Heesom performed independent mass spectrometry and verified data acquisition findings.

Dr. P. Eggleton: Conception and design of the work, literature review, data collection, data analysis and interpretation, critical revision of the article, final approval of the version to be published, responsibility for data accuracy and integrity. Dr. Eggleton was responsible for the design and methodology of the laboratory work conducted, supervising Mr. Clarke and seeking independent verification from Dr. Heesom. He was responsible for the integrity of the data and accuracy of data analysis alongside Dr. Hutchinson.

Chapter 5

Dr. D. Murphy: Conception and design of the work, literature review, data collection, data analysis and interpretation, drafting the article, critical revision of the article, final approval of the version to be published. I supervised and partook in data collection, conducted data analysis, generated hypotheses to test, designed the methodology in conjunction with Dr. Hutchinson, drafted the manuscript, submitted for publication and revised in response to peer review as corresponding author.

Dr. K Bellis: Data collection, data analysis and interpretation, final approval of the version to be published. Dr. Bellis assisted in data collection and interpretation of

results, and assisted in draft revision.

Dr. D. Hutchinson: Conception and design of the work, data interpretation, critical revision of the article, final approval of the version to be published. Dr. Hutchinson was consulted on all aspects in the role of supervisor, assisting in methodological design and revision in response to peer review.

Chapter 6

Dr. D. Murphy: Conception and design of the work, literature review, data collection, data analysis and interpretation, drafting the article, critical revision of the article, final approval of the version to be published. I supervised and partook in data collection, conducted data analysis, generated hypotheses to test, designed the methodology in conjunction with Dr. Hutchinson, drafted the manuscript and submitted for publication.

Dr. E. Sinha-Royle: Data collection, data analysis and interpretation, final approval of the version to be published. Dr. Sinha-Royle assisted in data collection and interpretation of results, and assisted in draft revision.

Dr. C. Harrington: Data collection, revision of the article, final approval of the version to be published. Dr. Harrington conducted urinary cadmium analysis along with Dr. Taylor from provided urinary samples to approved methodology.

Dr. D. Hutchinson: Conception and design of the work, data analysis and interpretation, critical revision of the article, final approval of the version to be published. Dr. Hutchinson was consulted on all aspects in the role of supervisor, assisting in methodological design and redrafing aspects. He was responsible for supervising data collection of cohort 2.

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And finally, to the fascinating, friendly and welcoming rheumatoid arthritis population of Cornwall, to whom this work is dedicated. Your good humour and tolerance in adversity is truly inspiring and I sincerely hope that your contribution this work will help to reduce the burden of rheumatoid arthritis disease for future generations.

Definitions

ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
Anti- CarP	Anti-carbamylated protein antibody
Anti- CCP	Anti-cyclic citrullinated protein antibody
BR	Bronchiectasis
BRRA	Bronchiectasis and rheumatoid arthritis
COPD	Chronic obstructive pulmonary disease
CT	Computed tomography
CTPA	Computed tomography pulmonary angiogram
Da	Dalton
DNA	Deoxyribose nucleic acid
EULAR	European League Against Rheumatism
HLA	Human leukocyte antigen
HSP70	Heat shock protein 70
iBALT	Inducible bronchial associated lymphoid tissue
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgGH	Immunoglobulin G heavy chain fragment
IgGHFc	Fc binding site region of immunoglobulin G heavy chain fragment
IgM	Immunoglobulin M
IQR	Interquartile range
IMD	Index of multiple deprivation
IRAS	Integrated Research Approval System
NIHR	National Institute for Health Research
OR	Odds ratio
PE	Pulmonary embolism
PAD	Peptidylarginine deaminase
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SE	Shared epitope
SIR	Standardised incidence ratio
VGDF	Vapour, gas, dust and fumes

1.1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an inflammatory disease, primarily targeting the small joints of the hands [1]. Individuals with RA develop debilitating fatigue and joint inflammation, resulting in weakened grip and development of joint deformities impairing physical function [2]. Global RA prevalence is estimated at 0.3–1.0% [3]. In the UK, a 2013 study of RA patients with disease duration of 10-15 years reported an annual direct health care cost of approximately £3000 per patient [4]. Overall UK direct health cost of RA was estimated at £700 million in 2010, with a further £8 billion lost to the UK economy when productivity loss and state benefit requirements were considered [5].

1.1.2 Inhalation in rheumatoid arthritis

The lung is established as a primary site for RA disease development [6], although the exact aetiology remains unclear. Genetic susceptibility plays a role, however low concordance rates of 9.1% (95% Confidence Interval [CI] 1.9 to 24.3) in monozygotic twins have been found [7], suggesting that environmental factors are significant in RA pathogenesis. Cigarette smoking is arguably the most important environmental trigger factor for RA, with a strong association between ever smoking in monozygotic twin pairs demonstrating an odds ratio (OR) of 12.0, (95% Confidence Interval [CI] 1.78–513) for RA development [8]. Smoking is associated with triggering a measurable immune response in RA through the development of rheumatoid associated autoantibodies. It is associated with development of both rheumatoid factor (RF) and antibodies to citrullinated peptide antigens (ACPA) rather than RF- and ACPA- negative RA [9], which suggests that inhalation may trigger autoantibody development.

Further inhaled environmental risk factors for RA have been suggested including exposure to: silica [10-14], construction work [14-16], asbestos [17,18], mineral oils [19,20] farming and pesticide exposure [15,17,20,21], electrical and electronics work [15,16], textiles [22], and roadside dust [23,24]. Specific occupations have been associated with ACPA positive RA [15,16,19,21,23]. Citrullination of proteins in the lungs, as found in the longevity-associated RA risk

identified in cigarette smoke inhalation [25,26], could equally occur in other inhalational exposures [26], which show an increased risk for RA when seen in combination with cigarette smoking [14]. Such exposures predominantly relate to occupation in male populations, and was noted anecdotally amongst the male RA population of Cornwall, UK. Evidence exists linking RA risk to female occupational inhalation, such as recently seen in textile workers [22] and inhalational exposures in the wider environment [21,23,24].

1.1.3 Autoantibody generation and clustering

A history of smoking, specific human leukocyte antigen (HLA-DRB1) alleles that code a “shared epitope” (SE), and ACPA have emerged as the “trinity” of RA pathogenesis [27]. However, in routine clinical practice, the use of both RF and ACPA remains, and for good reason: the presence of RF and ACPA in healthy individuals increases the risk of RA development over and above ACPA alone [28]; and a positive RF and ACPA confer a far poorer radiological prognosis in established RA compared to either of these autoantibodies alone [29]. The 2010 ACR/EULAR RA classification criteria [30] include a criterion that scores highly for those individuals with a strongly positive RF or ACPA (scoring 3 points), acknowledging that a strongly positive RF is of equal weight in RA diagnosis to a strongly positive ACPA.

Furthermore, an important association between the two RA autoantibodies was observed in the rheumatoid- associated chronic lung condition of bronchiectasis. a prospective study following 122 bronchiectasis (BR) patients demonstrated that the presence of both RF and ACPA in the sera of patients with bronchiectasis was significantly more prevalent than in healthy controls and predicted the development of RA [31]. From this, it was suggested that there may be a common autoantigen; the potential antigenicity of RF (that is, heavy chain fragments of immunoglobulin G (IgGH)) triggers B cell activation in the lung, whereby post-translational modification to IgGH orchestrates a local (lung) and distant (joint) immune response that involves the production of multiple autoantibodies, potentially triggering RA [32].

1.1.4 Cadmium and rheumatoid arthritis

RA-associated inhalational exposures involve the inhalation of vapours, gas, dust or fumes; and the potential exists for the direct inhalation of toxic elements such as cadmium, or by adsorption of such toxins onto inhaled substrates from concurrent environmental heavy metal co-exposure (most commonly through cigarette smoke). Cadmium has been described as the most important toxin in inhaled cigarette smoke [33]. Cadmium is found in cement dust, wood dust, and coal dust, and is associated with the finishing of textiles as a pigment and stabilising agent. Cadmium can enter the food chain through air, water, soil and plants, agricultural runoff and in industrial effluent. The process of lung adsorption is seen in, but not exclusive to, silica dusts, whereby toxic heavy metals such as cadmium bind directly onto the inhaled intra-pulmonary substrate, dramatically increasing total body levels. Once cadmium enters the body it may be stored and accumulated in tissues over a number of years, building up in concentration and toxicity [33]. Adsorption potentially explains the pronounced interaction of silica dust and smoking co-exposure amongst exposed workers (OR 14.9; 95% CI 5.32-37.84) [12], and the RA risk attenuation seen in silica and non-silica dust exposed never smokers [14].

Other environmental RA risk factors include: lower socio-economic class, low formal education levels and residing close to main roads [34]. Intriguingly, these risk factors are also associated with high levels of cadmium exposure [35].

Further evidence for the role of cadmium in driving inflammatory arthritis has been demonstrated in an animal model. Rat lung instilled with cadmium-containing silica nanoparticles has demonstrated greater expression of pro-inflammatory cytokines and granuloma formation than in lungs exposed to cadmium alone or silica nanoparticles alone [36]. All exposures demonstrated parenchymal inflammation, granuloma formation, cytokine expression, and stromal fibrogenic reactions.

Environmental cadmium contamination is a long term health concern, with an historical antecedent and a modern industrial equivalent. For example, toxic blood cadmium levels have been demonstrated in 85% of children living in a Turkish coal mining area (mean serum cadmium 13.1 μg/L) [37]. Recent South Korean literature reports that the RA prevalence odds ratios rise with increasing cadmium concentrations [38].

1.1.5 Historical and geographical clinical context

1.1.5.1 Cornish socio-economic deprivation

It is well established that better health associates with improving socio-economic status, with modelling of general socio-economic, cultural and environmental conditions as the main determinants of health being widely cited [39] and systematically reviewed [40]. Work-related ill health was the fourth most prevalent cause of total disease burden in the pre-2004 European Union [41], with work and work-related psychosocial factors disproportionately affecting those at the lower end of the socio-enonomic gradient [40]. This is of relevance as Cornwall is a relatively deprived area of the UK: Indices of Multiple Deprivation 2015 data show that Cornwall currently ranks 143 out of 326 local authority areas for deprivation (1= highest population proportion living in the most deprived neighbourhoods) [42]. However, this overall ranking masks a polarisation between relatively more and less deprived areas, with 5% of neighbourhoods in Cornwall among the most deprived in England. This is particularly pertinent given the historical industrial decline that has occurred in parts of Cornwall. The former mining areas which have formed the backbone of Cornwall's major towns continue to show a marked difference in deprivation to the more affluent areas. Combined analysis of neighbourhoods the former mining areas of Camborne/ Redruth, St Austell/ China Clay, West Penwith, Bodmin and Liskeard reveals 31/128 (24.2%) neighbourhoods ranking in the in the 20% most deprived in England. This is significantly higher than the 13/198 (6.6%) remaining neighbourhoods in the rest of Cornwall's predominantly non-mining areas, $p< 0.00001$ [42].

1.1.5.2 Cornish metal mining and ill health

Cornwall's mining history predates the first notes on ill health of miners by Hippocrates (c. 430 BC, "a man from the mines being pale, livid and breathing with difficulty" [43]). Diodorus Siculus described well established mining of trading of tin in this part of the Ancient British Isles in the first century BC [44], and it has been suggested that the "pthsis" suffered by ancient miners was a form of silicosis caused by inhalational exposure [45]. Primarily ores of copper and tin were extracted in Cornwall, accelerating in production from the 16th century [43]. Though these were the dominant ores extracted, others were sought including arsenic, iron, lead, cadmium, tungsten, uranium, zinc, antimony, gold, lithium, rare earths, manganese, silver and molybdenite, and the discovery of "new" metals such as titanium [46]. Production exploded through the 18th and 19th centuries, with Cornish miners accessing deep underground seams through the technological advancement of steam-driven pumping through engineers such as

Newcomen, Trevithick and Woolf. However, conditions were reported as particularly poor in Cornish mines. Cornish miners of all ages showed excess mortaility when compared to Cornish non-miners and to Northern UK coal miners, with more than double the mortality rates of these comparison groups in all age bands above the age of 45 [47]. In 1832, Charles Thackrah, a Leeds physician and widely cited pioneer of occupational medicine, drew attention to dust inhalation as a cause of morbidity and mortality amongst miners, including suggestions for watering to the rock, regular bathing facilities and ventilation [48].

A Royal Commission into the Condition of Mines in Great Britain, reported in 1864, led directly to the first legislation to improve conditions, primarily ventilation, enacted in 1872 [47]. Further rising mortality in Cornish mines through the 19th century led to a specific investigation by JS Haldane in 1904, which followed eradication procedures to cure hookworm infection amongst miners, who had previously been blamed for their own poor health due to poor hygiene [47]. Haldane concluded that inhalation of dust particles, complicated by potential tuberculous infection, was the cause of excess mining mortality. This was evidenced by the dramatic increase in the mortality amongst heavily dust exposed machine drill operators (compared to those not working machine drills, with lower levels of dust exposure): amongst miners aged 25-35 in the Redruth area during 1900-1902, a six- fold increase in machine driller mortality was observed [47]. Research into the suppression of dust in mining environments to minimise the effects from inhalational exposure in the early part of the twentieth century is thoroughly described by Holman's history of silicosis, used as a term for inhalational lung disease [48]. Measures such as advancing ventilation design, wetting equipment for dust suppression, automatic drifting drill heads and longer piston drills all reducing the need for miners to be close to the drilling surface are discussed. However, the profitability of deep mining in Cornwall fell throughout the twentieth century, culminating in the global collapse of tin price control in 1985/6 [49]. Cornish metal mines did not survive, with the last Cornish tin mine at South Crofty closing in 1998, making direct industrial exposure a largely historical antecedent. However, the scale and duration of mining in Cornwall has left a legacy of contamination, with elevated levels of elemental concentrations noted in residential soil [50], household dust [50], and private water supplies [51], in addition to the previously mentioned legacy of social deprivation through post-industrial decline [42].

1.1.5.3 Cornish kaolin mining and ill health

More pertinent to this thesis, primarily due to ongoing activity within the Cornish economy and a potential exposure affecting the present day Cornish workforce, is the extraction of China Clay (kaolin). Kaolin has been mined around the St.

Austell area of Cornwall for over 250 years. It is used as a substrate in ceramic, paint, building, pharmaceutical and paper industries. A broad term given to a range of naturally occurring substances, predominantly made up of aluminium silicate $Al_2Si_2O_5(OH)_4$, it contains impurities of other metal oxides and is found in association with ancillary minerals [52]. It is water insoluble and when heated, kaolin develops cationic charges between layers, attracting anionic heavy metals which can then be adsorbed resulting in contamination [53]. Importantly, Kaolin does not react as free silica, and as such the effects of respiratory disease related to kaolin exposure cannot be accurately described as silicosis.

Kaolin is mined by directed water jets, streaming and separation pumping, following which it is dried to a moisture content of 1% by milling. It is during the drying and handling phases of production of this “dry” substrate that inhalation of microscopic kaolin particles occurs [54]. Kaolinosis describes the pulmonary fibrotic changes seen in kaolin workers, of which it has been suggested that 23% have abnormal chest radiographs [55].

A review of 68 kaolin workers in South West UK found a common pattern of small, rounded opacities 1.5-3mm on chest radiography, predominantly at the bases but affecting all zones [56]. Intriguingly, 3% (2/68) cases also had seropositive rheumatoid arthritis, with demonstrable large peripheral pulmonary nodules typical of Caplan Syndrome. This contrasts with a much lower rate of 0.4% seen in the coal workers of South Wales where this specific type of inhalation-related lung disease was discovered [57].

Anecdotal over-representation of RA males in Cornwall with occupational kaolin dust exposure, particularly with progressive, nodular disease, was noted, and marked the starting point for the formulation of this thesis and investigation therein.

1.1.6 Development timeline and ethical approval

I commenced this research whilst a senior house officer in the Rheumatology Department, Royal Cornwall Hospital. I registered the initial data collection as an internal clinical audit, began the data collection, and wrote up the initial clinical case reports, one of which is included at the end of this chapter as a published example [58]. The narrative of the patient detailed in this published case report was a typical example, providing the starting point for the overall hypothesis and aims, developed from detailed histories of individual patient experiences of RA in Cornwall, UK.

As the project grew, I co-ordinated and led an interdisciplinary team of junior doctors, nurses and secretarial support in data collection. To allow time for this project to develop, I outlined my hypotheses and ideas for research to the Royal College of General Practitioners Peninsula Deanery, who were kind enough to allow me to take an “out of programme” research year from general practice training to dedicate specific time to this project. I also was given a year-long honorary research fellow/ staff grade doctor clinical post within the Rheumatology Department, Royal Cornwall Hospital. Figure 1 summaries the progression of the study development as detailed below.

Figure 1. Flow diagram of study progression

The initial discussion to investigate this phenomenon as a research project centred on taking detailed occupational and smoking histories of individual patients with RA under follow up at the Royal Cornwall Hospital, UK. The hypotheses (specifically detailed above) were presented at a local meeting of the National Rheumatoid Arthritis Society, attended by over 100 members, the majority of whom were RA patients. Subsequent interest following the meeting led to further discussion as to the development of a protocol that would be acceptable to patients in terms of its design and acceptability. From initial ideas, this was refined and presented to the Committee of the Cornwall Arthritis Trust, a local charity supporting arthritis patients.

Specific feedback was offered on the wording of consent forms, questionnaire design and arrangements for follow up on patients who may not have been able to complete written questionnaires. The subsequent protocol, questionnaires and patient information sheets are contained within Appendix 1. Patient involvement in this process was invaluable for how best to manage and undertake the research in a way that was minimally intrusive for patients in their regular clinical care. Also, at the recommendation of the patients, a search was undertaken to review the literature on the possible associations between rheumatoid arthritis with occupational exposures and/or tobacco smoking, detailed in Chapter 2 [59].

Detailed data collection on rheumatoid arthritis patient smoking and occupational histories began in February 2015, registered and ethically approved as a clinical audit at the Royal Cornwall Hospital, Truro, Cornwall.

As hypothesis generation progressed, I wrote the Heath Research Authority protocol IRAS ID 194833: Dust, Cadmium and Rheumatoid Arthritis (Appendix 1), submitting to the Integrated Research Application System in November 2015. I attended the Ethics Review at the South West Research Ethical Committee to answer questions. I authored all aspects including the patient information sheets (Appendix 1) and I obtained Ethics permission for the data collection at peripheral GP sites for non-disease controls (Appendix 1), to obtain specific blood and urine sampling of patients to investigate autoantibodies and cadmium.

The project was submitted initially as a postgraduate dissertation by publication to the University of Exeter Medical School in March 2016, funded by the Cornwall Arthritis Trust.

Review by the South West Regional Ethical Committee (UK) was completed in May 2016, with approval being granted in August 2016 as project IRAS ID 194833 (Appendix 1). A simultaneous application was made to store samples at the National Institute for Health Research (NIHR) Exeter Clinical Research Facility, Exeter, UK, for the potential use in future studies (Appendix 2), approved in July 2016 under portfolio number CRF 249. Approval for a new collection of serum, extracted DNA and urine was obtained. Patient consent and sample collection took place via the clinical team in the Rheumatology Department, Royal Cornwall Hospital, to August 2017. I set up clinics across peripheral sites in Cornwall,

collecting data and samples with assistance from departmental junior doctors, medical students and nurses. I arranged secretarial support in clinic administration and data input, and formulated a service level agreement to process samples between the Clinical Chemistry Department, Royal Cornwall Hospital, the Clinical Research Facility (Exeter), and the Supra-Regional Assay Service Trace Elements Laboratory, Guildford, Surrey.

Interim data analysis of collected information on autoantibody levels of RA patients led to the fascinating results seen in Chapter 3 [60], in that ACPA-positive rheumatoid arthritis was primarily determined by RF titre and the shared epitope rather than smoking *per se*, published in July 2017. In analysing the impact of environmental exposures on autoantibody generation, wider consideration was given to how such autoantibodies might be generated, and how their generation might be linked. Further hypothesis generation led to preliminary laboratory work on investigating the underlying pathophysiological mechanisms linking RA autoantibodies, detailed in Chapter 4 [61], published in July 2017.

Key findings on specific over-represented exposures such as wood dust [62] and kaolin dust [63] were presented in poster form and oral presentation at the European League Against Rheumatism annual conference in June 2017.

Collation and analysis of smoking and occupational data as detailed in Chapter 5 [64] took place up to December 2017, written up for publication and published in April 2018. Analysis of results relating to nodular patients and urinary cadmium took place to June 2018, with Chapter 6 [65] submitted for publication in August 2018.

Further analysis of cadmium levels and recruitment of non-RA control groups for comparison is ongoing.

1.2 Published case report: Rheumatoid Pulmonary Nodules and Significantly Elevated Urinary Cadmium in a Kaolin (China Clay) Worker: Could Cadmium Adsorption onto Occupationally Inhaled Dust Explain Caplan's Syndrome?

As an example of a typical Cornish male rheumatoid arthritis case, this published report is included as a way of introducing the hypotheses that led to the development of this research project. The narrative history of this patient case report demonstrates the starting point from which my hypotheses were formed, developing from detailed histories of individual patient experiences of RA in Cornwall, UK. [58].

Citation: Murphy D, Marshall R, Harrington C, Taylor A, Hutchinson D (2017) Rheumatoid Pulmonary Nodules and Significantly Elevated Urinary Cadmium in a Kaolin (China Clay) Worker: Could Cadmium Adsorption onto Occupationally Inhaled Dust Explain Caplan's Syndrome?. J Rheum Dis Treat. 2017;3:057.

1.2.1 Introduction

Caplan’s syndrome, first described in 1953 amongst Welsh coal miners, classically occurs in individuals with both rheumatoid arthritis (RA) and coal dust exposure [57]. It is characterised by development of pulmonary nodules 0.5-5cm throughout the lung field, distinct from silicosis [57,66]. This is of contemporary interest as lung inflammation is now considered to be an important site of RA initiation.

Regional differences in the prevalence of Caplan’s syndrome have been reported in pneumoconiosis patients, from 0.4-1.5% [66]. This raises an intriguing question; what is the specific component of coal dust responsible for the development of rheumatoid pulmonary nodules and the development of RA?

We hypothesise that kaolinite mineral adsorption predisposes to increased pulmonary cadmium levels as a result of both a direct cadmium load and enhanced pulmonary cadmium adsorption from cigarette smoke.

We describe a kaolin worker with Caplan's syndrome and elevated urinary cadmium levels and discuss the relevant literature to support the hypothesis that cadmium can trigger rheumatoid pulmonary nodule formation and RA development.

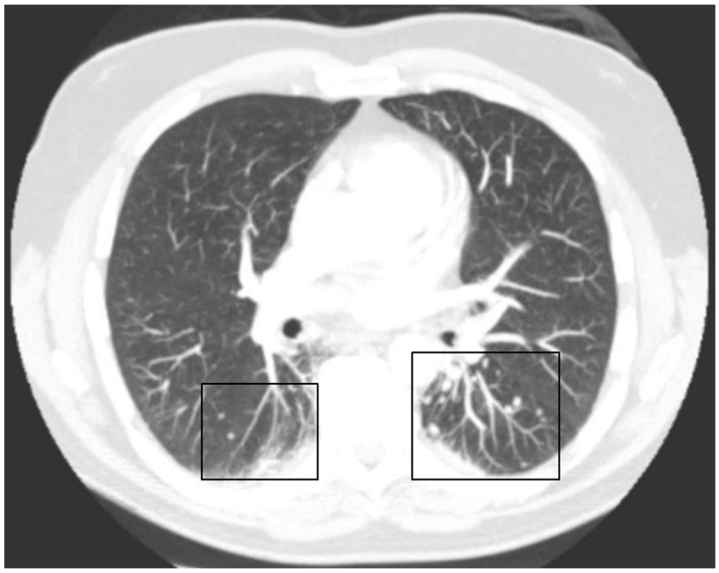
1.2.2 Case report

A 65 year old male from Cornwall, UK, developed nodular RA aged 32. Rose-Waaler testing at diagnosis was 1/2048 and a forefoot arthroplasty was undertaken within a year. He had worked for 16 years in kaolin drying refineries in Cornwall, UK, with consequent dust inhalation. He accumulated 20 pack years of cigarette smoking to age 30 years.

Treatment with prednisolone was commenced. Disease progression required multiple surgical interventions. Further disease modifying treatments included: penicillamine 1983-1985; myocrisin 1985-1992; sulphasalazine 1992-1996; and methotrexate from 1996 onwards. Aged 44, he ceased working in the Kaolin refinery as employers refused to grant adequate sickness leave.

Aged 55 he presented with a pulmonary embolus (PE), confirmed on CT pulmonary angiography (CTPA). Further CTPA imaging excluded recurrent PE as a cause of escalating shortness of breath symptoms. Radiology review of sequential CT chest imaging (2006-2008) demonstrated multiple pulmonary nodules consistent with Caplan's syndrome (Figure 2). Subsequent development of rheumatoid vasculitis has been treated effectively with rituximab.

Figure 2. Horizontal CT single slice: Caplan's nodules. Black frames highlight multiple well circumscribed >0.5cm Caplan's nodules throughout lower lobes bilaterally, more marked on left than right.



Contemporary serology levels demonstrated a rheumatoid factor of 481.7 iu/ml (0-10 normal range) and an anti-cyclic citrullinated peptide antibody of >500 u/ml (0-17 normal range).

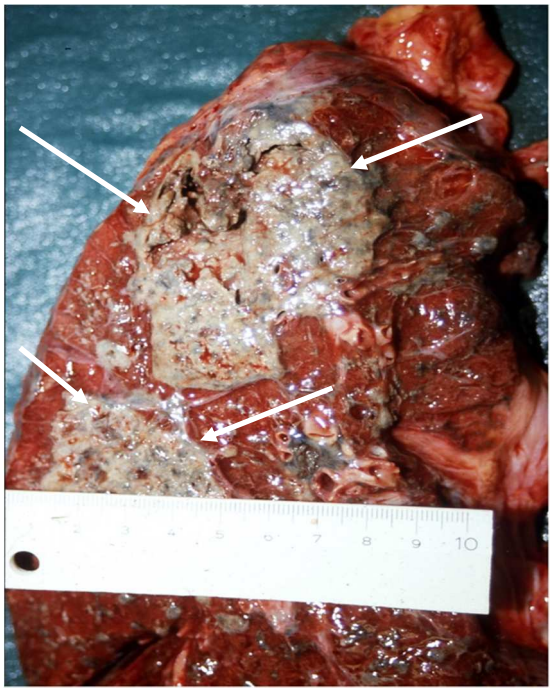
Given our interest in the role of occupation in RA development with reference to cadmium inhalation, a urinary cadmium level was undertaken, (0.66 µmol/mol creatinine, UK median 0.17, 95th centile 0.57) [67]. We suggest occupational kaolin dust inhalation has resulted in the significantly raised urinary cadmium level reported here.

1.2.3 Discussion

A diagnosis of Caplan's syndrome is evident by a diagnosis of RA, occupational dust exposure and radiological evidence of multiple pulmonary nodules. Kaolin workers are at high risk of Caplan's syndrome [56]. Kaolinosis is a complication of kaolin exposure, distinct from silicosis. Caplan's syndrome prevalence in kaolinosis is over seven times higher than originally reported in pneumoconiosis claimants (3% vs. 0.4%) [56,57].

Inhalation of fine kaolin dust can occur, particularly in drying phases of production. An experienced Cornish pathologist described being able to remove kaolin from the lungs of workers at post mortem "with a tablespoon" (Figure 3).

Figure 3. Post mortem lung tissue sample from a kaolin worker showing macroscopic kaolinosis.



Kaolin related employment in Cornwall declined to 900 people in 2014. However, 52/700 (7.4%) RA males under follow up at the Royal Cornwall Hospital have worked for >1 year in the kaolin industry, 12 times more than expected based on current occupational data. Likewise in Staffordshire (UK) underground coal miners have been demonstrated to have a significantly increased risk of RA development (odds ratio 8.47, 95% CI 2.59-27.66) [68].

Kaolin and coal dusts share a predisposition for a common contaminant: elemental cadmium. Cadmium content of coal varies, with a strong association noted between the kaolinite mineralisation of coal and cadmium content [69]. Far higher cadmium levels have been observed in West African kaolin (11.2-15.9 mg/kg), suggestive of local contamination by adsorption [70]. Mineral kaolinite is formed by geological feldspar decomposition, comprising a $(Si_2O_5)^{2+}$ tetrahedral layer and an $(Al_2[OH]_4)^{2+}$ octahedral layer bonded together by shared oxygen atoms between adjacent silicon and aluminium atoms [71]. Substitution of Si^{4+} by Al^{3+} gives rise to “permanent” active sites for adsorption in surface tetrahedral sheets, with “variable” pH-dependent adsorption occurring on alumina faces and crystal edges via direct covalent bonding. This renders kaolin as extremely effective at adsorbing heavy metals in environmental contamination [71].

Within coal, occurrence of trace elements such as cadmium is importantly dependent on the principal mineral species, in addition to coal age and individual elemental characteristics. We suggest kaolinite as an adsorption substrate in coal as a reason for Caplan's syndrome. Given that the original source for cadmium in coal is adsorption onto fine particle clay matter at formation [72], we suggest that interaction between the adsorption characteristics of principal mineral species and trace element contamination explains the variance of Caplan's syndrome found in global mining populations. Bituminous coals may display higher affinity for formation of organo-metal complexes and organic acid salts, thereby potentially containing higher cadmium levels at extraction or point of inhalation. However, we suggest that mineral capacity for further adsorption to inhaled dust causes the hitherto unexplained risk interaction seen in dust and cigarette smoke co-exposure [14]. This phenomenon explains the pronounced interaction of silica dust and current smoking >20 pack years co-exposure amongst exposed workers (OR 14.9, 95%CI 5.32-37.84) [11]. The phenomenon of an inhaled substrate predisposing to cadmium adsorption is observed in bitumen asphalters: smokers demonstrated a six-fold increase in serum cadmium compared to either non-smoking colleagues or control smokers [73]. Further evidence is seen in an animal model. Rat lung instilled with cadmium-containing silica nanopaticles demonstrated greater expression of pro-inflammatory cytokines and granuloma formation than lung exposed to cadmium alone or silica nanoparticles alone [36]. All exposures demonstrated parenchymal inflammation, granuloma formation, cytokine expression and stromal fibrogenic reactions.

Cadmium contamination of coal is a long term health concern. Toxic blood cadmium levels have been demonstrated in 85% of children living in a Turkish coal mining area (mean serum cadmium 13.1 μg/L) [37]. Recent South Korean literature reports RA prevalence odds ratio increasing by 1.62 per 1μg/L increase in serum cadmium [38].

We postulate that kaolin dust in this ex-smoker's lungs adsorbed cadmium from cigarette smoke, increasing intra-pulmonary cadmium concentration, stimulating inflammation and disease development.

This case highlights the importance of occupational dust exposure in RA, and further studies are underway to determine if kaolin workers have high levels of bodily cadmium and an increased RA risk. We highlight the process of adsorption as an overlooked factor, explaining the interaction of silica and non-silica based dust and cigarette smoking seen in RA.

1.3 Study hypotheses and aims

Hypothesis 1: RA is precipitated by inhaled environmental exposures, and patients with multiple inhalation exposures are more likely to have elevated autoantibody levels to both rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA).

> **Aim:** To conduct a literature search into inhalational exposures in RA with reference to occupation (Chapter 2) [59].
>
> **Aim:** To collect and analyse data on autoantibody levels in RA patients [60,61], and to compare autoantibody levels in patients exposed to cigarette smoke and/or vapour, gas dust and fumes (VGDF), (Chapters 5 and 6) [64,65].

Hypothesis 2: There is a common autoantigen in many RA patients to explain autoantibody clustering of both RF and ACPA.

> **Aim:** To investigate the possibility of a common autoantigen to explain autoantibody clustering (Chapters 3 and 4) [60,61].

Hypothesis 3: The presence of nodular RA is associated with raised rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA) levels irrespective of the smoking history.

> **Aim:** Utilising the findings of Chapter 3 [60], to further analyse the relationship between subcutaneous nodules**,** rheumatoid autoantibodies and smoking (Chapter 6) [65].

Hypothesis 4: RA smokers exposed to VGDF develop pulmonary nodules, adsorbing heavy metal toxins into lung granulomata rather than into the systemic circulation.

> **Aim:** Utilising the findings and of Chapters 3 and 5 [60,64], to compare urinary cadmium levels amongst nodular and non-nodular rheumatoid arthritis patients, analysing for differences in exposures (Chapter 6) [65].

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Chapter 2. Is Male Rheumatoid Arthritis an Occupational Disease? A Review.

Citation: Murphy D, Hutchinson D. Is Male Rheumatoid Arthritis an Occupational Disease? A Review. Open Rheumatol J. 2017;11:88–105.

2.1 Abstract

Background: Rheumatoid arthritis (RA) is a systemic, inflammatory disease with an estimated global prevalence of 0.3–1.0%. An unexplained association exists between low formal education and the development of RA independent of smoking. It is established that RA is initiated in the lungs and that various occupations associated with dust, fume and metal inhalation can increase the risk of RA development.

Objective: The objective of this review is to evaluate published clinical reports related to occupations associated with RA development. We highlight the concept of a “double-hit” phenomenon involving adsorption of toxic metals from cigarette smoke by dust residing in the lung as a result of various work exposures. We discuss the relevant pathophysiological consequences of these inhalational exposures in relation to RA associated autoantibody production.

Method: A thorough literature search was performed using available databases including Pubmed, Embase, and Cochrane database to cover all relative reports, using combinations of keywords: rheumatoid arthritis, rheumatoid factor, anti-citrullinated peptide antibody silica, dust, fumes, metals, cadmium, cigarette smoking, asbestos, mining, bronchial associated lymphoid tissue, heat shock protein 70, and adsorption.

Conclusion: We postulate that the inhalation of dust, metals and fumes is a significant trigger factor for RA development in male patients and that male RA should be considered an occupational disease. To the best of our knowledge, this is the first review of occupations as a risk factor for RA in relation to the potential underlying pathophysiology.

2.2 Introduction

2.2.1 Background

Rheumatoid arthritis (RA) is a common autoimmune inflammatory disease with an estimated global prevalence of 0.3–1.0% [1], and primarily targets the small joints of the hands [2]. The overall cost to the individual with RA as a consequence of fatigue, joint inflammation and the subsequent development of joint deformities are impaired physical functioning, reduced work productivity and activities of daily living [3]. The overall economic cost of RA to society is substantial. A study of RA patients in Norfolk UK with longstanding disease of 10-15 years reported an annual cost of approximately £3000 per patient (2013 prices) for direct health care alone [4]. Therefore studies identifying potential causes of RA that are avoidable are of huge importance to at risk individuals and also wider society.

Literature has established the lung as a primary site for disease development [5], although the exact aetiology remains unclear. Whilst genetic susceptibility plays a role, concordance rates of 9.1% (95% CI 1.9 to 24.3) in monozygotic twins have been found [6] suggesting that environmental factors are of great importance regarding the pathogenesis of RA. Cigarette smoking has emerged as an important risk factor for RA, with a strong association between ever smoking and RA in monozygotic twin pairs demonstrating an odds ratio (OR) 12.0, (95% CI 1.78–513) [7]. Smoking, as the most important environmental trigger factor for RA, associates with rheumatoid factor (RF) and antibodies to citrullinated peptide antigens (ACPA) rather than RF and ACPA negative RA [8]. Further inhaled environmental risk factors for RA have been suggested including exposure to silica [9-13], construction work [13-15], asbestos [16,17], mineral oils [18,19] farming and pesticide exposure [14,16,19,20], electrical and electronics work [14,15], textiles [21] and roadside dust [22,23]. Specific occupations have been associated with ACPA positive RA [14,15,18,20,22]. Citrullination of proteins in the lungs, as found in the longevity-associated RA risk identified in cigarette smoke inhalation [24], could equally be applied to other inhalational exposures [25]. Such exposures predominantly relate to occupation in male populations, though evidence exists linking RA risk to female occupational inhalation, such as recently seen in textile workers [21] and inhalational exposures in the wider

environment [20,22,25,26].

2.2.2 Key question

Why do people exposed to workplace dust or fumes in addition to cigarette smoke demonstrate a greatly elevated risk of RA?

2.2.3 Hypothesis

In short, we suggest that RA should be considered an occupational disease as a consequence of dust and fume inhalation in the workplace, as such exposures stimulate immune tolerance breakdown. Furthermore, these exposures form a lung substrate that can adsorb heavy metals from other environmental sources such as cigarette smoke.

We argue that metals have the potential to induce lymphoid tissue in the lung, generating ACPA and RF production locally. We highlight the process of adsorption as critical for RA disease development in men and discuss the pathophysiology of autoantibody generation resulting from occupational inhalation in stimulating RA disease development.

2.3 Methods

A literature search was performed using Pubmed, Embase, and Cochrane databases and to cover all relative reports. Titles and abstracts of identified articles were screened for eligibility and relevance to the key question and a priori hypothesis generation as detailed above. Duplicates and reports generated that were not relevant to the study aims were removed. Reference lists of relevant articles were searched for further evidence by hand around mechanisms of action and further hypothesis refinement.

2.3.1 Occupational exposure

Initial searches were conducted using the following keywords: “occupation OR mining OR silica”, in combination with disease specific term “Rheumatoid

arthritis". Subsequent searches of disease and exposure detail were made using the terms "Rheumatoid factor OR anti-citrullinated protein antibody/ies", AND "silica OR dust OR fumes OR metals OR cadmium OR asbestos OR mining". A separate search was made using the combination terms "rheumatoid AND smoking AND occupation". Following hypothesis generation and in the process of refinement, further searches were made using the combination terms "Rheumatoid arthritis AND bronchial associated lymphoid tissue OR adsorption OR heat shock protein 70" (Figure 4).

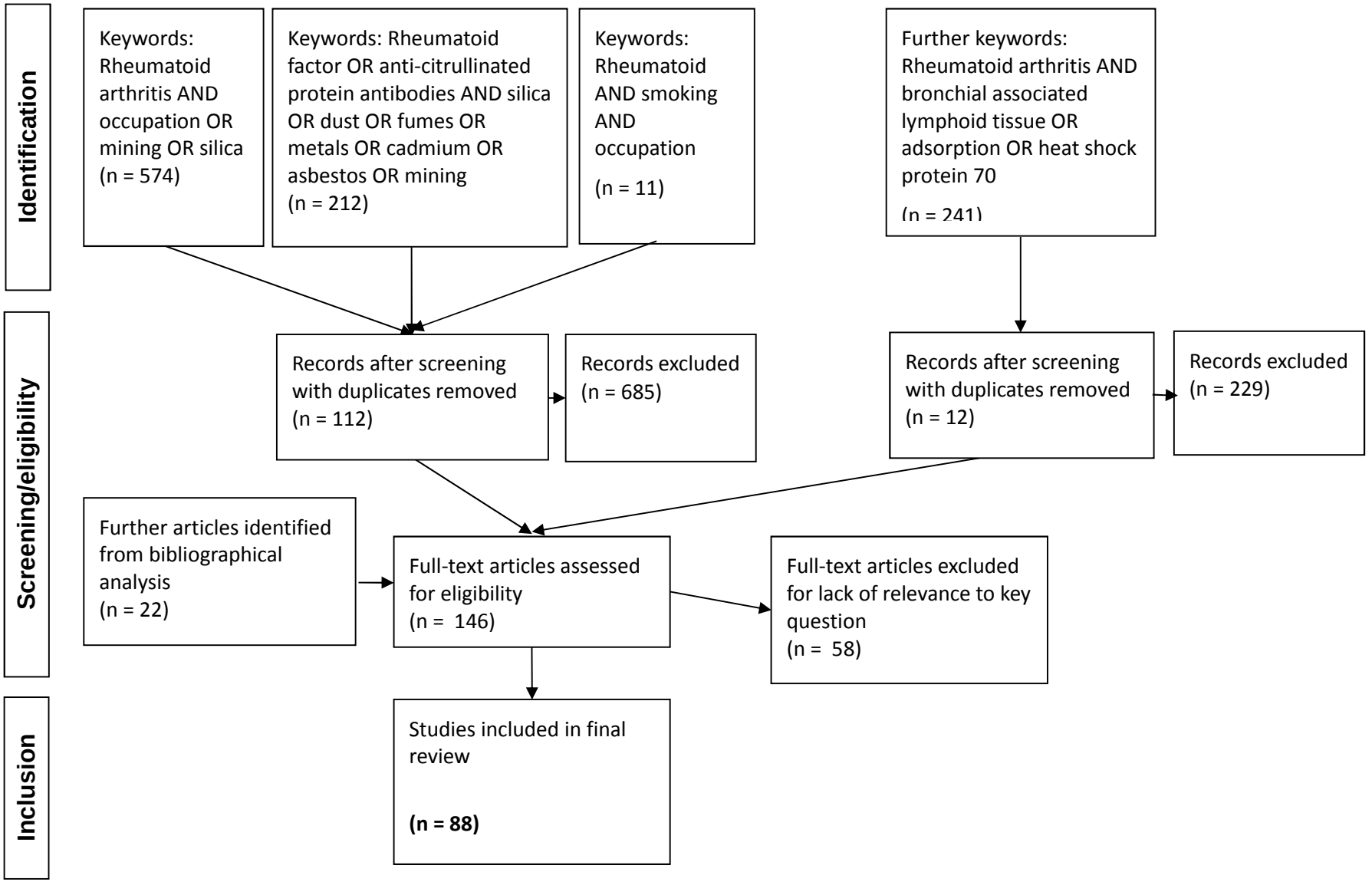
2.3.2 Appraisal

For occupational exposures, studies were examined for information relevant to output fields of sex, country of study, follow up period and exposure type. Reported or calculable RA risk being statistically significant for increased RA risk (with lower 95% confidence interval greater than 1.0) was used for inclusion.

2.3.4 Integration

For critical review purposes, statistically significant data pertaining to increased RA risk in male occupational exposures was integrated into a single table (Table 1), for descriptive comparison. Data not reaching statistical significance was not included in the table, though elements of non-statistical significance are included in the discussion. Heterogeneity in study populations, comparison groups and outcome measures precluded further pooled analysis or systematic review.

Figure 4. Literature retrieval flowchart



2.4 Inhalational workplace exposures.

2.4.1 Mining/ Quarry workers

Mining has long been associated with RA [27]. A cohort follow up study of 1026 Finnish granite quarry and processing yard workers from 1940-1981, demonstrated RA incidence rate ratio (defined as award to disability pension for RA), over five times higher than expected, compared to general population statistics. Silica exposure was postulated as the primary mechanism of aetiology [9]. Interestingly, RA was seen in the absence of frank silicosis, suggesting silicosis per se does not account for the observed increased risk of RA and an alternate pathophysiology is likely.

Stolt et al [11] found elevated ACPA+ RA risk in silica exposed Swedish quarry workers compared to unexposed controls (OR 1.67, 95% CI 1.13–2.48), but no increased risk of ACPA- RA when compared to unexposed controls. This was particularly evident amongst men exposed to stone dust and rock drilling. In terms of outcome, Swedish miners and quarry workers have also demonstrated the highest and most consistent standardised incidence ratios (SIR) across three cohorts of patients hospitalised due to RA (SIR 1.4, 95% CI 1.0-1.9 for 43 RA miners as per 1960 and 1970 census data) [14].

An association was seen in mortality odds ratio between postulated silica exposure and RA (OR 1.19, 95% CI 1.12-1.25) on analysis of death certificate data from 27 USA states from 1982-1995 [12]. Comparing 15/1237 RA silicotics with 20/6185 RA controls, this study was limited by the death certificate record of occupation rather than complete occupational history, and diseases/conditions contributing to cause of death. Categorisation of silica exposure was made based on occupation type rather than duration or intensity of exposure. However, stringent exclusion criteria would suggest bias towards the null hypothesis, suggesting that the significant risks presented may be an underestimate.

Mining has long been associated with extra-articular RA manifestations such as pulmonary nodules. Caplan’s syndrome, first described in 1953, classically

occurs in coal dust exposed RA cases, characterised by development of pulmonary nodules 0.5-5cm throughout the lung field, distinct from silicosis [27]. Given that the lung is an important site of RA initiation [5], this is of contemporary interest in RA aetiology. Radiological changes of Caplan's syndrome can precede RA development, and regional differences in the incidence of Caplan's syndrome in coal miners have been noted [28]. Underground coal miners in Staffordshire, UK, demonstrated a significantly increased risk of RA development (OR 8.47, 95% CI 2.59 to 27.66) [29]. Workers exposed to Kaolin (China clay) dust demonstrate the highest prevalence of Caplan's syndrome ever reported, 12 times higher than the prevalence noted in coal dust associated pneumoconiosis claimants (2/68, 3% [30] vs. 37/14000, 0.26% [27]). This raises intriguing questions as to the common components of inhaled dust responsible for RA pulmonary nodule development, and suggests that both dusts are implicated in RA development.

2.4.2 Construction workers

Elevated RA risk has been demonstrated amongst construction workers. Li *et al*. found significantly elevated risk amongst three cohorts of general construction workers studied, with SIR of 1.4 (95% CI 1.2-1.6), for RA hospitalisations 1964-2004 comparing to census data for occupations over census periods covering 1960-1970 [14]. No elevated SIR was seen amongst woodworkers or bricklayers. A recent abstract on the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) study of 3295 new RA cases in defined Swedish geographical areas compared to 4912 controls, suggested an OR of 2.6 (95% CI 1.3-4.9) for bricklayers and concrete workers [15]. However, this abstract does not detail actual numbers in the trades listed, and whilst the authors report that results are adjusted for smoking, no methodology is given. Furthermore, the authors included patients aged 18-70, and only report "last occupation" listed. Given the longevity of risk from the known inhalational insult of smoking [24], some acknowledgement of risk longevity conferred by other inhalational exposures would be expected. With the notable exception of mineral oil exposure, polarisation of RA risk estimates occurs when a latency period is used in occupational exposures [18], indicating a long induction period between exposure and RA development. Ergo, an upper age cut off of 70 years may miss those

occupationally exposed cases over the age of 50, who may not present until well into their 70s and crucially will not account for multiple occupational co-exposures.

Asbestos exposure, commonly found in historical building trade populations, was found to be an RA risk factor amongst 12/74 incident cases compared to 19/382 referents in multivariate analysis of lifelong occupational exposures (OR 2.5, 95% CI 1.0-6.8) [16]. Further evidence is found in a case-control study of community residents and workers exposed to asbestos contaminated vermiculite in Montana, USA, who demonstrated a strikingly elevated RA risk in those aged over 65 (OR 3.23 (95% CI 1.31-7.96) [17]. Interestingly, no significant increased risk was seen in those aged under 65, further highlighting the need to acknowledge a latency period between exposure and disease development.

2.4.3 Electrical workers and workers exposed to metal fumes.

Electrical workers demonstrated a non-significant, though consistent elevated RA hospitalisation risk (OR 1.1-1.3, (95% CI 1.0-1.3) [14]. Ilar et al suggest an OR of 1.8 (95%CI 1.0-3.1) for ACPA+ RA, and OR 2.1 (95%CI 1.1-4.0) for ACPA- RA, amongst electrical and electronics workers [15]. In the UK, electricians represent a subset of construction industry workers with multiple co-exposures. Inhalation of silica, non-silica inorganic, and organic dusts from physical interactions with building industry substrates. Activities such as drilling and chiselling out concrete, wood and gypsum plasterboard are likely to be relevant. Further specific aspects to electrical work involve handling and assembly of componentry that exposes workers to metal fumes. Toxic elements (such as cadmium) have been sequentially reduced and eliminated from solder, plastics and brazing fillers in modern European applications [31]. However, a longitudinal study of ten workers exposed to silver solder in the UK demonstrated the longevity of historical heavy metal fume exposure [32].

Other direct metal fume exposed trades demonstrate increased RA risk. An increased RA risk was reported in 29/74 Swedish mechanics, sheet metal workers and welders (OR 1.8, 95% CI 1.0-3.4) [16]. A single cohort of Li et al's study [14] showed a significantly elevated SIR of 1.2 (95%CI 1.1-1.4) amongst

foundry workers, with two large cohorts of mechanics and metal workers demonstrating slight, though insignificant, SIR of 1.1 (95%CI 1.0-1.1) and 1.0 (95% CI 1.0-1.1) respectively. Ilar et al [15] suggest an OR of 2.6 (OR 1.0-7.4) for ACPA+ RA in smelters and metal foundry workers. A retrospective cohort study of workers recycling scrap metals at an electrical arc furnace in Italy noted 3/331 incident cases of RA over a 20 year follow up period, a dramatically increased RR of 6.18 (95%CI 2.00-19.02), when compared to 420/20332 incident RA cases in the same town over the same period [33]. Despite the relatively small sample size and lack of directly comparable working group, this cohort demonstrated an appreciably elevated RA risk, thought due to foundry dust exposure containing a range of potential toxins including heavy metals [34].

2.4.4 Mechanics and workers exposed to mineral oils

In an analysis of 281 cases to 507 referents aged 25-75 years presenting to a single secondary care hospital in southeast Sweden, an increased though non-significant RA risk was seen amongst machine and engine repairers (OR 2.1, 95% CI 0.8-5.6), with specific exposure to hydraulic oils demonstrating an OR of 1.8, (95% CI 0.7-4.3) [35]. Li et al found that engine and motor operators demonstrated consistent though insignificant SIR elevation across three cohorts (SIR 1.1-1.2, 95% CI 1.0-1.5 combined) [14].

A report on the EIRA study compared 135 RA males exposed to different types of mineral oil to 132 controls, comparing seropositive and seronegative RA [18]. Exposure to any mineral oil demonstrated an increased risk (OR 1.3, 95%CI 1.0-1.3), with motor oil exposure (OR 1.2, 95%CI 0.9-1.8) carrying higher risk than others. When subdivided by autoantibody positivity, significantly increased risk to any mineral oil exposure was only seen in seropositive disease (ACPA+ OR 1.6, 95%CI 1.1-2.2). No significant increased risk for asphalt exposure was seen, unlike findings elsewhere (OR 14.0, 95%CI 1.2-99.9), albeit in limited numbers (4 cases: 1 referent) [16]. Limited correction for smoking was made via “ever” and “never” smoker categories. The authors noted a possible interaction between smoking and mineral oil exposure, but report an inability to reach a firm conclusion due to small numbers (ACPA+ RA attributable portion due to interaction= 0.5, 95%CI -0.2-1.2). No attributable portion was reported for RF+

RA.

2.4.5 Farm workers

Farming has long been associated with increased RA risk. A small but persistent SIR was noted in 821 RA farmers in Li at al's combined cohort as per 1960-1970 data (SIR 1.1, 95% CI 1.1-1.2) [14]. A further study of incident cases in Sweden found an OR 2.4 (95% CI 1.1-5.2) amongst 20 farm worker RA cases to 41 referents [16]. Interestingly, this study examined potential exposures which farm workers may encounter. On multivariate analysis with adjustment for age and smoking, no statistically significant increased risk was found for exposure to pesticides, farm animals, mineral oils or organic dusts. However, subsequent pooled analysis of specific occupational exposures with at least 50 exposed subjects amongst 176 cases and 630 referents, with adjustment for age and smoking, showed statistically significant increased risk amongst those exposed to crops and forage (OR 3.2, 95% CI 1.6-6.7 for >20 years exposure), and for fertilisers (OR 3.0, 95% CI 1.3-3.8). No dose-duration relationship was observed.

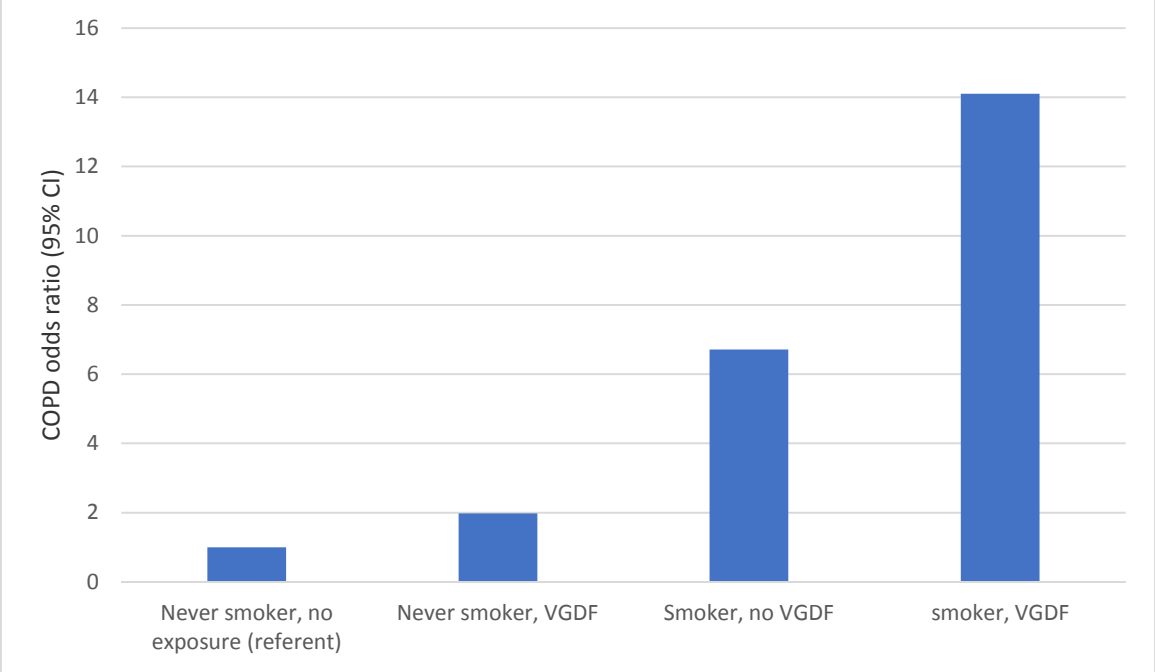
A longitudinal American study of 23570 spouses of pesticide license applicants from 1993-1997 found no association of RA with growing up on a farm or years living on a farm, on structured follow up interview in 2010 [19]. Detailed exposure analysis of 271 RA cases revealed increased risk from lifetime pesticide use (OR 1.4, 95% CI 1.0-1.6), and application of chemical fertilizers (OR 1.7, 95% CI 1.1-2.7). Amongst specific pesticides examined, an elevated but non-significant risk was seen for DDT (OR 1.9, 95% CI 0.97-3.6). Significant risks were seen for maneb/ manecozeb fungicides (OR 3.3, 95% CI 1.5-7.1), and chemical fertilizers (OR 1.7, 95% CI 1.1-2.7).

2.5 Discussion

Low formal education has consistently been found to be associated with RA [36-38]. All the occupations listed in Table 1 require no formal academic qualifications and it is noteworthy that no study as yet has considered an individual’s occupation as a confounding factor when considering low formal education levels as a risk factor for RA development. Likewise the stark contrast in mortality reported by

Pincus et al [39] in RA patients over a nine year period with a low formal education (45% mortality) compared to those with the highest formal education (5% mortality) has not been explained. However, occupations undertaken by working class individuals that expose them to vapours, gas, dust or fumes greatly increases the risk of chronic obstructive pulmonary disease (COPD) particularly in smokers, with an OR 14.1 (9.33-21.2) compared to never smokers with no such work exposures [40]. The “double-hit” of occupational exposure and smoking combined risk was far higher than the OR of 6.71 (4.58-9.82) in those smokers without such work exposures (Figure 5).

Figure 5. COPD risk: interaction between smoking and vapour/gas/dust/fume (VGDF) exposure. Adapted from Blanc et al [40].

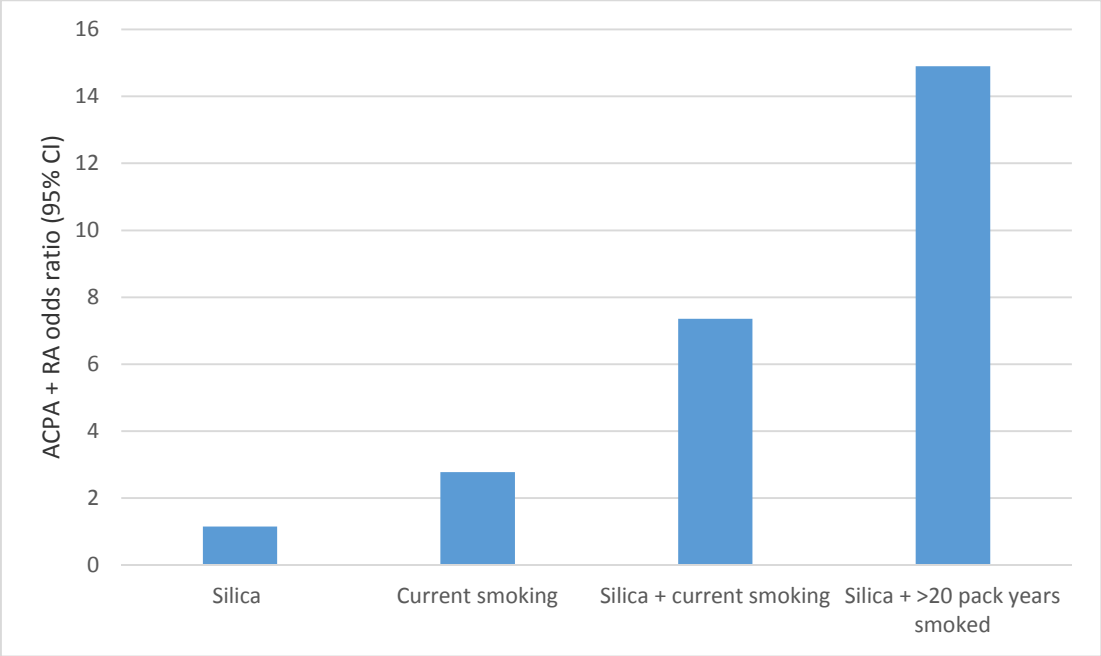


Whist the contribution of occupational exposures to chronic obstructive airways disease has been acknowledged [41], these occupations demonstrate only modest odds ratio rises in non-smokers. This is exemplified by Blanc’s study where significant but only modest differences were seen in never smokers when combined into broad categories of vapours, gas, dust and fume exposure in the longest held occupation (OR 1.98 (1.26-3.09) [40]. The impact of these exposures becomes particularly apparent when occurring in combination with smoking (Figure 5). This phenomenon is highly relevant to RA as men who work in dusty trades are highly likely to have smoked as the vast majority of male RA patients

have been smokers (77%) [8].

Occupational risk alongside smoking delivers a substantial “double-hit” for disease development. For example, silica dust exposure and smoking combined confer an increased risk of ACPA positive RA [11,15,18]. Interestingly, a pronounced risk interaction of silica dust and cigarette smoking for ACPA+ RA development was seen amongst co-exposed workers: silica only, OR 1.15 (95% CI 0.42-3.15); current smoking, OR 2.78 (95% CI 1.77-4.38); silica + current smoking OR 7.36 (95% CI 3.31-16.38), rising to OR 14.9, (95% CI 5.32-37.84) for >20 pack years smoked (Figure 6). This risk exceeded the expected separate effects of silica exposure and current smoking, indicating an interaction between these exposures (attributable proportion due to interaction = 0.60 95% CI 0.26-0.95). No explanation for this important interaction has been proposed in the literature.

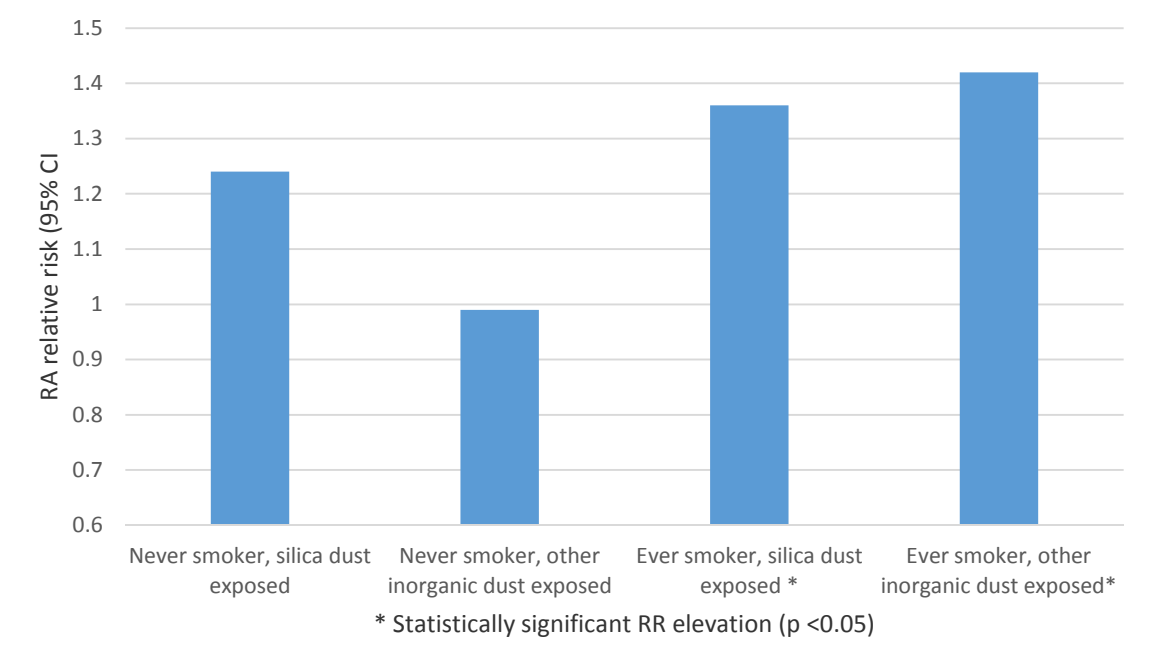
Figure 6. Risk interaction of ACPA+ RA in Swedish quarry workers with smoking and silica dust exposure, compared to unexposed never smoking controls, adapted from Stolt et al [11].



Recent analysis of 240,983 construction industry workers in Sweden suggested that “other inorganic” dusts are independent RA risk factors, in addition to silica [13]. Among ever smokers, both silica and other inorganic dust exposure were associated with increased RA risk: RR 1.36 (95% CI 1.11-1.68) and 1.42 (95% CI

1.17-1.73) respectively. However, no increased risk was seen amongst dust-exposed never smokers (Figure 7). This poses interesting questions, both as to the risk posed by inorganic dusts other than silica, and to the pathophysiology of the risk interaction of dust and cigarette smoke co-exposure.

Figure 7. Relative RA risk: interaction between dust exposure and smoking amongst Swedish construction workers, adapted from Blanc et al [13].



Here we suggest that silica and non-silica inorganic dusts act as an adsorber of heavy metals entering the lung via cigarette smoke. Cigarette smoke contains the metals aluminium, cadmium, chromium, copper, lead, manganese, mercury, nickel, selenium, vanadium and zinc. Cadmium has emerged as the most important of these metals in terms of increased levels relative to non-smokers and the associated increased risk of cardiovascular disease and COPD [42].

It has been hypothesised that inhalation of the metal cadmium links the established risk of smoking for RA and many of the occupations demonstrated to have an elevated RA risk [43]. Recent South Korean literature reports a significant RA OR rise of 1.62 per 1µg/L increase in serum cadmium, and a smaller significant OR rise in serum lead. [44]. Importantly cadmium has recently been demonstrated to citrullinate intracellular cytokeratins [45], which may potentially lead to immune tolerance breakdown and ACPA generation. Furthermore in micromolar concentrations (1–10 µM range), cadmium is associated with a pro-

inflammatory state [46], and high dose administration to Wistar rats has been shown to exacerbate collagen induced arthritis disease development, demonstrating pro-inflammatory cytokine expression [47].

2.5.1 Limitations of evidence

Over the course of an occupational lifetime, many workers will be exposed to multiple agents. For example, in less skilled labouring construction workers, co-exposure to silica dust, inorganic non-silica dust and wood dust is common. This is particularly true in poorer populations, where trade sub-specialisation and adherence to health and safety legislation will be less evident. As previously described, lower socio-economic status is linked with RA [36-38]. Given the longevity of increased risk for RA found in smoking [24], occupational exposure to dust or fumes may not present until long after exposure cessation.

Therefore, studies may be confounded by multiple inhalational insults, even when correcting for smoking. Blanc et al [13] excluded 40645 workers who were exposed to wood dust, gas or fumes to reduce confounding in their analysis of silica vs. non-silica dust exposed construction workers. In the above study, capturing data from occupational health service records for unionised construction workers existing from 1968-1993, culminated in enrolment of approximately 80% of the eligible workers. Analysis of the “missing” 20% and the excluded, co-exposed workers would be interesting; one would suggest predominance towards lower socio-economic status groups. Such workers are more likely to reside in unskilled “itinerant” trades, and thus become excluded from analysis. Non-unionised workers are not mentioned, which may have limited relevance in Swedish construction but is highly relevant when generalising findings elsewhere. We suggest many co-exposed cases of interest may have been excluded, underestimating risk.

As with construction workers, farmers perform a heterogenous mix of tasks in their work which can result in inhalation exposures. Soil type, rainfall and wind patterns affect aerosolisation of particles. Oil exposures from machinery vary, along with solvent, concrete, cement and general building dust exposure from the variety of tasks that some farmers perform. Metal fumes from welding have been

associated with increased RA risk amongst spouses of farmers [20]. We have previously suggested that heavy metal-laden particle inhalation may be an RA risk factor for farmers using pesticides and fertilisers [26]. Maneb and mancozeb fungicides are approximately 21% manganese by weight. Their use associates with increased levels in residential house dust, with concentrations declining with distance from use. Long-term Mancozeb use has shown evidence of wider environmental contamination [48]. Additionally, cadmium exposure from phosphate fertiliser may account for the RA risk seen in farmers. Rates of cadmium contamination in soil can range from 0.3 to 1.2 g/ha from contaminated fertiliser application [49], though this specific risk is largely of historical significance due to controls on the use of such fertilisers. Detailed information of exposure type and duration and intensity is needed to refine the potential risks that farmers face.

Swedish case referent studies utilising census defined occupations and hospitalisation records dominate the literature for occupational risk in RA. Whilst illuminating, such studies do have limitations. Patients in manual jobs may present to hospital more readily as they may be unable to complete tasks that would not present a problem in sedentary occupations, leading to possible risk overestimation in hospitalisation data. Symptomatic RA patients pre-census may have already switched from “heavier” manual occupations to lighter trades, whilst similarly symptomatic patients in those lighter trades may have been able to continue in the same occupation. As such, single cohort registry data may underestimate risk in heavier, manual trades, and overestimate risk in lighter occupational categories in which working conditions can be adapted to meet the needs of RA patients. Comparing occupation and socioeconomic status based on historical census occupation data, Li et al's [14] study was limited to those requiring hospital treatment, therefore misses non-hospitalised disease occurrence. Linking RA hospitalisation to preceding census occupation may reflect selection bias towards ill health, and is a criticism that can be levelled at all large cohort surveys measuring disease incidence via hospitalisation records. In having a cohort of patients who held the same job in 1960 and 1970, this study includes patients who held the same occupation over a ten-year period, and may mitigate against occupational selection bias. Given these caveats, one may expect sedentary occupations to predominate, and the mix of active technical and

mechanical occupations seen here and elsewhere is therefore likely to be an underestimate. Demonstrating elevated RA risk over different census reference periods, a strong argument can be made for miners, engine and motor operators, construction workers, electrical workers and farmers having an increased risk of RA.

Extrapolating Swedish data to represent populations around the world has limitations. Cigarette smoking in Sweden has a fascinating reverse social class gradient on analysis of 55,000 participants in the 1960 census, with both long term and heavy smoking being twice as common amongst non-manual workers as manual workers, and ten times more common than in those working in farming and agriculture [50]. This trend is opposite to the UK, where smoking has consistently shown a lower socio-economic class predominance; in 1982, 49% unskilled working men smoked, compared to 20% non-manual professionals [51]. Inhalational co-exposure to dust and cigarette smoke is therefore less likely in the Swedish populations reported than in UK equivalents.

2.5.2 Pathophysiologic considerations of vapour, gas dust and fume inhalation in relation to RA development

2.5.2.1 Inducible bronchial associated lymphoid tissue (iBALT)

The lung is now considered a principal site for the development of ACPA and RF positive RA [5]. A study of established RA demonstrated evidence of iBALT more commonly than in inflammatory lung disease without RA [52]. iBALT has the appearance of ectopic lymphoid follicles similar to those observed in rheumatoid joints [53]. iBALT contains numerous B cell follicles containing germinal centres and follicular dendritic cells. Lymphocyte lung infiltration was more frequently found in ACPA-positive RA patients (50%) as compared with ACPA-negative RA patients (17%) and controls (13%). Critically germinal centres, B cells and plasma cells were only found in the lungs of ACPA-positive RA patients [54].

Cigarette smoking is strongly associated with the development of iBALT as the expression of iBALT was significantly more common in smokers than non-

smokers (82% (14/17) v 14% (2/14) respectively) [55]. Furthermore silica has also been observed to generate iBALT in a murine model [56]. Rheumatoid pulmonary nodules have also been observed to contain lymphoid aggregates containing B lymphocytes and, in some cases, demonstrate characteristic features of lymphoid follicles [57]. The initial histological examination of the rheumatoid pulmonary nodules in the lungs of Welsh miners with Caplan's syndrome eluded to the presence of germinal centres with lymphoid collections in the outer collagen layer [58], which have never been described in peripheral nodules [59]. Pulmonary rheumatoid nodules, therefore, have the potential to generate rheumatoid associated autoantibodies and it is unsurprising that pulmonary nodules have been noted to precede the development of RA, invariably being associated with seropositive rather than seronegative disease [60]. For example, the presence of pulmonary rheumatoid nodules in Welsh coal miners who had no history, signs, or symptoms of RA was associated with a high prevalence of positive rheumatoid factor tests (60%). The potential importance of pulmonary nodules may have been over looked as on plain chest x-rays lung nodules were present in only 0.3% of RA patients [61]. However a study utilising CT imaging of the lung demonstrated pulmonary nodules in 22% of RA patients and subpleural micronodules and/or pseudoplaques in a further 17% of patients [62].

We suggest that accumulation of metals in the lung may predispose individuals to rheumatoid pulmonary nodules. The histology of a rheumatoid nodule is that of a granuloma with a palisade of cells proven to be a dual population of macrophages and fibroblastic cells clustered around a central necrotic core of fibrin. [63]. The macrophages are activated and have the appearance of epithelial cells and are termed epithelioid histiocytes [64]. Occasionally these histiocytes coalesce to form multinucleated giant cells in the rheumatoid nodule. Metal inhalation has been reported to be associated with the development of pulmonary macrophage derived multinucleated giant cells [65]. Metals such as beryllium are also linked to the formation of pulmonary nodules with histological features similar to rheumatoid nodules and can cause a chronic pulmonary granulomatous disease termed berylliosis which may arise long after exposure has ceased with a reported latency period of up to 40 years [66]. Raised levels of the heavy metals cobalt and chromium are associated with complications associated with metal on metal hip replacements. One of these complications includes the formation of

tertiary lymphoid tissue around the implant and this tissue is identical to that observed in joints of RA patients [67]. There appears to be an association between significant bodily levels of metals such as copper and cadmium in RA independent of smoking [68,69]. These data suggest that RA patients are exposed to metals and that heavy metals can induce the development of ectopic lymphoid tissue.

Further animal model evidence strengthens the “double-hit” hypothesis in terms of granuloma formation. Rat lung instilled with cadmium-containing silica nanopaticles demonstrated greater expression of pro-inflammatory cytokines and granuloma formation than lung exposed to cadmium alone or silica nanoparticles alone [70]. Nanoparticle use per se may be of interest as an evolving occupational risk, as inhaled silica and carbon nanoparticles, aerosolised in electronics componentry and production of lightweight materials, can induce lung citrullination and activate peptidyl arginine deaminase [71]. Given that numerous metals are found in cigarette smoke [42], one possible explanation for the association between cigarette smoking and iBALT development is the accumulation of metals in the lungs of smokers; a process likely to be accentuated by the presence of various occupational dusts.

2.5.2.2 Enhanced ACPA generation

It is well documented that the generation of ACPAs occur prior to the development of RA and greatly increase the risk of disease development [72]. Examination of bronchoalveolar lavage fluid from RA detected ACPAs and the levels of these antibodies were increased in patients that had more well-developed iBALT [52]. These data suggest that ACPA antibodies are produced locally in the lung by plasma cells contained within iBALT. In addition to silica and cadmium inducing iBALT, both nanoparticles have been observed to citrullinate intracellular proteins such as cytokeratins [45, 71]. In the case of silica, citrullination occurred via the peptidylargininedeiminase (PAD)-dependent mechanism [71].

2.5.2.3 Rheumatoid factor production

Rheumatoid factor is an autoantibody directed against the rheumatoid binding

site of the Fc of immunoglobulin G (IgG) [73]. Rheumatoid factor positivity is associated an enhanced risk of RA development [74]. Rheumatoid factor is more frequently observed in non-RA smokers [75], and individuals with lung disease associated with exposure to coal dust [60], asbestos [76] and silica [77]. Revealing the processes that result in RF generation may prove insightful for understanding how cigarette smoke and industrial inhalational exposures increase the risk of RA development.

Newkirk et al [78] described a mechanism by which cigarette smoke induces either IgM RF or IgA RF to be generated by B cells. This mechanism is dependent on the generation of an IgG immune response to heat shock protein 70 (HSP 70). Complexes of IgG-HSP70 double bind to RF expressing B cells via an interaction with the B cell receptor and CD91 with the subsequent production of IgM RF and IgA RF [78]. Therefore, environmental insults that associate with the induction of HSP 70 with a secondary IgG response are likely to be important with regards to the generation of IgM RF and IgA RF. HSP 70 expression is enhanced in the rheumatoid joints and an IgG autoantibody response to HSP 70 is significantly raised in recent onset RA (53.0%) compared to normal controls (4%) [79]. Interestingly exposure to dusts alongside heat and noise in the work place can generate a significantly enhanced immune response to HSP 70 (40%) compared to office workers (19%) [80]. Likewise in smokers of North American Indian origin (an ethnic group with a very high risk for RA) there is a reported significantly increased prevalence of IgG anti-HSP70 positive individuals (40%) as opposed to non-smokers (5%) [78]. Cadmium and other metals (copper and mercury) are strongly linked to the upregulation of HSP 70 [81], as is silica [82]. No studies to date have correlated RF levels in RA with dual exposures to various dusts and cigarette smoke and the relationship between RF and IgG HSP 70 autoantibodies.

2.5.2.4 Adsorption in dust exposure

All the occupations discussed involve inhalation of dust, fumes or particles, with the potential for adsorption of toxic elements such as heavy metals, from concurrent environmental co-exposure (most commonly through cigarette smoke). This process is seen in, but not exclusive to, silica dusts, with adsorption

of toxic heavy metals such as cadmium directly onto the intra-pulmonary substrate inhaled previously, dramatically increasing total body levels. We suggest a hypothesis of adsorption of trace elements in vitro onto previously inhaled substrates as a cause of increased, interactive RA risk seen in sequential inhalational exposures. Cadmium has been described as the most important toxin in inhaled cigarette smoke [42]. Adsorption explains the pronounced interaction of silica dust and current smoking >20 pack years co-exposure amongst exposed workers (OR 14.9, 95%CI 5.32-37.84, Figure 6), described by Stolt [11], and the attenuation of risk seen by Blanc [13], in silica and non-silica dust exposed never smokers.

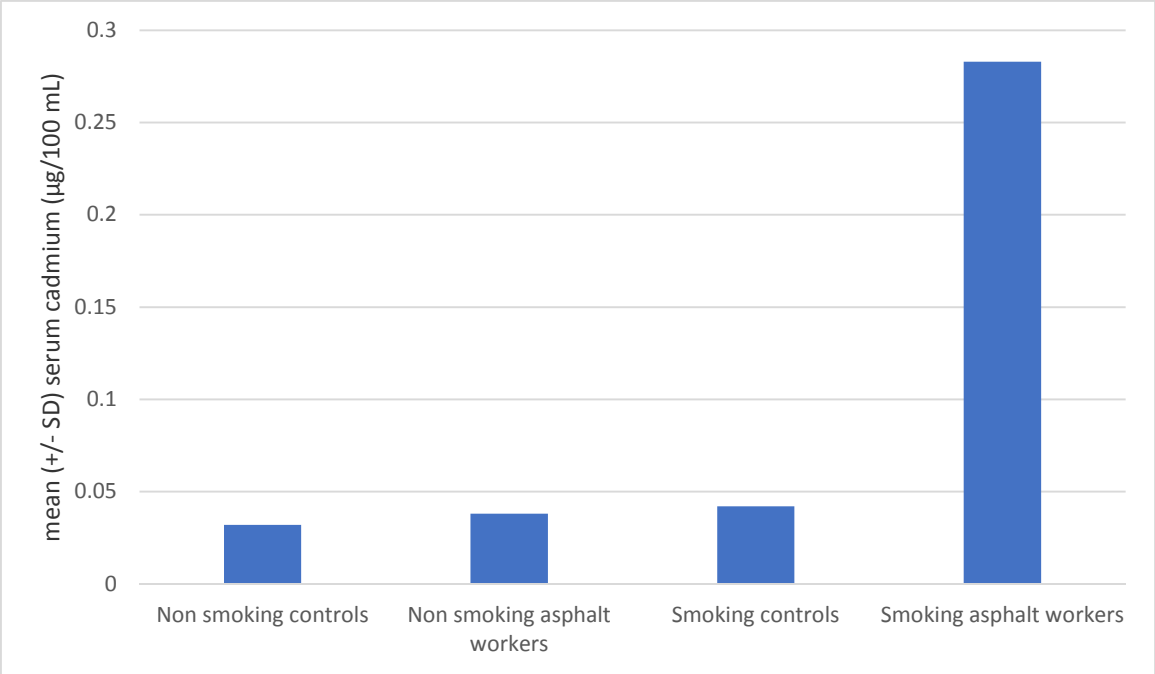
Quantification of the exact adsorption capacity of dust varies. In laboratory analysis of a single known adsorption substrate, sorption depends heavily on experimental conditions such as pH, metal concentration, ligand concentration competing ions, and particle size. This is before consideration of the differing adsorption capacities of multiple dusts that workers may be exposed to. For example, a general builder may inhale fine sand, gypsum, cement, and sanded wood. Occupational exposure matrices have a limited ability to capture individual variability within this due to heterogeneity of tasks and substrates, with consequent potential for misclassification bias [83].

Adsorption capability generally results from a net negative charge on structural particles of substrates, attracting and binding positively charged heavy metal particles. Binding potential is enhanced by increasing surface area, seen in finer dust particles [84]. Within mineral dusts, occurrence of trace elements depends on principal mineral species of the substrate and individual characteristics of the sorption particle. For example, bituminous coals may display a high affinity for formation of organo-metal complexes and organic acid salts, thereby potentially containing higher trace element levels in situ. Conversely, the aluminiosilicate mineral content of fine dust from anthracite coals has greater potential for further adsorption of heavy metals due to surface charges [85]. Various organic dust particles have quantifiably demonstrated adsorption of heavy metals, such as cotton, wood, wool, moss, and waste from paper and seafood industries [84]. Of non-silica inorganic dusts, commercial gypsum and industrial gypsum by-product, both widely used in the construction industry for plasters and plasterboards, have

demonstrated adsorption of lead and cadmium in solution via sulphate binding [86]. An interesting analysis on cadmium binding via adsorption demonstrated interaction between sand, cement and clay to which construction workers would be exposed. Clay addition to sand-cement mix (a common process for enhancing mortar plasticity), exposed more ion exchange sites and altered pH, enhancing uptake capacity of cadmium via ion exchange of iron, magnesium and aluminium oxides bound in cement [87].

We believe it is the “double hit” of inhaled particles with capacity for further adsorption from subsequent inhalation of trace elements that imparts excess risk. Most commonly, this would take the form of inhaled occupational dusts with the ability to adsorb trace elements from cigarette smoke. Further evidence for this compound exposure is demonstrated in Turkish bitumen asphalters, who demonstrated a six-fold increase in serum cadmium compared to either non-smoking colleagues or control smokers [88], Figure 8.

Figure 8. Interaction of asphalt exposure and cigarette smoking on serum cadmium levels among Turkish asphalt workers, adapted from Atasoy at al [88].



2.6 Implications for treatment

It is well established that cigarette smoking reduces the clinical response to both methotrexate and tumour necrosis factor (TNF) inhibitors (89,90). Cigarette smoking is strongly associated with the titres of RF [91], and it is noteworthy that a RF titre < 20 IU/ml was highly predictive for remission or low disease activity in RA patients with established disease receiving TNF inhibitors [92]. In this study RA patients with a RF titre < 20 IU/ml were significantly more likely (OR 18.9, 95 % CI 10.79-38.36) to be in remission at 12 months than RA patients with a RF titre >20 IU/ml. This is important as our own observations have demonstrated that wood dust exposed carpenters have significantly higher RF titres than non-dust exposed RA cases. Control never smokers (n=40) were noted to have RF titre of 16 IU/ml (IQR 6.7–47.2) compared to carpenter never smokers (n=8) RF titre of 86.4 (IQR 19.5–230.3), p=0.04 [93]. It is therefore conceivable that individuals who have been exposed to dust in the work place have a blunted response to methotrexate and TNF inhibitors by virtue of high RF titres, generated by inhalational insults as discussed above.

2.7 Conclusion

A variety of occupations have demonstrated increased risk for RA, primarily identified through Swedish case referent and linkage studies, and involve exposure to inhalational particles, in the form of dust, fumes or both. Global health and safety legislation, working patterns and smoking habits vary considerably. Studying populations where such co-exposures are common may demonstrate further areas of enhanced RA risk.

Interaction is seen between occupational RA risk and cigarette smoking, and we present information to support a hypothesis of heavy metal adsorption onto inhaled dust particles to explain this. Further work is needed to analyse the ability of inhaled dusts to stimulate immune tolerance breakdown in the causation of RA.

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Table 1. Occupational exposures previously found to demonstrate significantly increased RA risk in males

Author(s), publication year and reference no.	Country, period of employment and follow–up	Study population	Exposure type	Cases	Controls	Reported RA risk (95% CI)	Comments
Klokars et al 1987 [9]	Finland 1940–1981	1026, Cohort	Granite quarry and processing yard workers	35	7.5		Comparison with age matched Finnish population statistics for all workers
Stolt et al 2010 [11]	Sweden 1996–2006	577 RA cases, 659 age, gender, location matched controls	Silica: stone dust, rock drilling, stone crushing	54	69	1.39 (1.13–2.48)	ACPA+ RA. ACPA– and combined RA failed to reach significance
Stolt et al 2010 [11]	Sweden 1996–2006	577 RA cases, 659 age, gender, location matched controls	Silica: stone dust, rock drilling, stone crushing, current smokers	21	13	7.36 (3.31–16.38)	ACPA+ RA, silica and smoking interaction

Author(s), publication year and reference no.	Country, period of employment and follow–up	Study population	Exposure type	Cases	Controls	Reported RA risk (95% CI)	Comments
Calvert et al 2003 [12]	USA 1982-1995	4839231 death certificates from 27 states, silica exposed cases matched to non-silica	Silica	15/1237	20/6185	3.75 (19.2-7.32	Mortality odds ratio. OR 1.19 (1.12-1.25) following conditional logistic regression
Li et al 2008 [14]	Sweden 1964–2004	13820 hospitalisations for RA, cohort	Miners and quarry workers	175	-	1.7 (1.5–2.0)	Comparison with standardised incidence ratio (SIR) by occupation category in 1960 census
Li et al 2008 [14]	Sweden 1964–2004	13820 hospitalisations for RA, cohort	Miners and quarry workers	145	-	1.8 (1.6–2.2)	Comparison with SIR by occupation category in 1970 census
Turner and Cherry 2000 [41]	North Staffordshire, UK	6353 male pottery workers, case–referent	Underground mining	9/43	5/172	8.47 (2.59–27.66)	Referents matched on age, sex and pottery exposure

Author(s), publication year and reference no.	Country, period of employment and follow–up	Study population	Exposure type	Cases	Controls	Reported RA risk (95% CI)	Comments
Li et al 2008 [14]	Sweden 1964–2004	13820 hospitalisations for RA, cohort	Construction workers	498	-	1.2 (1.1–1.4)	Comparison with standardised incidence ratio (SIR) by occupation category in 1960 census
Li et al 2008 [14]	Sweden 1964–2004	13820 hospitalisations for RA, cohort	Construction workers	561	-	1.3 (1.2–1.5)	Comparison with SIR by occupation category in 1970 census
Li et al 2008 [14]	Sweden 1964–2004	13820 hospitalisations for RA, cohort	Construction workers	210	-	1.4 (1.2–1.6)	Comparison with SIR by occupation category in 1960 and 1970 census
Ilar et al 2016 [15]	Sweden, unreported	3295 incident RA cases, 4912 controls	Bricklayers and concrete workers	Unreported	-	2.6 (1.3–4.9)	Abstract only publication

Author(s), publication year and reference no.	Country, period of employment and follow–up	Study population	Exposure type	Cases	Controls	Reported RA risk (95% CI)	Comments
Blanc et al 2015 [13]	Sweden, 1968–1993	240983 unionised construction workers enrolled in occupational health service 1968–1993	Silica dust, ever smokers	160/52419	273/108400	1.36 (1.11–1.68)	
Blanc et al 2015 [13]	Sweden, 1968–1993	240983 unionised construction workers enrolled in occupational health service 1968–1993	Non–silica inorganic dust, ever smokers	202/132583	273/108400	1.42 (1.17–1.73)	

Author(s), publication year and reference no.	Country, period of employment and follow–up	Study population	Exposure type	Cases	Controls	Reported RA risk (95% CI)	Comments
Li et al 2008 [14]	Sweden 1964–2004	13820 hospitalisations for RA, cohort	Electrical workers	282	-	1.2 (1.1–1.3)	Comparison with SIR by occupation category in 1960 and 1970 census
Ilar et al 2016 [15]	Sweden, unreported	3295 incident RA cases, 4912 controls	Electrical workers	unreported	-	2.1 (1.1–4.0)	ACPA– RA Abstract only publication
Olsson et al 2004 [16]	Sweden 1996-1998	74 incident male RA cases, 382 referents	Electricians, electromechanical workers, service personnel	9/74	18/382	3.4 (1.2-9.4)	
Li et al 2008 [14]	Sweden 1964–2004	13820 hospitalisations for RA, cohort	Smelters and metal foundry workers	271	-	1.2 (1.1–1.4)	Comparison with SIR by occupation category in 1970 census
Cappalletti et al 2016 [45]	Trentino, Italy 1979-2009	331 exposed workers, cohort	Electric arc furnace workers	3/331	420/20332	6.18 (2.00-19.02)	Compared to incident cases in same town over same period

Author(s), publication year and reference no.	Country, period of employment and follow–up	Study population	Exposure type	Cases	Controls	Reported RA risk (95% CI)	Comments
Olsson et al 2004 [16]	Sweden 1996-1998	74 incident male RA cases, 382 referents	conductors, freight, transport workers	3/74	2/382	17.8 (1.5-207.8)	
Li et al 2008 [14]	Sweden 1964–2004	13820 hospitalisations for RA, cohort	Engine and Motor operators	383	-	1.2 (1.1-1.3)	Comparison with standardised incidence ratio (SIR) by occupation category in 1960 census
Svedrup et al 2005 [18]	Sweden 1996-2003	407 incident male RA cases, 486 controls	Mineral oil exposure	93	132/486	1.6 (1.1-2.2)	ACPA + RA, any mineral oil exposure. Specific cohort size not given for ACPA+RA
Olsson et al 2000 [47]	Sweden 1980-1995	102 RA cases, 248 referents	Asphalters	3/102	1/248	14.0 (1.2-799.0)	
Li et al 2008 [14]	Sweden 1964–2004	13820 hospitalisations for RA, cohort	Farmers	821	-	1.2 (1.1-1.2)	Comparison with SIR by occupation category in 1960 and 1970 census

Author(s), publication year and reference no.	Country, period of employment and follow–up	Study population	Exposure type	Cases	Controls	Reported RA risk (95% CI)	Comments
Olsson et al 2004 [16]	Sweden 1996-1998	74 incident male RA cases, 382 referents	Farmers	20/74	41/382	2.4 (1.1-5.2)	
Parks et al 2016 [20]	USA 1993-2010	23570 spouses of pesticide applicants, cohort	Farming: maneb/mancozeb perticides	10/271	351/23570	3.3 (1.5-7.1)	
Parks et al 2016 [20]	USA 1993-2010	23570 spouses of pesticide applicants, cohort	Farming: chemical fertilisers	23/132	2540/24018	1.7 (1.1-2.7)	

Chapter 3. Anti-citrullinated protein antibody positive rheumatoid arthritis is primarily determined by rheumatoid factor titre and the shared epitope rather than smoking per se.

Citation: Murphy D, Mattey D, Hutchinson D. Anti-citrullinated protein antibody positive rheumatoid arthritis is primarily determined by rheumatoid factor titre and the shared epitope rather than smoking per se. PloS one. 2017;12:e0180655.

3.1 Abstract

Objective: To analyse the relationship between rheumatoid factor (RF) titre, smoking and HLA-DRB1 alleles coding a “shared epitope” (SE) in relation to anti-citrullinated protein antibody (ACPA) positivity in rheumatoid arthritis (RA).

Methods: RA patients (n=658) attending rheumatology clinics in Cornwall, UK (cohort 1) were stratified according to RF and ACPA titre, and smoking pack years at diagnosis. A further 409 RA patients from North Staffordshire, UK (cohort 2) were studied to confirm the relationship between RF levels, smoking and ACPA positivity in relation to SE status.

Results: In cohort 1 there was a trend ($p<0.01$) of increasing ACPA positivity rates with increasing levels of RF without statistically significant differences between patients who had never smoked and smokers (never smoked: 15/71 (21%) RF -ve, vs. 43/64 (67%) RF weak +ve, vs 88/100 (88%) RF strong +ve, ever smoked: 18/70 (26%) RF -ve vs. 66/83 (80%) RF weak +ve vs. 196/210 (93%) RF strong +ve). No significant gender difference was observed. No significant difference between smoking and ACPA positivity was seen in RF negative patients. Smoking >20 pack years conferred an increased risk of anti-CCP positive RA (158/200 (79%)), compared to having never smoked (146/235 (62%), p=<0.01), but this increased risk correlated with smokers’ RF positivity as the principal determinant on subsequent regression analysis of cohort 2. In cohort 2, ACPA positivity rates significantly increased with RF positivity and carriage of 1 or 2 SE alleles ($p<0.01$). Little or no relationship was observed in patients lacking SE.

Conclusions: ACPA positivity in RA strongly associates with increasing RF titre independent of smoking. This relationship is dependent on carriage of SE alleles. There is no relationship between ACPA and smoking in RF negative patients.

3.2 Introduction

Over the last 2 decades, smoking, HLA-DRB1 alleles that code a “shared epitope” (SE), and anti-citrullinated protein antibodies (ACPA) have emerged as the trinity of RA pathogenesis [1]. However, in routine clinical practice the use of both RF and ACPA remains, and for good reason: the presence of RF and ACPA in healthy individuals increases the risk of RA development over and above ACPA alone [2], and a positive RF and ACPA confers a far poorer radiological prognosis in established RA compared to either of these autoantibodies alone [3]. The 2010 ACR/EULAR RA classification criteria [4] includes a criterion that scores highly for those individuals with a strongly positive RF or ACPA (scoring 3 points), acknowledging that a strongly positive RF is of equal weighting in RA diagnosis to strongly positive ACPA. RF and ACPA co-exist in RA more than would be expected by chance alone: a study of established RA (n=784) in Sheffield, UK, observed that 93% of RF patients were also ACPA positive [3]. The clustering of RF and ACPA is more pronounced with high titre RF as a study of 102 RA patients observed that 61% of RA patients with a RF >50 U/ml were ACPA positive (the expected frequency is 18.5% if RF>50 U/ml and ACPA occurred independently of each other) as opposed to only 25% of RA patients with a RF <50 U/ml [5]. This raises the possibility that a common process generates high titre RF and ACPA and determines a poorer prognosis in RA.

An important recent pooled analysis of 2234 RA patients casts significant doubt on the concept that smoking specifically associates with ACPA alone as RA ever smokers (n=1318) were found to have no significant association with single ACPA positivity, (OR 0.83, 95% CI 0.56-1.24), compared to ACPA and RF double seropositivity (OR 1.4, 95% CI 1.06-1.84) [6]. Moreover, this paper included a large cohort (n=9575) of non-RA cases. We suggest that in this non-RA cohort a much higher frequency of double autoantibody seropositivity for RF and ACPA exists than would be expected if RF and ACPA developed independently. Clustering of RF and ACPA was more pronounced in ever smokers (7 fold higher than expected) than never smokers (3 fold higher than expected), but was evident in the never smokers nonetheless. Accordingly, we have investigated this clustering of RF and ACPA in both smokers and never smokers for the first time. To date no studies have addressed if this co-existence is increased in those

strongly positive for RF irrespective of smoking. Surprisingly given the obvious clinical importance ascribed to a strongly positive RF, a literature search revealed no studies investigating the relationship between the presence and levels of a positive RF, cigarette smoking, carriage of the SE alleles and the frequency of ACPA in RA. We therefore set out to answer the following two important questions: Is there a relationship between RF levels at RA diagnosis and the likelihood of testing positive for ACPA irrespective of the smoking history of the patient? Is there a relationship between the presence of the shared epitope, RF and ACPA irrespective of the smoking history of the patient?

3.3 Methods

Two cohorts of RA patients were studied. Cohort 1 consisted of males and females attending routine rheumatology clinics at the Royal Cornwall Hospital, Cornwall, UK from February 2015 to August 2016. The data was anonymised at source, collected as part of project IRAS ID 194833, approved by South West Regional Ethical Committee. This cohort was selected to achieve an intended total cohort size of six hundred gender matched patients for analysis. Patients reviewed in clinic with a new or existing diagnosis of RA during the study period were included for analysis, with 60 patients subsequently excluded (see below). 298 male and 300 female RA patients were included to provide a suitable cohort size for serological subgroup analysis, with approximately equal numbers of each gender selected to detect potential differences in smoking rates, age of onset, and serological status rather than to represent the overall male to female prevalence ratio. Recruitment ceased when the target cohort size was reached. Data was recorded by clinicians in a standard questionnaire via face-to-face interview. Missing data was obtained by follow up telephone conversation. Age, age of RA onset, disease duration, smoking history prior to disease development, RF levels at disease onset and subsequent ACPA levels were recorded as part of routine clinical practice. Testing for SE status does not form part of routine clinical practice and was therefore unavailable. All patients fulfilled the 2010 ACR/EULAR RA criteria at diagnosis [4].

RF was measured with Tina-quant Rheumatoid Factors I1 Test System, by Roche Diagnostics Corporation. A value of < 14 IU/ml was considered as negative as per manufacturer guidelines. ACPA was measured by Roche Modular Analytics Second Generation E170 Anti-CCP analysis, with a negative value of <17 U/ml as per manufacturer guidelines.

As per guidelines, a negative RF or ACPA was defined as a level within the normal range, a weakly positive RF or ACPA <3 times the upper limit of normal and a strongly positive RF or ACPA >3 times the upper limit of normal [4]. Six percent (18/318) of females and 12% (40/340) of males were excluded during the collection period due to >20 years between smoking and RA diagnosis. A further 2/340 males were excluded due to incomplete data on subsequent review. Social deprivation analysis was undertaken through the UK government validated Index

of Multiple Deprivation (IMD) [7], a deprivation rank score of 32,844 neighbourhoods weighted by income, health/disability, education, housing/service access, crime and living environment, converted to equal deciles.

Table 2. Cohort 1 demographic data.

	Males **n = 298**	**Females** **n = 300**
Median age (yrs, IQR)	64 (55-73)	64 (52-72)
Median age at disease onset (yrs, IQR)	54 (46-63)	54 (41-62)
Median disease duration (yrs, IQR)	7 (4-12)	7 (2-12)
RF + (%)	232/298 (78%)	225/300 (75%)
ACPA + (%)	217/298 (73%)	208/300 (69%)
Mean IMD (SD)	4.36 (1.69)	4.38 (1.79)

A second cohort (n=409) of RA patients consisted of 154 males and 255 females attending rheumatology clinics at the Haywood Rheumatology Centre in North Staffordshire, UK, in whom SE status had been determined. Patients were recruited consecutively from a clinic established to monitor the effects of disease modifying anti-rheumatic drugs including hydroxychloroquine, sulfasalazine, D-penicillamine, gold and methotrexate. Sample collection occurred from 1994 to 2002 as part of a study to investigate the relationship between genetic factors and outcome in RA. Ethical approval was obtained from the North Staffordshire local research ethics committee and written informed consent was provided by all patients. All patients had established disease (median disease duration 9.2 years), fulfilling 1987 RA criteria at time of diagnosis [8]. A full smoking history was obtained on 386/409 patients. Smokers were defined as smoking >1 cigarette (or equivalent)/day for >1year, and categorised according to pack years smoked [9]. All patients had been genotyped for HLA-DRB1 as previously described [10].

Table 3. Cohort 2 demographic data.

	Males **n = 154**	**Females** **n = 255**
Median age (yrs, IQR)	59 (49 – 65)	58 (50 -68)
Median age at disease onset (yrs, IQR)	49 (40 – 56)	48 (38 – 56)
Median disease duration (yrs, IQR)	9.0 (5 -12)	9.3 (5 – 13)
RF + (%)	107/154 (69.4%)	140/255 (54.9%)
ACPA + (%)	124/154 (80.5%)	183/255 (71.8%)

IgM RF was measured by nephelometry at disease onset while ACPA was measured subsequently using a commercially available anti-CCP2 ELISA (Axis-Shield, Dundee, Scotland). RF levels were reported in International Units (IU). A level > 60 -180 IU/ml was considered weakly positive and a level >180 IU/ml was strongly positive. ACPA measurements above 5 units/ml were considered positive.

3.3.1 Statistical analyses

Chi square tests and multivariate logistic regression analysis were used to examine the relationships between ACPA, IgM RF, HLA-DRB1 shared epitope and cigarette smoking. Analyses were adjusted for age, sex and disease duration where appropriate. Mann-Whitney U testing was performed to analyse differences in RF titre. Given the heterogeneity between different cohorts, no pooled analysis was made.

All analyses were carried out using the Number Cruncher Statistical System for Windows (NCSS60).

3.4 Results

3.4.1 Cohort 1

Twenty four percent (141/598) of patients were RF negative. The proportion of RF negative patients did not differ significantly with gender, with 75/300 (25%) females and 66/298 (22%) males testing negative. A weakly positive RF was observed in 77/300 (26%) females and 70/298 (23%) males, while a strongly positive RF was observed in 148/300 (49%) females and 162/298 (54%) males.

Table 4. Cohort 1 relationship between smoking, RF and ACPA.

	Median RF	ACPA neg (%)	ACPA pos (%)	Odds ratio (95% CI)
Never smoker, RF neg	neg	56 (79)	15 (21)	1.0 (reference) *
1-10 PY, RF neg	neg	13 (87)	2 (13)	0.57 (0.06-3.0) †
11-20 PY, RF neg	neg	13 (76)	4 (24)	1.2(0.2-4.5) ‡
>20 PY, RF neg	neg	26 (68)	12 (32)	1.7(0.6-4.6) #
Never smoker, RF weak pos	22 (17-31)	21 (33)	43 (67)	7.6(3.3-17.9) *
1-10 PY, RF weak pos	25 (19-29)	2 (12)	14 (88)	26.1(4.9-249.4) †
11-20 PY, RF weak pos	25 (21-31)	4 (15)	22 (85)	20.5(5.6-90.8) ‡
>20 PY, RF weak pos	27 (17-30)	11 (27)	30 (73)	10.2(3.8-27.6) #
Never smoker, RF strong pos	115 (71-227)	12 (12)	88 (88)	27.4(12.6-59.3) *
1-10 PY, RF strong pos	139 (76-277)	5 (13)	34 (87)	25.4(7.8-94.1) †
11-20 PY, RF strong pos	153 (67-495)	4 (8)	46 (92)	42.9(12.3-182.0) ‡
>20 PY, RF strong pos	166 (92-283)	5 (4)	116 (96)	86.6(27.9-307.6) #

Cochrane- Armitage test for OR trend: * $P < 0.0001$, † $P < 0.0001$, ‡ $P < 0.0001$, # $P < 0.0001$.

Seventy one percent (425/598) of patients were ACPA positive, with 393/425 (93%) demonstrating strong positivity as per ACR/EULAR criteria [4]. There was no significant difference in ACPA positivity between males and females (217/298 (73%) vs. 208/300 (70%), p=0.32). There was a significant trend of increasing ACPA positivity rates with increasing levels of RF without significant differences between patients who had never or ever smoked (never smoked: 15/71 (21%) RF-ve, vs. 43/64 (67%) RF weak +ve, vs 88/100 (88%) RF strong +ve. Ever smoked: 18/70 (26%) RF-ve vs. 66/83 (80%) RF weak +ve vs.196/210 (93%) RF strong +ve) (Table 1). There was no relationship between ACPA positivity and smoking in patients who were RF negative (OR (95% CI), 1.3 (0.4 – 5.1)).

Sixty one percent (363/598) of patients had ever smoked, and males were more likely to have ever smoked than females: 214/298 (72%) vs. 149/300 (50%), OR (95% CI) 2.6 (1.8 – 3.7), $p < 0.0001$. Smoking >20 pack years was observed in 200/598 (33%), and was significantly different between males and females: 130/298 (44%) vs. 70/300 (23%), OR (95% CI) 2.5 (1.8 – 3.7), $p < 0.0001$. Patients with >20 pack years demonstrated significantly increased risk of ACPA positive RA compared to patients who had never smoked (158/200 (79%) vs. 146/235 (62%), OR (95% CI) 2.3(1.5-3.6), $p < 0.0001$).

There was a significant difference in the proportion of strongly RF positive patients between those who had never smoked (100/235, 43%) and those who had smoked >20 pack years 121/200 (61%), (OR (95% CI), 2.1 (1.4 – 3.1), p=0.0002.

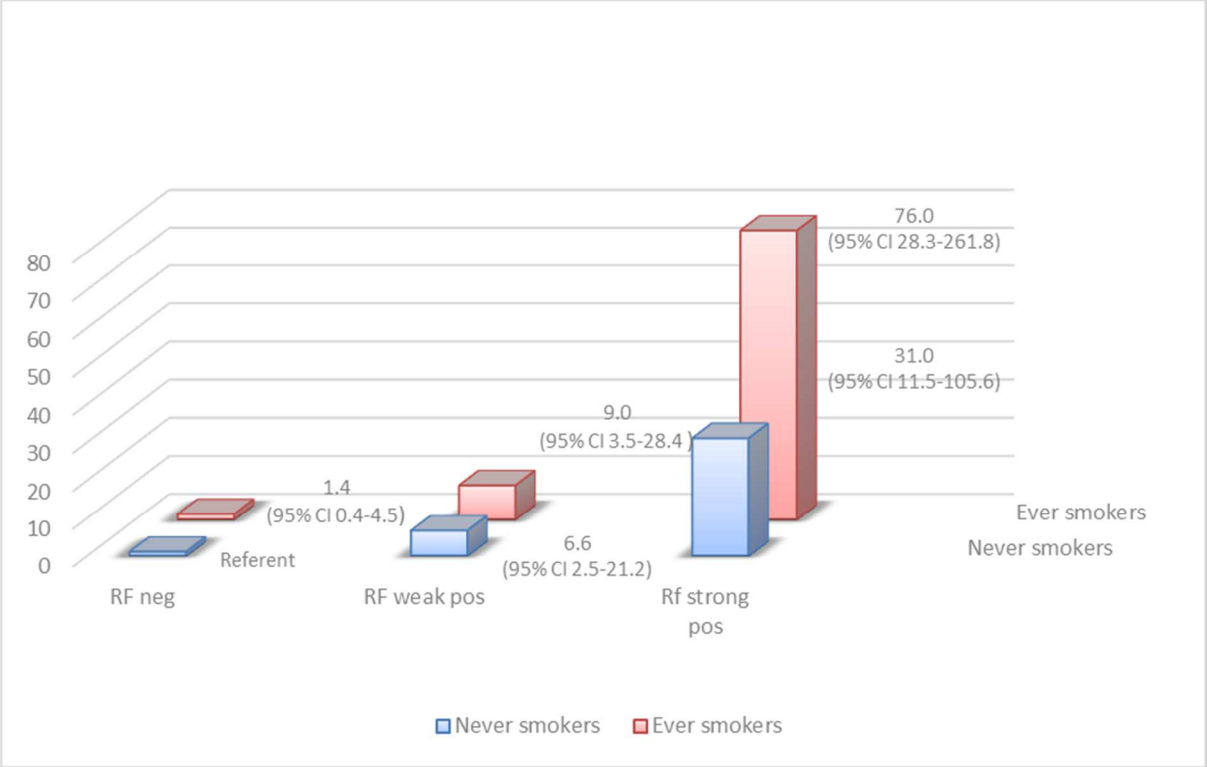
No significant differences were seen in ACPA positivity amongst weakly positive RF patients who had never smoked (43/64) and weakly positive RF ever smokers (66/83), irrespective of smoking status or pack years smoked (OR (95% CI), 1.9 (0.8 –4.3)).

Strongly RF seropositive never smokers demonstrated ACPA seropositivity predominance (88/100, 88%), as did strongly RF seropositive smokers of >20 pack years (116/121, 96%, Table 4). However, this numerically higher rate of ACPA positivity in strongly RF seropositive >20 pack year smokers is likely to be explained by significantly higher median RF titres of 166 (IQR 92-283), compared to a median RF in never smokers with a strongly positive RF of 115 (IQR 71-226), p=0.008.

Given the above, and the dominant ACPA positive prevalence (88%) in RF strongly positive never smokers compared to just 32% of RF negative smokers of >20 pack years, odds ratio (OR) risk increases were calculated for both ever smokers (as a combined cohort) and never smokers, with RF negative, weakly positive and strongly positive titres (RF negative never smokers as referent). Amongst ever smokers (n=365), a stepwise increase in OR is seen, rising from 1.3 (95% CI 0.6-3.1) for RF negative, to 14.5 (95% CI 6.2-34.2) for RF weakly positive, to 52.3 (95% CI 22.4-124.2) for RF strongly positive cases. Whilst not

as dramatic, the OR rise for never smokers (n=235) shows a similar trend; OR 7.6 (95% CI 3.3-17.9) for RF weakly positive, rising to 27.4 (95% CI 11.2-68.7) for strongly positive cases (Figure 9).

Figure 9. Odds ratio risk of ACPA positivity in RA



From the strength of relationship seen for OR increases with RF titre irrespective of smoking, a further cohort (cohort 2, North Staffordshire) was analysed to determine whether ACPA was determined by increasing titres of RF with reference to SE status.

3.4.2 Cohort 2

Nearly 40% (162/409) of patients were RF negative in a second cohort of patients in which the SE status had been determined. Seventy five percent (307/409) of patients were ACPA positive (Table 5). There was no significant difference between males and females in the proportion of RF positive or ACPA positive patients. Sixty one percent (235/386) of patients had ever smoked, with a significant difference between males and females (males, 123/150 (82%) vs females, 120/236 (51%), OR 4.3 (95% CI 2.6-7.4), $p < 0.0001$). As in cohort 1,

there was no relationship between ACPA positivity and smoking in RF negative patients (37/74 (50%) never smoked, v 45/73 (61.6%) ever smoked; OR 1.6 (95% CI 0.8–3.3).

Table 5. Cohort 2 relationship between RF levels, ACPA and shared epitope status (n=409).

	SE=0			SE=1			SE=2		
	ACPA neg	ACPA pos	**OR (95% CI)**	ACPA neg	ACPA pos	**OR (95% CI)**	ACPA neg	ACPA pos	**OR (95% CI)**
RF	n (%)	n (%)		n (%)	n (%)		n (%)	n (%)	
Negative	23 (59.0)	16 (41.0)	**Referent**	29 (43.3)	38 (56.7)	**2.3(1.1-5.6)**	15 (26.8)	41 (73.2)	**4.8(2.0-12.8)**
Weak +ve	7(30.4)	16(69.6)	**3.1(1.0-11.4)**	7(11.9)	52(81.1)	**10.0(3.5-33.8)**	5(10.9)	41(89.1)	**10.7(3.4-43.1)**
Strong +ve	6(37.5)	10(62.5)	**2.3(0.6-9.4)**	8(13.1)	53(86.9)	**9.0(3.3-28.8)**	2(4.8)	40(95.2)	**23.1(5.5-200)**

We found that the proportion of ACPA positive patients increased with RF positivity in patients carrying 1 or 2 SE alleles but there was only a weak or no relationship in patients lacking a SE allele (Table 5). In RF positive patients the number of copies of the SE (either 1 or 2) was not significant in determining ACPA positivity. However, in RF negative patients, the rate of ACPA positivity appeared to have a greater dependence on number of SE copies, although it did not achieve significance (57% vs 73%, p=0.06).

Multivariate logistic regression analysis confirmed that RF positivity was strongly associated with anti-CCP positivity (Table 6). Ever having smoked was only weakly associated with ACPA positivity, (Model 1) whereby RF positivity and the presence of the SE were significantly associated. Similar associations were seen in models in which having smoked for >20 years (Model 2) or accumulating >20 pack years (Model 3) were compared with never having smoked, though the association with ACPA was not significant in Model 3.

Table 6. Cohort 2 multivariate logistic regression analysis of variables associated with anti-CCP positivity in RA (n=386).

	Model 1			Model 2			Model 3	
	OR (95% CI)	P		OR (95% CI)	P		OR (95% CI)	P
Age	1.00 (0.98 -1.02)	NS	**Age**	1.00 (0.98 -1.02)	NS	**Age**	1.00 (0.98 – 1.02)	NS
Male sex	1.32 (0.74 – 2.34)	NS	**Male sex**	1.72 (0.80 – 3.68)	NS	**Male sex**	2.32 (1.05 – 5.12)	0.04
Duration (per yr)	1.03 (0.98 – 1.07)	NS	**Duration (per yr)**	1.03 (0.98 – 1.07)	NS	**Duration (per yr)**	1.03 (0.98 – 1.07)	NS
Shared epitope	3.00 (1.67 – 5.37)	0.0002	**Shared epitope**	2.26 (1.06 – 4.79)	0.03	**Shared epitope**	2.45 (1.16 -5.17)	0.02
RF	4.23 (2.54 – 7.06)	<0.0001	**RF**	6.09 (3.21 – 11.54)	<0.0001	**RF**	4.41 (2.35 – 8.25)	<0.0001
Ever smoked	1.73 (1.01 – 2.94)	0.04	**Years smoked >20**	2.31 (1.15 – 4.68)	0.02	**Pack years > 20**	1.83 (0.90 – 3.69)	0.09

3.5 Discussion

We have noted several interesting observations on the determination of ACPA positive RA. Smoking was associated with ACPA positivity only in RF positive patients, and especially in patients who were strongly RF positive. Furthermore, there was a strong relationship between increasing RF levels and ACPA positivity in RA, irrespective of smoking (Table 2). Underpinning this relationship is carriage of the SE. In RA patients who did not carry the SE, we found only weak, or no relationship between RF levels and the frequency of ACPA positivity. This suggests that the co-existence of 2 RA-associated autoantibodies cannot be explained by a propensity for antibody production *per se* as the frequency of ACPA positivity in patients without the SE who were RF negative was not significantly different to those who were RF strongly positive.

There appeared to be a difference in the importance of the number of SE alleles between RF negative and RF positive patients. In those patients who were weakly or strongly RF positive, the number of copies of the SE (either 1 or 2) was not significant in determining ACPA positivity. However, in RF negative patients, the rate of ACPA positivity appeared to have a greater dependence on number of SE copies. If larger studies confirm a significant difference this may pave the way for investigating distinct SE-associated mechanisms giving rise to a positive ACPA in patients positive or negative for RF.

The modest relationship between smoking and ACPA positivity appeared to be due to an increased propensity for smokers to be RF positive. In logistic regression analysis, one model suggested that smoking may be an independent risk factor for ACPA positivity in RA. However, this relationship was far less apparent than the dominant relationship between RF positivity and ACPA positivity. Smoking appeared to demonstrate a significant relationship between the development of both RF and ACPA in the patients studied. There was a clear relationship between smoking in terms of pack years and RF, and ACPA positivity in RA. There did not appear to be a relationship between smoking in terms of pack years smoked and positivity for ACPA alone. Significantly fewer patients with >20 pack years and weakly positive RF were ACPA positive compared to never smokers with positive RF of any titre, further strengthening the argument that the principal determinants of ACPA positivity are RF and SE carriage rather

than smoking per se. However, these sub-groups were relatively small and larger studies are required to investigate these sub-groups further.

We excluded 58/658 patients who had ceased smoking >20 years prior to RA onset. Previous studies have demonstrated no increased risk of RA in individuals who have stopped smoking before this time point [11,12]. This cohort demonstrated similar seropositivity trends to RA patients who had never smoked (data not shown). We excluded these patients so that the calculated association between smoking and ACPA positivity was not under represented.

It is noteworthy that the prevalence of RF negative RA was low in cohort 1 (24%). Cornwall has been noted as the poorest region of England [13] and the low prevalence of RF negative RA may reflect a high proportion of socially deprived individuals in this cohort; the majority of the patients were in the most deprived 40% of UK society as 180/300 (60%) females and 173/298 (58%) males had an IMD decile score of 4 or lower. RF positivity associates with social deprivation in other European RA populations [14,15].

This study does have limitations due to its cross-sectional nature. Different classification criteria were used for each cohort. However, as both cohorts are of patients with established RA (median disease duration for cohort 1= 7yrs, cohort 2= 9yrs), we do not feel that utilisation of the 1987 RA criteria for cohort 2 and the 2010 criteria for cohort 1 of importance as it is well established that both classification criteria identify patients with established RA. The 2010 classification criteria identifies patients with RA at an earlier time point than the 1987 criteria, however at 5 years of disease duration the number of patients identified by either criteria are the same [16].

Patient notes were analysed to ascertain RF levels at the time of diagnosis in cohort 1. There is the potential for RF titres to vary with disease duration and treatment. A study of 640 inflammatory polyarthritis patients from the Norfolk Arthritis Register demonstrated that over a five-year period, RF status changed in 17% of patients [17]. Critically, this change in RF status was strongly linked to baseline anti-CCP status in this study. The status of RF was 8 times more likely to change in patients with a discordant baseline autoantibody status (RF+/ACPA-

or RF-/ACPA+) than in those who were either double negative or double positive (OR 8.2; 95% CI, 4.9 to 13.7; p<0.00001). Thus, RF-negative individuals who seroconverted to RF positive were more likely to be ACPA positive at baseline than were those who remained RF negative (OR, 7.6; 95% CI, 3.8 to 15.3); similarly, those who converted from RF positive to RF negative were more likely to be ACPA negative at baseline than were those who remained RF positive (OR, 7.4; 95% CI, 3.5 to 15.6). Ergo, we feel our data has the potential to have underestimated the relationship between RF and ACPA as the RF test was only undertaken once at the time of diagnosis as per usual clinical practice. ACPA positivity in the same study remained remarkably constant over 5 years of follow up, with only 2% of ACPA negative patients seroconverting to positive, and 4.6% ACPA positive individuals becoming negative between baseline and 5 years. We therefore believe that the cross-sectional nature of ACPA testing would not significantly influence the findings reported here. The authors suggested that repeated testing of ACPA or RF in inflammatory arthritis patients should not be undertaken in routine clinical practice, which also reflects our clinical practice.
Established literature suggests that therapies that target the adaptive immune response, such as rituximab and abatacept, can significantly reduce anti-CCP2 IgG levels [18]. No such reductions in anti-CCP2 IgG levels have been noted in patients treated with methotrexate, tumour necrosis factor inhibitors or tocilizumab. In cohort 1, 18/598 (3%) had an ACPA of below 140 U/ml, with a theoretical possibility of changing from a strongly positive to a weakly positive or negative ACPA through treatment with rituximab or abatacept. However, only 5/598 (0.8%) patients had an ACPA test after treatment with such agents. Whilst we acknowledge that treatment in a cross-sectional cohort may lead to possible misclassification bias due to the mechanism of action of specific disease modifying therapies, the numbers affected in this study are insignificant.

We feel these results can be reconciled with a landmark Swedish study identifying smoking and carriage of the SE alleles as risk factors for ACPA positive RA [1]. In this study carriage of the SE was significantly associated with ACPA positive/ RF negative RA rather than RF positive/ ACPA negative RA. Consequently, the authors suggested that the primary process in RA involved anticitrulline immunity influenced by an interaction between smoking and carriage of the SE. However, in this study only a very small proportion of the ACPA positive RA cases were RF

negative (13%) and the clear majority (87%) were double positive for both RA autoantibodies (far more than would be expected by chance alone). Ergo, given the overall strong association between the carriage of SE alleles and ACPA positivity it follows that the SE is principally associated with ACPA positive / RF positive RA. Likewise, the observation that smoking interacts with the SE to generate ACPA positivity overall in RA is factually correct; more precisely this interaction is between smoking, the SE and both RA autoantibodies. This has recently been proven to be the case as pooled analysis of 2234 RA patients has suggested that smoking interacts with the SE to generate multiple RA associated antibodies rather than ACPA alone [6].

We have suggested that the citrullinated heavy chain of immunoglobulin G (IgG) is the dominant antigen in RA and generated by B cells in the lungs of smokers; a process facilitated by SE alleles with the B cell acting as both an antigen presenting cell and the source of the antigen [19]. This process need not be exclusive to the lung as the rheumatoid joint could be the host for B cell activation and therefore could theoretically occur in both smokers and never smokers.

Citrullination of the Fc region of IgG may generate an antibody response specific to RA, consequently generating both a strongly positive RF and ACPA response. This hypothesis is supported by the recent finding of citrullinated IgG in the synovial tissues of RA patients, with IgG as a target of peptidylarginine deiminase (PAD) 2 and PAD4 activity in RA synovial biopsy tissues [20]. Another recent study observed over 80 different citrullinated peptides in the RA synovial fluid including the heavy chain of IgG. [21]. Furthermore, the demonstration of bispecific antibodies against cyclic citrullinated peptides and IgG in RA suggests that citrullinated IgG may generate both a RF and ACPA response [22].

We do not consider that ACPA positivity is likely to have given rise to a subsequent RF response in the RA patients studied here as a study of the RF isotypes (IgM, IgG and IgA) in individuals who subsequently developed RA were more likely to be present before the development of various ACPAs (fibrinogen, α-enolase, triple helical collagen type II, filaggrin and vimentin). Previous data suggests the frequency of ever being positive for IgM-RF in individuals who developed RA, but were negative for the above ACPA was 16–31% [23].

Recent analysis of specific immunoglobulin subtypes to ACPA, specifically IgG types 1-4 to anti-CCP, has been demonstrated [24]. It has been recently suggested that IgG1 and IgG4 anti-CCP antibodies associate with the shared epitope, and IgG2 associates with smoking. However, low patient numbers in analysis of individual subtype classes limit the applicability of this data. Furthermore, other evidence suggests that the shared epitope has been associated with elevated IgG3 and IgG2 ACPA titres. Selective support of the Th1 response among RA patients homozygous for HLA-DRB1*04 has been suggested in IgG3 subtypes [25].

Our study does not aim to address specific IgG subclasses [24] and IgA responses [26] to ACPA. This would be worthy of further study taking into account RF levels at diagnosis, and the smoking history of patients.

3.6 Conclusion

These results raise important questions about the relationship between RF and ACPA in RA and the potential mechanisms underlying this relationship. We feel that processes involving the simultaneous or sequential generation of both RF and ACPA need to be considered in further studies investigating RA pathogenesis irrespective of smoking.

3.7 Chapter 3 References

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Chapter 4. Lysine carbamylation and arginine citrullination at the Fc region of immunoglobulin G potentiates a risk factor for rheumatoid arthritis development.

Citation: Hutchinson D, Clarke A, Heesom K, Murphy D, Eggleton P. Carbamylation/citrullination of IgGFc in bronchiectasis, established RA with bronchiectasis and RA smokers: a potential risk factor for disease. ERJ Open Res 2017;3:0018-2017.

4.1 Abstract

Objective: To investigate the mechanisms by which bronchiectasis (BR) triggers rheumatoid arthritis (RA). BR strongly associates with concurrent rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibody (anti-CCP) positivity and RA development. Anti-carbamylated protein antibodies (anti-CarP) have also been observed in BR patients. Given that immunoglobulin G (IgG) is the sole RF antigen, we hypothesize that post-translational modifications to IgG explain clustering of RA associated autoantibodies in both RA and BR.

Methods: Protein analysis was undertaken on 22 individuals. Four individuals had a diagnosis of BR and subsequently developed RA up to 18 months following blood sampling. Four individuals had a diagnosis of RA and four individuals with both BR and RA. Ten were healthy controls. The distribution of lysine (Lys) carbamylation and arginine (Arg) citrullination within the heavy chain of IgG (IgGH) from individuals was undertaken by mass spectrometry and immunoblotting.

Results: This is the first report of citrullinated IgG. Citrullination and carbamylation of IgG appeared to be universal. However, citrullination of Arg and/or carbamylation of Lys in the RF binding site of IgG CH2 domain was observed in 5/12 (41.6%) patients (1 BR, 2 RA and 2 BRRA), but in no control subjects (0/10, 0 %).

Discussion: These results suggest that post-translationally modified IgGHFc is a potential universal antigen in RA. This may explain the clustering of RA

associated autoantibodies in RA and in individuals at risk of RA. Furthermore, post-translational IgGHFc modifications affect polarity, altering the binding to the Fc receptors of inflammatory cells in RA.

4.2 Introduction

The lung is now considered a site for RA development. Bronchiectasis (BR) is a particularly strong risk factor for RA. We prospectively followed 122 BR patients: four developed RA over an 18 month period [1]. The presence of both RF and anti-CCP in the sera of patients with BR was significantly more prevalent than in healthy controls and predicted the development of RA. Interestingly, a significant association between the two RA autoantibodies was observed. We noted that 4/31 (13%) RF positive BR patients were strongly anti-CCP positive, versus 0/91 (0%) RF negative BR patients [1]. These results demonstrate a significant clustering of RF and anti-CCP in those BR patients who later develop RA.

BR provides a non-smoking model of RA development given the absence of smoking in the majority of the patients studied (. A pathological characteristic shared between BR and RA is the presence of tertiary lymphoid structures. Bronchial associated lymphoid tissue (BALT) was first described in BR [2] and identical lymphoid tissue is also present in the synovium of RA patients [3]. Smoking, another important risk factor for RA development, associates with BALT development and the presence of BALT is associated with both RF and anti-CCP in established RA with pulmonary disease [5].

A study of early RA (<1 year) observed lymphocyte infiltration more frequently in anti-citrullinated peptide antibody (ACPA) positive patients (9/18, 50%), compared to ACPA-negative patients (1/6, 17%), on mucosal lung biopsy [5]. Furthermore, germinal centres, B cells and plasma cells were exclusive to ACPA-positive patients. This suggests that BALT development potentially initiates RA and is not necessarily a secondary consequence of disease activity in established RA. Recently, anti-CarP were described as a third autoantibody system in RA. Again, these RA specific autoantibodies are more frequent in BR (3/80, 3.8%) compared to healthy controls (0/36, 0%) [6].

Given this evidence, we have recently suggested that the potential antigenicity of

the heavy chain of immunoglobulin G (IgGH) is triggered by B cell activation in the lung. This process involves post-translational modification to IgGH orchestrating a local (lung) and distant (joint) immune response that involves the production of RF, ACPA and anti-CarP, triggering RA [7]. Accordingly, we have studied the sera of 22 individuals: four BR patients prior to RA development, eight RA patients (four with BR and four without), and 10 healthy controls, analysing individual IgGH for sites of potential citrullination and carbamylation.

4.3 Methods

4.3.1 Patient population

Sera from patients and controls were selected from the BRACRA (Bronchiectasis, Asthma, Control, Rheumatoid Arthritis) study: a prospective, multicentre, case–control, observational study, conducted to determine the relationship between bronchiectasis and RA development. The overall study design, including assays used for the determination of RF and anti-CCP levels and approval has been previously reported [1]. All the RA patients fulfilled the ACR 2010 classification criteria for RA and the definition of a negative, low positive and high positive RF and anti-CCP is as per the above classification criteria [8].

4.3.2 Study design

Serum samples from four BR seroconverts (anti-CCP positive prior to developing RA), four RA smokers without overt lung disease, four BRRA never smokers and ten control subjects (five never smokers and five current/ever smokers) were studied.

Equal serum protein loads as determined by nanodrop spectrometry were separated on 8-16% SDS-PAGE gradient gels. A protein band identified by immunoblotting with anti-human citrulline antibody, contained numerous citrullinated proteins in the region of 37-50 kilodaltons (KDa). The bands were excised from Bio-safe™ Coomasie stained gradient gels and subjected to in-gel tryptic digestion using a DigestPro automated digestion unit (Intavis Ltd.). The resulting peptides were fractionated using an Ultimate 3000 nanoHPLC system.

Tandem mass spectra were acquired using an LTQ- Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against the UniProt Human database (131351 entries) using the SEQUEST algorithm. Search criteria routinely included carbamidomethylation of cysteine (+57.0214) as a fixed modification and oxidation of methionine as a variable modification. In addition, citrullination (+0.984Da) at Arg, Asn and Gln and carbamylation (+43.006Da) at Lys, Met, Arg, Ser, Thr and Tyr, were included as variable modifications in two separate searches. Only peptides where citrullination at Arg and carbamylation at Lys were ranked 1 in the respective searches (indicating that those residues were the most likely sites of modification) were considered. This identified IgG heavy chain as one of the most abundant proteins identified as being citrullinated and carbamylated. The amino acid sequence of IgG was obtained from the Uniprot database and then submitted to a phyre2 search (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) to obtain models in 3D. The UCSF Chimera protein viewing software (http://www.rbvi.ucsf.edu/chimera/) was used to annotate the amino acid modifications of interest to highlight the citrullinated and carbamylated sites on each IgG molecule.

To confirm the presence of citrullination and carbamylation in the light and heavy chains of IgG from our test subjects, IgG was purified from individual sera by protein G affinity chromotography using an AKTA FLPC system (GE healthcare) employing unicorn 5.1 software. Protein aliquots of IgG were run on SDS-PAGE under reducing conditions to separate the 25 kDa light (IgGL) and 50 kDa heavy chain (IgGH), then immunoblotted and probed with primary anti-citrulline (ab100932) or anti-carbamyl-lysine (ab175132) specific antibodies. Post-translational modifications were detected by probing the blot with 1:15000 dilutions of IR Dye800-labeled anti-rabbit secondary antibody (Li-Cor P/N 925-32213) followed by infrared imaging (Li-Cor Odyssey infra imaging system).

4.4 Results

Identification of citrullination and carbamylation residues of IgG in the blood of

BR, RA and BRRA patients and controls. We examined the degree of carbamylated Lys (hcit) and citrullinated Arg (cit) residues in the proteins recovered from the blood of our patient and control subject cohorts (Table 7).

Table 7. Demographics of individual subjects and identification of individual post-translational modifications in the RF binding site in the CH_2 domain of the Fc region of their serum IgG

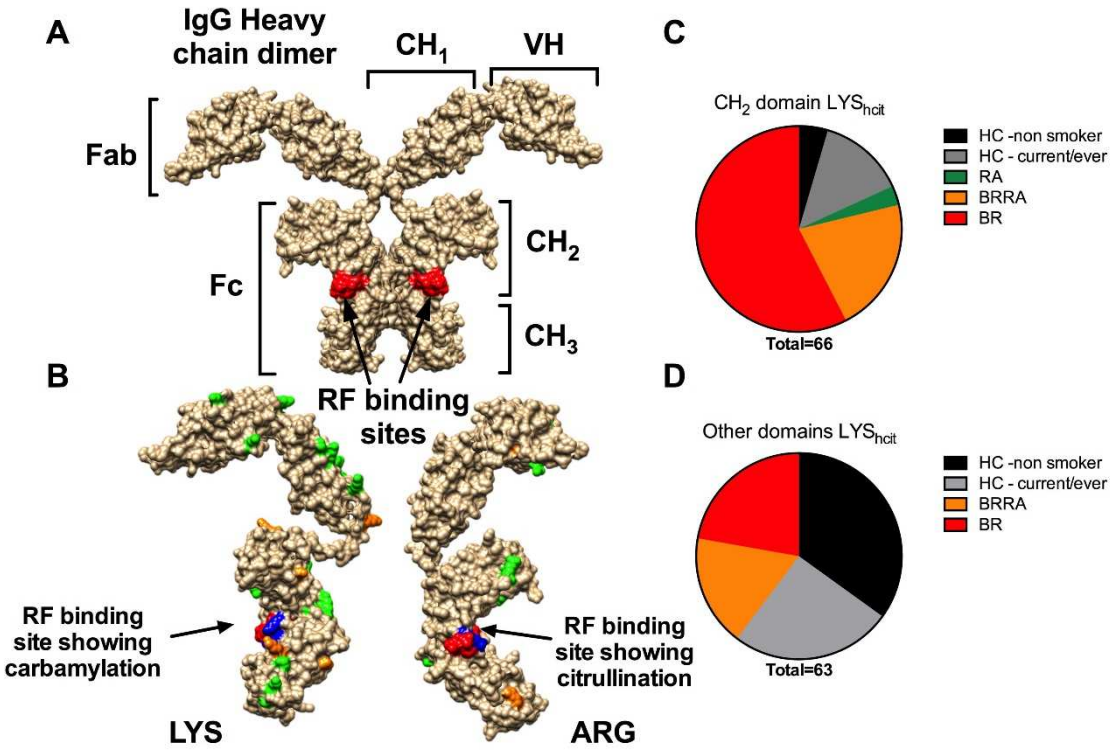
ID	Cohort	Age	Gender	Smoking	Anti-CCP Actual Result	ACR Anti-CCP Interpretation	RF Actual result (IU/ml)	ACR RF Interpretation
1*	BR	72	F	Never	522	High +ve	56.2¶/§	High +ve
2*	BR	67	F	Ex	43	High +ve	16	Low +ve
3*	BR	77	M	Never	340	High +ve	22	Low +ve
4*	BR	71	F	Ex	92	High +ve	130	High +ve
5	BRRA	75	F	Never	88	High +ve	27.5§	Low +ve
6	BRRA	81	F	Never	600	High +ve	130	High +ve
7	BRRA	60	F	Never	229	High +ve	130¶	High +ve
8	BRRA	66	F	Never	340	High +ve	130	High +ve
9	RA	52	F	Current	197	High +ve	74.6§	High +ve
10	RA	38	F	Current	6.9	Negative	78.1¶	High +ve
11	RA	44	F	Current	340	High +ve	130	High +ve
12	RA	62	F	Current	1.5	Negative	8.4	Negative
13	Control	53	F	Never	1	Negative	<7.0	Negative
14	Control	80	F	Never	1	Negative	<7.0	Negative
15	Control	68	F	Never	1	Negative	<7.0	Negative
16	Control	40	F	Never	1	Negative	<7.0	Negative
17	Control	80	F	Never	1	Negative	<7.0	Negative
18	Control	37	F	Current	1	Negative	7.8	Negative
19	Control	54	F	Current	1	Negative	<7.0	Negative
20	Control	52	F	Current	1	Negative	<7.0	Negative
21	Control	81	M	Ex	1	Negative	<7.0	Negative
22	Control	56	F	Current	1	Negative	<7.0	Negative

*Bronchiectasis patients who went on to develop RA 12-18 months - post sampling.

Key to post translational modifications observed:- §hcit of Lys at RF binding site; ¶cit of Arg at RF binding site

We focussed on the distribution of hcit and cit modifications relevant to IgG, especially the CH2 domain that contains the RF, C1q and FcR binding peptides (Fig. 10A). Using mass spectrometry, we found 8/34 Lys carbamylated and 3/12 Arg citrullinated (Fig. 10B) in the IgGH region. We identified modified arginines most frequently in the variable region and CH3 domains of IgG in patients and control subjects alike, but only observed citrullinated Arg and/or carbamylated Lys modifications (Fig. 10B) in the RF binding site of IgG CH2 domain of 5/12 (41.6%) patients investigated (1 BR, 2 RA and 2 BRRA), but in no control subjects (0/10, 0%) see Table 7. Furthermore, the degree of carbamylation of the CH2 domain varied between cohorts with BR>BRRA>HC>RA (Fig. 10C). In contrast, the degree of carbamylation in other IgGH domains was very similar for all cohorts (Fig. 10D).

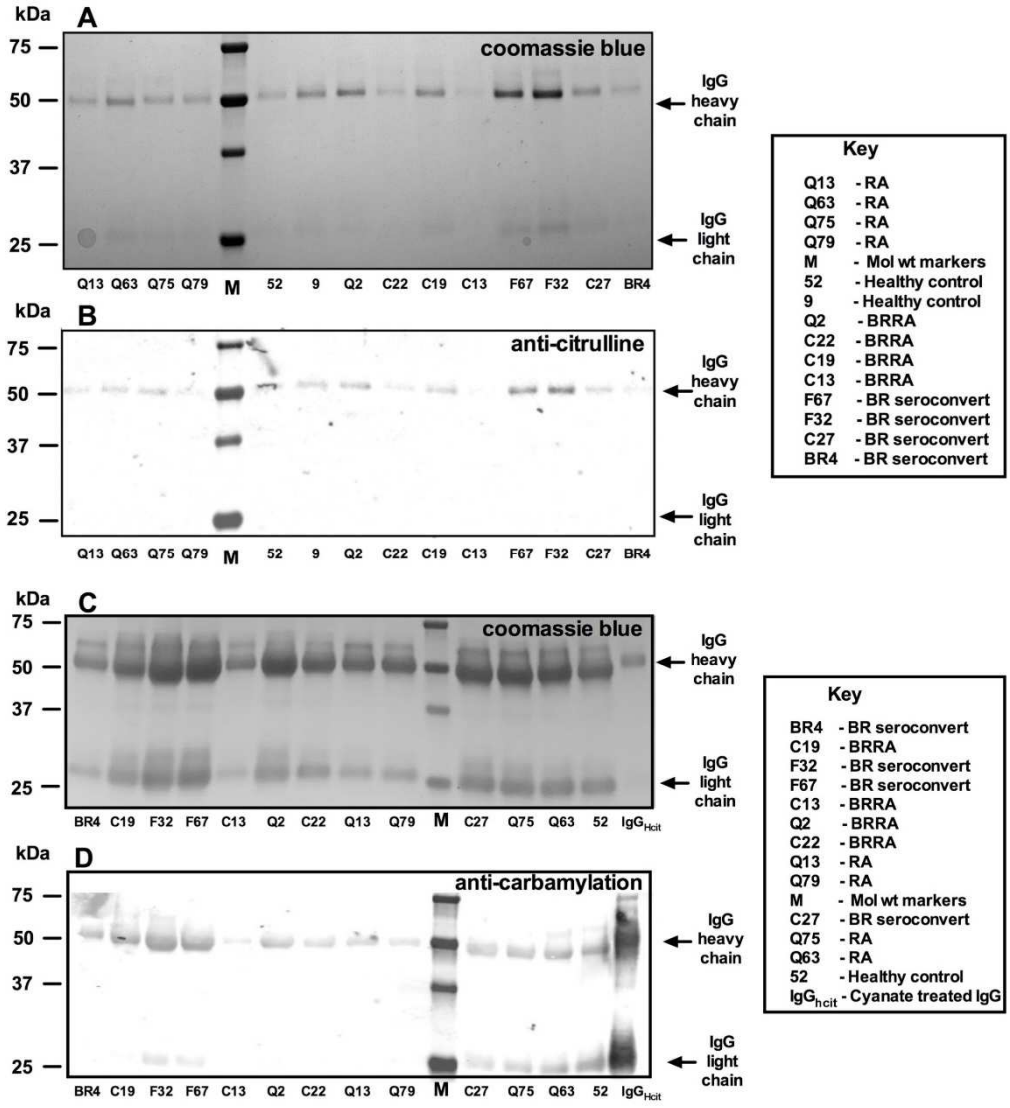
Figure 10. Lysine carbamylation and arginine citrullination in IgGH.



A. Schematic model of IgGH dimer showing RF binding sites (red).
B. IgGH fragment showing post-translational modification of Lys (blue – left model) or Arg (blue – right model), detected at the RF binding site (red-both models) in individual patients. Green- other unmodified Lys (left model) or Arg (right model). Orange- other modified Lys (left model) or Arg (right model).
C. Proportion of LYS_{hcit} peptides (n = 66) found in the CH_2 domains of individual cohorts of patients and control subjects.
D. Proportion of LYS_{hcit} peptides (n = 63) found in the IgG VR, CH_1 & CH_3 domains of individual cohorts of patients and control subjects.

The presence of citrullination and carbamylation IgGH and/or IgGL was tested by immunoblotting the sera with anti-citrulline and anti-carbamyl-lysine antibody (Fig. 11A and C). The presence of citrullination was confined only to the 50 kDa IgGH of all subjects (Fig. 11B). Similarly the IgGH in all subjects were carbamylated, but we also observed some carbamylation in some patient and control IgGL (Fig. 11D).

Figure 11. SDS-PAGE and immunoblots of purified IgG for citrullination and carbamylation.



A. Representative Coomassie blue stained isolated total IgG from patients and control sera used to blot for citrulline.
B. Immunoblot of IgGs probed with anti-citrulline.
C. Representative Coomassie blue stained isolated total IgG from patients and control sera used to blot for carbamylation.
D. Immunoblot of IgGs probed with anti-carbamyl-lysine.

4.5 Discussion

This study has demonstrated for the first time that citrullinated and carbamylated IgGH is present in the sera of RA patients and also a pre-RA BR patient. We suggest our findings extend the findings of carbamylated IgG in synovial fluid of RA patients [9]. Citrullinated IgG was observed in healthy subjects and RA patients. However, specific citrullination and carbamylation of the RF binding site in the IgG CH2 domain was only observed in patients with seropositive RA (4/8) with or without apparent lung disease and a patient with BR who subsequently developed seropositive RA (1/4). This would suggest that post-translational modifications of the RF binding site of the region of IgG are of importance in the development of RA. This study is limited by the small sample size and only represents a snap shot in time of IgGH in the sera of patients and healthy controls. However, an antibody response is a legacy of exposure to a particular antigen. The development of an antibody against the post-translationally modified Fc region of IgG may be of value as a specific diagnostic marker.

These findings are of importance as they suggest that IgG is a potential universal antigen in RA. The association between the Fc region of IgG and RF has long been recognised. However, evidence is emerging that a citrulline antibody response against IgG exists in RA as bispecific antibodies against cyclic citrullinated peptides and IgG have been observed in RA [11]. The specific post-translational changes noted at IgGHFc in this study could explain the clustering together of the three known RA autoantibodies in RA [12].

An animal study demonstrated that citrullinated filaggrin is highly arthritogenic, but only with co-exposure to carbamylated lysine residues [11]. This suggests a “double hit” of citrullinated and carbamylated antigen is required to trigger immune-mediated arthritis. We suggest that an antigen such as IgG that is both citrullinated and carbamylated could also elicit RA by the mechanisms described in the above study [11]. Supporting this, IgG complexes isolated from RA synovial fluid have triggered arthritis in an animal model [12].

The relative abundance of IgG in RA serum (mean 8.9mg/ml [13]) compared to other putative RA associated autoantigens such as calreticulin (median 10.3

ng/ml [14]), is likely to be of significance in breaching immune tolerance.

Post-translationally modified IgGH is not necessarily exclusive to the BR lung; it could potentially arise in BALT as a result of smoking, in other mucosal surfaces and in the joint. The importance of post-translational modification of IgGHFc is not only in the development of a neo-antigen, but in a fundamental change to the polarity of the Fc region and a potential enhanced interaction with the Fc receptor. Citrullination of arginine and carbamylation of lysine at the Fc region will change the electrical charge (from positive to neutral). This may alter binding and/or signalling with the FcγRIIB1 of B cells and FcγRIIIB receptors of follicular dendritic cells, stimulating development of autoimmune disease via tertiary lymphoid tissue development. We suggest that post-translationally modified IgGHFc enhances the ligand activity of IgGH by permitting binding to specific Fc receptors containing positively charged amino acids at critical sites of the Fc receptor [15]. Ergo, post-translationally modified IgGH has the potential to be an autoantigen and an enhanced ligand, which sets it apart from other citrullinated autoantigens.

4.6 Conclusion

Further, larger studies are needed to confirm if post-translational modifications to the Fc region of IgGH are present in the sera of individuals who have developed RA to determine if the presence of a specific antigen and ligand is unique to RA.

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Chapter 5. Vapour, gas, dust and fume occupational exposures in male rheumatoid arthritis patients resident in Cornwall (UK) and their association with rheumatoid factor and anti-cyclic protein antibodies: a retrospective observational study.

Citation: Murphy D, Bellis K, Hutchinson D. Vapour, gas, dust and fume occupational exposures in male rheumatoid arthritis patients resident in Cornwall (UK) and their association with rheumatoid factor and anti-cyclic protein antibodies: a retrospective clinical study. BMJ Open 2018;8:e021754

5.1 Abstract

Objectives: To quantify exposure to vapour, gas, dust and fumes (VGDF) and smoking in male rheumatoid arthritis (RA), and investigate impact on rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA) levels.

Design: A retrospective observational study.

Setting: The Royal Cornwall Hospital Trust, UK. A single university hospital setting.

Participants: 726 males followed up between February 2015 to August 2016, fulfilling RA diagnostic criteria.

Main outcome measures: Prevalence of VGDF exposure and smoking prior to RA diagnosis. Determination of association between VGDF, smoking and autoantibody levels.

Results: 546/726 (75%) had been exposed to VGDF for >1 year. 561/726 (77%) had been smokers. Only 58/726 (8%) had no exposure to VGDF and had never smoked. A significant difference in RF levels was observed between unexposed and VGDF exposed never smokers (median RF 24 vs. 36, p=0.03), more marked when comparing unexposed to ≥2 VGDF exposures (median RF 24 vs. 57, p=0.02). A significant difference in RF levels was also observed between unexposed and VGDF exposed smokers (median RF 71 vs. RF 95 p=0.04), more marked when comparing unexposed to ≥2 VGDF exposures (median RF 71 vs. RF 113 p=0.01). A significant difference in RF titre was observed between never smokers ≥2 VGDF exposures and smokers with ≥2 VGDF exposures (RF 57 vs RF 113, p=0.02). No association of ACPA seropositivity rates or titres with VGDF

exposure was observed. Smokers with ≥2 VGDF exposures had a significantly lower age of RA diagnosis than smokers with no VGDF exposure (53 years vs 57 years p=0.03). All results remained similar when corrected for social class.

Conclusions: VGDF exposure increases RF levels. Combination exposure to smoking and VDGF results in higher RF levels, particularly with multiple exposures. These compelling findings demonstrate the importance of combined inhaled exposures in RF generation.

5.1.1 Strengths and limitations of this study

- Methodology allowed follow up of an entire male RA cohort, with fixed diagnostic criteria applied and non-response minimised.
- Rheumatoid factor titres were reported rather than seropositive or seronegative status.
- Adjustment for social deprivation and educational level was made.
- Heterogeneity was noted between the cohorts studied with regard to education and social deprivation.
- As a single centre retrospective study, it is subject to a range of possible biases, with further study necessary to confirm the results in a population of wider demographic diversity.

5.2 Introduction

Rheumatoid arthritis (RA) is an inflammatory disease which primarily targets the small joints of the hands [1]. Individuals with RA develop debilitating fatigue**,** joint inflammation, resulting in weakened grip and the subsequent development of joint deformities impairing physical function and dexterity [2].

The estimated global prevalence is 0.3–1.0% [3]. A UK study of RA patients with disease duration of 10-15 years reported an annual cost of approximately £3000 per patient (2013 prices) for direct health care alone [4]. The overall direct health cost estimated to be £700 million in 2010 in the UK, with a further £8 billion lost to the UK economy when work productivity loss and state benefit requirements are considered [5].

Identifying the underlying causes for progressive RA has centred on a combination of environmental and genetic risk factors that stimulate inflammation

via autoantibody production. The RA associated autoantibodies rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) are both associated with progressive joint damage in RA [6], and with mortality [7]. Smoking is a risk factor for the presence of the combination of RF and ACPA in RA and the number of pack years smoked is proportional to the RF titre, but not ACPA titre in RA [8]. Given the multifactorial basis for disease development, it is surprising that the effect of combined environmental exposures on autoantibody development has not been more widely investigated.

Case-control studies have observed an association independent of smoking between occupational dust and fume inhalation and the risk of male RA. This relevant to RA as in the UK approximately 28% of RA patients are male (reviewed in [9]).

Recent studies investigating the relationship between occupational exposures and chronic obstructive airways disease (COPD) have utilised self-report of any occupational inhalational exposures together and considered these exposures as an entity that can trigger COPD. Reported exposure to vapours, gas dust and fumes (VGDF) are independently, but modestly associated with COPD development [10]. VGDF exposure greatly increases the risk of chronic obstructive pulmonary disease (COPD) particularly in smokers, with an OR 14.1 (9.33-21.2) compared to never smokers with no such work exposures [10]. The combined risk of VGDF and smoking was far higher than the OR of 6.71 (4.58-9.82) in those smokers without such exposures.

The lung is considered an initiating site for RA development [11] and the occupational inhalational insults associated with male RA development and COPD are concordant [9]. Considering grouped inhalational exposures as the potential risk for RA development (rather than “last occupation” only), has not been previously undertaken.

We aimed to study the occupational and smoking histories of all male RA patients attending the rheumatology department in Cornwall, UK, to test a hypothesis that combinations of inhalational insults would interact to increase autoantibody levels in RA.

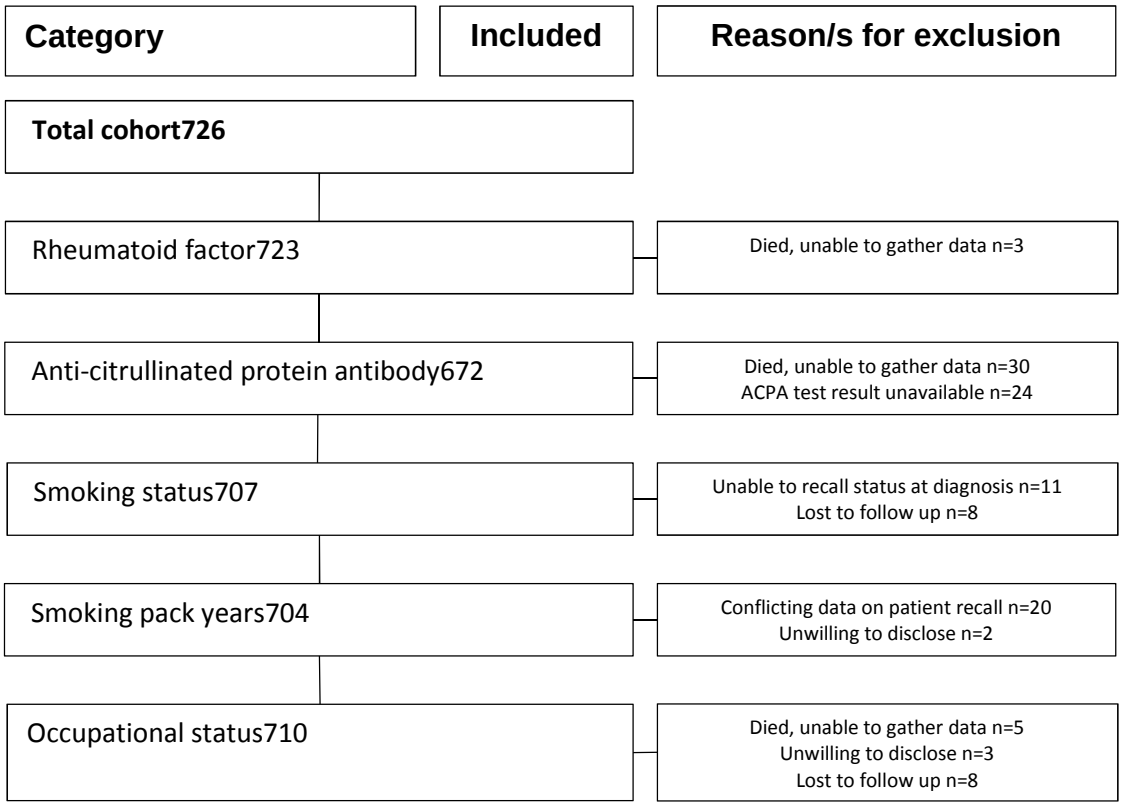
5.3 Materials and methods

5.3.1 Subjects

The data in this study was collected as part of project IRAS ID 194833, approved by South West Regional Ethical Committee (UK). The cohort consisted of males attending routine rheumatology clinics at the Royal Cornwall Hospital, Cornwall, UK from February 2015 to August 2016. Data was anonymised at source. Patients reviewed in clinic with a new or existing diagnosis of RA during the study period were included for analysis, initially invited to complete a written questionnaire. Data was recorded by clinicians in a standard questionnaire via face-to-face interview to determine current occupation, former occupation if retired, and previously held employments for >1 year. Answers to open questions on occupation and main duties for each response were recorded, to be coded using SOC 2010, The current standard occupational classification for the UK [12]. As in previous COPD literature [10], an item used in the European Community Respiratory Health Survey (ECRHS II) main questionnaire was utilised, with each case asked about self-reported exposures to vapours, gas, dust or fumes (VGDF) in the line of their work [13]. Missing data was obtained by follow up telephone administration of the same questionnaire. Written consent was obtained for patients enrolled as part of the IRAS ID 194833 project investigating the role of cadmium inhalation and the development of RA. Individuals were stratified for the number of self-reported VGDF exposures in each occupation held for >1 year (0, 1**,**≥2). Age, age of RA onset, disease duration, smoking history prior to disease development, RF levels at disease onset and subsequent ACPA levels were recorded as part of routine clinical practice. All patients fulfilled 2010 ACR/EULAR RA criteria at diagnosis [14].

726 patients were included for initial analysis. Of these, 22/726 (3%) had incomplete smoking data and were excluded from linear regression analysis. 3/726 (0.4%) had no verifiable RF data, and were excluded from RF analysis. 54/726 (7%) had no ACPA data and were excluded from ACPA analysis. 16/726 (2%) were excluded from occupational analysis; 5/16 died during the study period and therefore had incomplete occupational data, 3/16 did not want to disclose occupation in writing or on subsequent interview. 8/16 did not return initial questionnaires and were lost to follow up (Figure 14). Prevalence analysis of VGDF and smoking was analysed as a proportion of the entire identified cohort (n=726) to bias towards the null hypothesis.

Figure 12. Flow diagram of patients included for analysis with reasons for exclusion



5.3.2 Patient involvement

The initial concept of this research was developed from detailed histories of individual patient experiences of RA in Cornwall, UK. Initial hypotheses were presented at a local meeting of the National Rheumatoid Arthritis Society, attended by over 100 members, the majority of whom were RA patients. Subsequent interest following the meeting led to further discussion as to the development of a protocol that would be acceptable to patients in terms of its design and acceptability. From initial ideas, this was refined and presented to the committee of the Cornwall Arthritis Trust, a local charity supporting arthritis patients. Specific feedback was offered on wording of consent forms, questionnaire design and arrangements for follow up on patients who may not have been able to complete written questionnaires. Patient involvement in this process was invaluable for how best to manage and undertake the research in a way that was minimally intrusive for patients in their regular clinical care.

Dissemination of results to patients will take place via departmental displays post publication.

5.3.3 Autoantibody measurement

RF was measured with Tina-quant Rheumatoid Factors I1 Test System (Roche Diagnostics Corporation), with a value of < 14 IU/ml considered as negative as per manufacturer guidelines. ACPA was measured by Second Generation E170 Anti-CCP analysis (Roche Modular Analytics), with a negative value of <17 U/ml as per manufacturer guidelines.

5.3.4 Social deprivation analysis

Social deprivation analysis was undertaken through the UK government validated Index of Multiple Deprivation (IMD) [15], a deprivation rank score of 32,844 neighbourhoods weighted by income, health/disability, education, housing/service access, crime and living environment, converted to equal deciles.

5.3.5 Statistical analysis

Values are expressed as a median (interquartile range (IQR), or number (%). The Mann-Whitney U test was used for non-parametric comparisons of continuous

data of differing sample group sizes. All data were analysed using commercially available software (Microsoft Excel (Microsoft Corp) and Number Cruncher Statistical System for Windows (NCSS60)).

5.4 Results

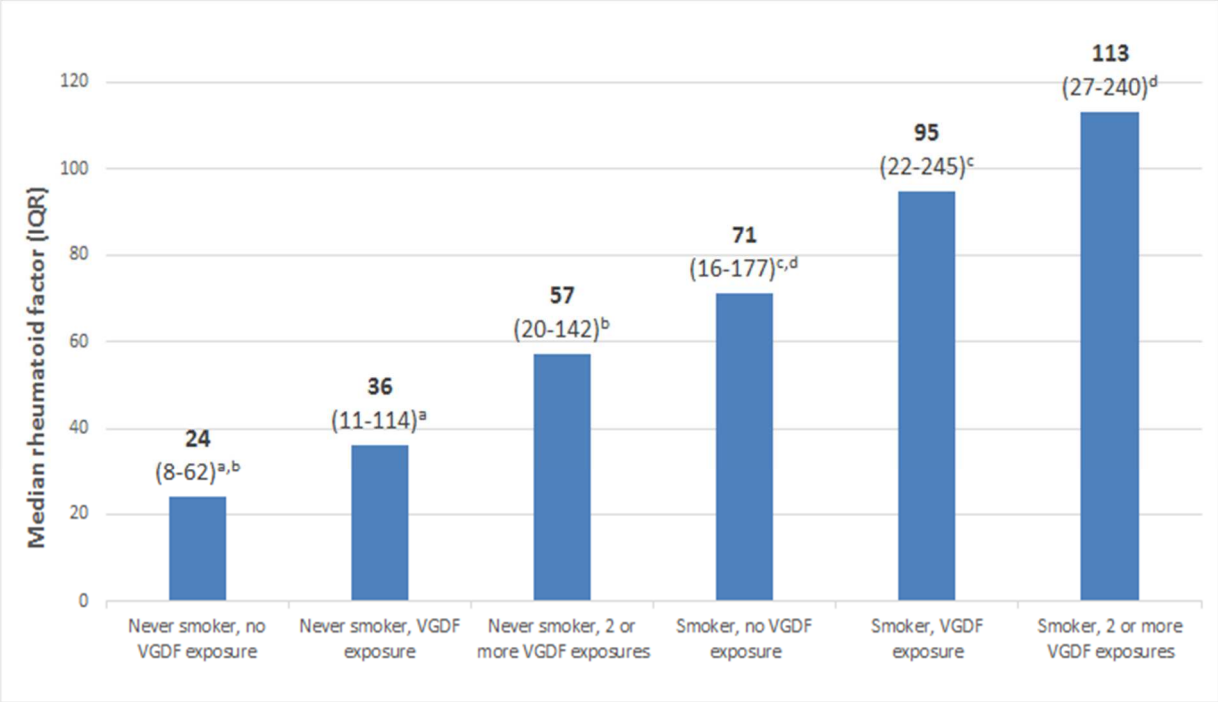
546/726 (75%) had been exposed to VGDF for >1 year, with 254/726 (35%) having 2 or more identified VGDF exposures. 561/726 (77%) had been smokers, with 349/726 (48%) having accumulated 20 pack years or more at the time of diagnosis. 58/726 (8%) had never smoked and had no exposure to VGDF (Table 8). No significant difference was seen in deprivation analysis (IMD) between VGDF and smoking exposed or unexposed cohorts, even when comparing never smokers with no VGDF exposure to smokers with ≥2 VGDF exposures (median IMD decile 4, IQR ranges 3-5 to 4-6, p=0.29).

Table 8. Patient characteristics for whole cohort n=726

Median age at diagnosis (IQR)	55 (45-64)
Median RF (IQR)	69 (17-201)
Median ACPA (IQR)	200 (1-428)
Median IMD (IQR)	4 (3-5)
Ever smoker	561/726 (77%)
≥20 pack years smoked	349/726 (48%)
VGDF exposure >1 year	546/726 (75%)
≥2 VGDF exposures >1 year	254/726 (35%)

545/710 (77%) had occupations with VGDF. In never smokers and ever smokers there was a significant dose response in terms of number of VGDF exposures and RF levels (Never smoker/ no VGDF, RF 24 vs never smoker/ VGDF, RF 36, p=0.03; Never smoker/ no VGDF, RF 24 vs never smoker/ ≥2 VGDF, RF 57, p=0.02, and smoker/ no VGDF, RF 71 vs smoker/ VGDF, RF 95 p=0.04; smoker/ no VGDF, RF 71 vs smoker/ ≥2 VGDF, RF 113, p=0.01, Figure 13).

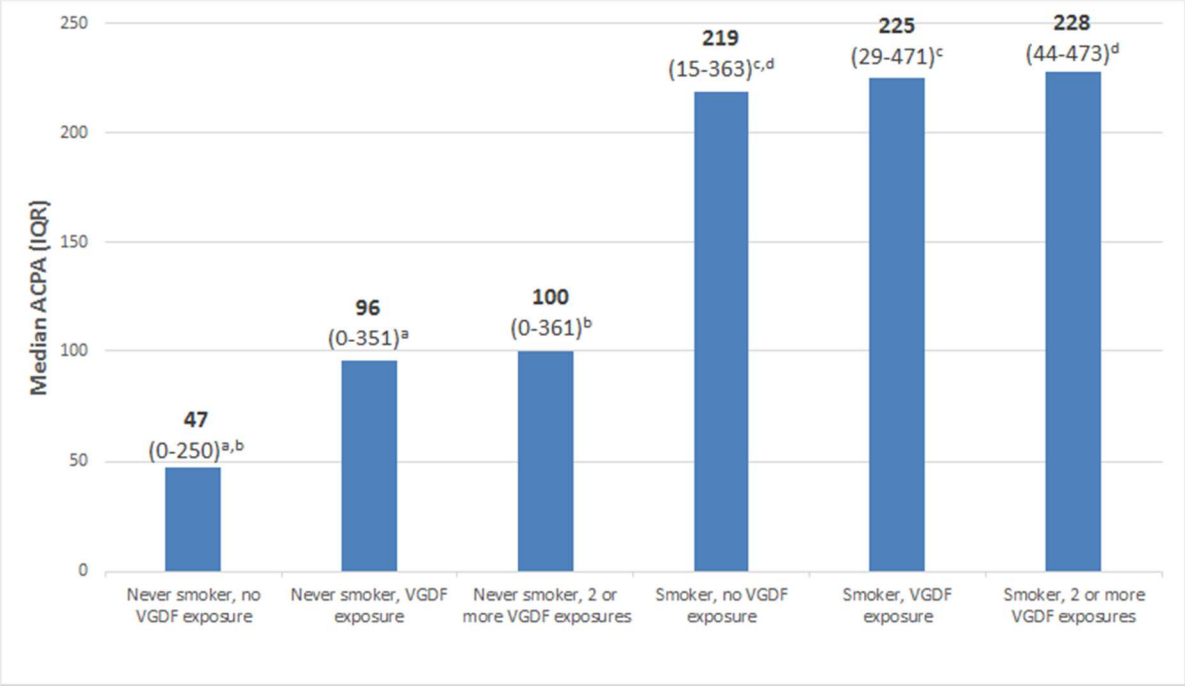
Figure 13. Median RF (IQR) by exposure group



^{a}p=0.03 ^{b}p=0.02 ^{c}p=0.04 ^{d}p=0.01

No titre trends or significant additive relationship was seen in ACPA titres with VGDF exposure (Figure 14), but smoking cohorts all had significantly higher ACPA titres than non-smoking counterparts with similar VGDF exposure (Table 9). No significant association was found in ACPA seropositivity rates amongst VGDF exposed or unexposed groups; ACPA for never smokers with 0, 1 and >2 VGDF exposures 50.9% v 52.0% v 58.7%, p (trend) = 0.44, nor in ever smokers 70.8% v 68.4% v 66.4%, p (trend) = 0.72.

Figure 14. Median ACPA (IQR) by exposure group



[a]p=0.34 (not sig) [b]p=0.33 (not sig) [c]p=0.41 (not sig) [d]p=0.22 (not sig)

Only 58/726 (8%) had never smoked and had no exposure to VGDFs, and had the lowest median RF titres (24, IQR 8-62). In never smokers with ≥2 VGDF exposures the median RF titre levels at RA diagnosis reflected ever smokers without VGDF exposure (57 vs 71). Smokers with a history of ≥2 VGDF exposures had the highest median RF titres (113 IQR 27-240), and had a significantly lower age of RA diagnosis than smokers with no VGDF exposure (53 years IQR 44-61 vs 57 years IQR 42-68 p=0.03, Table 9).

Table 9. IMD, age, RF and ACPA by smoking and VGDF exposure

	Median IMD (IQR)	Median age at diagnosis (IQR)	Median RF (IQR)	Median ACPA (IQR)
Never smoker, no VGDF exposure **n=58**	4 (3-5)	58 (42-68)	24 (8-62) [b,c]	47 (0-250)[f]
Never smoker, VGDF exposure **n=105**	4 (4-6)	54 (43-66)	36 (11-114)[b]	96 (0-351)[g]
Never smoker, 2 or more VGDF exposures **n=51**	4 (4-6)	54 (42-63)	57 (20-142)[c]	100 (0-361)[h]
Smoker, no VGDF exposure **n=120**	4 (3-5)	57 (42-68) [a]	71 (16-177)[d,e]	219 (15-363)[f]
Smoker, VGDF exposure **n=443**	4 (3-5)	54 (46-63)	95 (22-245) [d]	225 (29-471)[g]
Smoker, 2 or more VGDF exposures **n=203**	4 (3-5)	53 (44-61) [a]	113 (27-240)[e]	228 (44-473) [h]
[a] p=0.03, [b] p=0.03, [c] p=0.02 [d] p=0.04, [e] p=0.01, [f] p=0.02, [g] p<0.01, [h] p=0.03				

Further analysis by multivariate linear regression on the effect of pack years smoked and cumulative VGDF exposures on RF levels demonstrated significance (R^2 <0.03, p<0.0001).

In analysis of occupations listed, 33/726 (4%) had an occupation that required an educational level greater than standard UK secondary education with a school leaving age of 15 or 16 years. 10/33 (30%) were teachers or lecturers, 1/10 (10%) of which was a never smoker with no reported dust or fume exposure. 7/33 (21%) were engineering or surveying professionals with a range of reported VGDF

exposures. 7/726 (<1%) were professionally occupied never smokers with no dust or fume exposures.

By broad exposure types, construction dust (a mixed group of combinations of wood, cement/concrete, fine sand/rock, and gypsum, incorporating both silica, non-silica inorganic, and organic dusts) was the most common type of VGDF exposure, with 330/726 (45%) reporting such exposure. More prevalent exposures included diesel fumes (239/726, 33%), and oils including mineral oil exposure (207/726, 28%). Other exposures that were less prevalent but still existed in >10% of the total cohort included those reporting fume exposure as part of plastics manufacturing (98/726, 13%), metal fumes from electrical work (103/726, 14%), and specific dust exposure involved with rock mining (98/726, 13%), metals extraction (196/726, 27%), and wood related trades such as carpentry and boatbuilding (155/726, 21%,).

5.5 Discussion

5.5.1 Principal findings

We believe this is the first study to link occupations in combination with smoking to RA autoantibody development. Strikingly high numbers of cases in this study had been exposed to VGDF (75%) or had been smokers (77%). VGDF exposure combined with smoking leads to RA development at a significantly younger age. Whilst these findings are unique in an RA population, they reflect a trend of exposure risk as seen in COPD [10].

We have found compelling evidence that a combination of environmental exposures influences RF levels, more than observed in individual exposure. RF levels were highest in those with smoking and multiple VGDF exposures.

5.5.2 Strengths and weaknesses of this study

There is a relative lack of comparable RA cases that are unexposed to VGDF and smoking, giving limited ability to provide statistical significance via an adequate control group to analyse specific occupational exposures in which self-report of VGDF exposure was found. The relative homogeneity of educational levels and social depravation indices between groups in our study may explain why no significant differences were found here, though this may reflect how

inhalational exposures manifest in a male RA cohort. A wider unexposed RA population and a similarly matched, non- RA cohort would both be useful for further study.

Face to face interview with telephone follow up was designed to minimise non-response. At termination, only 8/726 (1%) were non-responders. Non-response, particularly in postal studies, is strongly correlated with low levels of formal education. Established literature links RA to low levels of formal education [16-18], with two UK studies observing a functional illiteracy rate of 15% [19,20]. Perhaps unsurprisingly, divergence in occupations related to literacy scores has been noted, with lower scores recorded in agriculture, construction, manufacturing and mining [21]. These occupations associate with male RA [9]. 95% of occupations held by individuals in this study required no formal academic qualifications beyond secondary education, therefore it was not possible to compare lower and higher levels of formal education for RA autoantibody prevalence. We suggest that further studies investigating the impact of socioeconomic status or formal education levels on male RA susceptibility, severity, comorbidity and mortality consider an individual's occupation as a confounding factor.

Previous database referent studies have chosen to include patients under the age of 70 [22]. Occupational disease can have a long latency period; asbestos exposure and mesothelioma development is associated with a latency of between 14 and 72 years (median 51 years), with the majority of cases presenting in the 7th and 8th decade of life [23]. Though we found a significantly earlier age of onset in our most severely co-exposed cohort, 104/726 (14%) cases studied were diagnosed over the age of 70. The risk of smoking and RA is evident up to 15 years after smoking cessation [24]. As polarisation of RA risk estimates occurs when a latency period is used in occupational exposures [25], using an age cut off of 70 does not allow an adequate latency period between exposure and disease development.

Much of the literature investigating occupational risk and RA has been undertaken in Sweden, in the form of case control/ case referent or linkage studies from large dataset analysis (reviewed in [9]). Studies investigating occupation and RA risk may only consider the last occupation undertaken by the

respondent [22], or occupation noted on the database studied. It is not uncommon for individuals with low literacy levels to move from one casual job to another, accruing different occupational exposures over time. The last recorded job may not reflect these exposures. In poorer populations, trade sub-specialisation and health and safety legislation adherence may be less evident. Throughout an occupational career, unskilled workers may have multiple exposures. Construction labourers, for example, may be co-exposed to silica dust, inorganic non-silica dusts, wood dust and diesel fumes.

We suggest that studies investigating individual occupational risk and RA via single job title analysis may be confounded by multiple inhalational insults, even when correcting for smoking and quantifying single job exposure risk through the use of job exposure matrices. In a well conducted analysis of silica *vs*. non-silica dust exposed construction workers, Blanc *et al*. [26] demonstrated that in ever smokers, both silica and other inorganic dust exposure were associated with increased risk of RA (RRs 1.36, 95% CI 1.11-1.68 and 1.42, 95% CI, 1.17-1.73, respectively), whilst in never smokers, neither exposure was associated significantly with an increased risk of RA. This large, longitudinal cohort registry study contained detailed information on occupations and exposure types. 40645/240983 cases were excluded due to co-exposure to wood dust, gas or fumes to reduce confounding. It would be interesting to study these types of multiple exposures to investigate enhanced RA risk.

5.5.3 Possible explanations and implications for clinicians and policymakers, unanswered questions and directions for future research

We suggest potential reasons why VGDF may associate with RF and not ACPA. Newkirk *et al* [27] described RF generation via upregulation of IgG-heat shock protein 70 complexes stimulating production of IgA and IgM RF. Both cigarette smoke [27] and harsh working environments including exposure to dust [28] induce IgG-heat shock protein 70 complexes, with potential synergistic action on the same pathway to induce an enhanced RF response. An ACPA response is clearly related to smoking, but the mechanism by which this occurs is unclear. We acknowledge that VGDF exposure appears to have less of an influence on this process.

Furthermore, there appears to be a discordant RA autoantibody response in COPD. This is important as COPD is related to VGDF exposure and smoking in combination [10], and may be pertinent to the population studied here. Newkirk *et al* [27] observed that 20/20 COPD cases were IgA RF positive (20/20), but 0/14 tested ACPA positive. Yang *et al* [29] found 29/70 COPD cases positive for RF, but 0/70 were ACPA positive. We suggest that COPD individuals either have an increased propensity for RF generation but not ACPA, or that the COPD lung differentially sequesters ACPA rather than RF.

This study cannot address if multiple work exposures to VGDFs increase the risk of developing RA. Further studies are needed to address this important question. An increasing titre of RF in the general population increases the incidence of RA development by 20-fold when comparing the lowest vs highest titre of RF [30]. We suggest that studies of RF in the general population and the risk of developing RA not only consider the smoking history, but also occupational inhalations.

It is well established that smoking blunts clinical response to RA treatment with both methotrexate and tumour necrosis factor (TNF) inhibitors [31,32], and higher levels of RF in RA patients with established disease receiving TNF inhibitors are much less likely to be in remission at 12 months follow up [33]. We suggest that RA patients exposed to VGDF will have a more pronounced blunting of response to conventional treatment due to high RF levels, generated by sequential inhalational insults.

Prospective, large scale multi-centre studies of RA patients utilising occupational classification criteria and job exposure matrices may help to determine if occupational exposures in never smokers increase RF levels, increase disease activity and affect treatment response. Cohort studies using similar detailed exposure classification criteria may compare respiratory mortality rates amongst RA and non-RA cases with the same exposures, as it has been demonstrated that exposures to both smoking and VGDF greatly increase the risk of COPD in the general population with an OR of 14.1 (9.33-21.2) compared to never smokers with no such work exposures [10]. If our findings are representative, this is likely to be highly relevant to the co-morbidity and mortality of male RA.

Given the single centre nature of this study, we cannot comment on how representative this male RA cohort compares to a wider RA population and

suggest that wider study is needed. We suggest that rheumatologists undertake a detailed occupational and smoking history when assessing patients with RA.

5.6 Conclusion

The overwhelming majority of RA men is this analysis of an entire University hospital male RA cohort had exposure to vapours, gas, dust or fumes and had been smokers before diagnosis. Men who had such exposures combined had significantly higher RF titres and were diagnosed at a significantly younger age. Given the prevalence of these exposures in an RA male cohort, further study is needed to uncover the pathophysiological mechanisms underlying these compelling findings as the environmental exposures driving the autoantibody generation seen here may affect response to treatment.

5.7 Chapter 5 references

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Chapter 6. Nodular rheumatoid arthritis (RA): A distinct disease subtype, initiated by cadmium inhalation inducing pulmonary nodule formation and subsequent RA–associated autoantibody generation.

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6.1 Abstract

Nodular rheumatoid arthritis (RA) patients have raised rheumatoid factor (RF) and anti–citrullinated protein antibody (ACPA) levels, and are more likely to smoke than RA patients without nodules. Subcutaneous and pulmonary rheumatoid nodules (granulomas) frequently co–exist. Pulmonary rheumatoid nodules develop prior to RA development and have the immunological machinery to generate RF and ACPAs. Pulmonary granulomas have been observed in animal models exposed to cadmium (Cd) inhalation. Cigarette smoke increases pulmonary Cd exposure. It has been suggested that dust and cigarette smoke co–exposure increases localised pulmonary Cd adsorption. We hypothesise that subcutaneous nodular RA represents a distinct disease subtype induced by pulmonary rheumatoid nodule formation and the generation of high levels of RA associated autoantibodies initiated by Cd inhalation via cigarette smoke.

Cohorts of RA patients attending rheumatology clinics in Cornwall, UK (total n=504) were studied to determine the prevalence of nodular RA, with matched analysis (age, gender and social class) to compare urinary Cd, RF and ACPA levels stratifying for nodular disease and smoking.

In cohort 1 45/303 (14.9%) of the RA patients under regular follow up had nodular disease. Of the RA smokers, 30/155 (19%) were nodular and of the RA non–smokers 15/148 (10%) were nodular. Smoking was significantly associated with nodular RA, odds ratio (OR) = 2.48 95% confidence interval (CI) 1.26–4.88, p=0.008. Raised urinary Cd levels were significantly associated with nodular RA in non–dust exposed individuals, OR 2.26 (95% CI 1.08–4.73), p=0.03 compared

to dust exposed individuals, OR 0.78 (95% CI 0.35–1.76), p=0.557, despite fewer pack years (py) at diagnosis (16 vs 20 py). Nodular RA smokers had significantly raised RF levels compared to RA smokers without nodular disease (median RF 171.5 (interquartile range (IQR) 48–394) vs median RF 31.7 (IQR 10.3–170.3), p<0.00001). RF positivity was significantly more prevalent in nodular RA smokers compared to RA smokers without nodular disease (84/89 (94%) vs. 141/199 (71%), OR=6.9 (95% CI 2.66–17.91), p<0.00001). ACPA levels were also significantly raised in nodular smokers compared to non–nodular smokers (median ACPA 250 (IQR 145–426) vs 116 (1–257.5), p<0.00001), as were ACPA positivity rates (83/89 (93%) vs 123/191 (64%), OR=7.65 (95% CI 3.17–18.4), p<0.0001).

These pilot results support the hypothesis that nodular RA represents a distinct disease subtype initiated by cadmium inhalation, which we suggest induces pulmonary rheumatoid nodule formation and generation of RA–associated autoantibodies.

6.2 Introduction

Rheumatoid arthritis (RA) is a relatively contemporary disease having been first described in early 19th century Paris [1]. Florid classical RA is a characteristic disease with the development of ulnar deviation, swan neck deformities and subcutaneous nodules [2]. However, even in untreated disease, these features do not always develop, suggesting distinct RA subtypes. Classical features of RA strongly associate with erosive disease [3] which has not been observed in extensive studies of European skeletal remains prior to the 19th century [4], suggesting that classical RA is triggered by a set of relatively contemporary environmental risk factors. Cigarette smoking is the most important identified environmental risk factor for seropositive RA development and accounts for a third of cases [5], associating with erosive and nodular disease when compared to RA without these classical features [6]. However, the association between smoking and subcutaneous nodular RA appears to be dependent on rheumatoid factor (RF) positivity [7].

Rheumatoid nodules are pathognomonic for RA. The presence of rheumatoid nodules previously formed part of the 1987 classification criteria for RA [8]. This is not altogether surprising given that in the 1960s nodules occurred in 30% of RA patients (n=516) in the UK [9]. Over time the prevalence of nodules in RA in the UK (1991–1993) has declined to 6% with established disease of 3 years duration [10]. Consequently rheumatoid nodules are no longer included in the updated 2010 classification criteria for RA [11].

Rheumatoid nodules can either be located subcutaneously and are usually apparent over the extensor surfaces of the elbow, over the dorsum of the hand and fingers [2] or develop in the lung and are referred to as pulmonary rheumatoid nodules [12]. Pulmonary rheumatoid nodules were initially thought to be rare in RA as a case series of RA patients (n=253) failed to identify a single case [13], however, in retrospect, chest X–rays have a marked insensitivity with regards to identifying such nodules. Subsequently, computed tomography (CT) imaging has identified a relatively high prevalence of pulmonary rheumatoid nodules (22–30%) in a number of RA case series [14–17].

There is a striking association between cigarette smoking and the development of pulmonary rheumatoid nodules. Walker [9] observed a high rate of smoking in RA patients with pleural effusion (pleural effusion is recognised to be one of the sequelae of pulmonary rheumatoid nodules). Seventy six per cent of the RA patients studied with pleural effusion were smokers compared to 55% of the total RA cohort studied. Subcutaneous nodules appeared to be associated with the development of pulmonary rheumatoid nodules as the pleural effusion cohort had an increased prevalence of subcutaneous nodules of 10/19 (53%) compared to the prevalence of subcutaneous nodule in the RA population without a pleural effusion 146/497 (29%). Interestingly it was observed that RA associated pleural effusion on occasions preceded the development of RA or presented simultaneously with RA [9].

Prior to this description of a high prevalence of subcutaneous nodules in association with pleural effusions in a non–mining RA cohort in Northern England [9], Caplan described pulmonary nodules in Welsh coal miners [18]. In stark contrast to subcutaneous rheumatoid nodules, it was apparent that pulmonary rheumatoid nodules occurred frequently without RA as a study of miners with pulmonary rheumatoid nodules observed that 82/168 (49%) did not have RA. Furthermore of these men, 71 were tested for the then contemporaneous RA latex test and 25 (34%) were positive. This compares to 0/32 control miners with no lung disease or simple pneumoconiosis [19]. Caplan observed the same phenomenon as Walker in that pulmonary rheumatoid nodules often preceded the development of RA [18]. Unsurprisingly coal mining has been observed to increase the risk of male RA with an odds ratio of 8.47 (95% CI 2.59 to 27.66) [20]. The prevalence of smoking was high in miners with pulmonary rheumatoid nodules; a study of lung function observed that 18/23 (78%) of miners with a documented smoking history were current smokers [21].

These historic studies are of contemporary relevance as it has been suggested that RA can be initiated in the lung via local RA associated autoantibody generation [reviewed in 22]. A highly relevant, important observation of the nature of pulmonary rheumatoid nodules has been made by Highton et al [23]. . Pulmonary rheumatoid nodules have the immunological “machinery” to generate RA associated autoantibodies, evidenced by the presence of B lymphocytes and

ectopic lymphoid follicles.[23]. Such histological changes have not been observed in subcutaneous rheumatoid nodules [24].

It has been suggested that cadmium (Cd) exposure may be implicated in the pathogenesis of seropositive RA as Cd associates with cigarette smoking, social deprivation, living within 30 metres of a main road, residency in certain geographical locations of the United States of America and a plethora of occupations associated with RA development [25]. This hypothesis is specifically relevant to pulmonary rheumatoid nodule (granuloma) formation as a number of animal studies have observed that Cd nanoparticles nebulised into the lung can initiate the development of pulmonary nodules [26–28]. Cigarette smoking is strongly associated with an increased lifelong exposure to Cd and this is reflected in raised urinary Cd levels [29]. Median, gender specific UK levels for urinary Cd have been determined [30].
However, the relationship between measurable bodily Cd levels and disease is complex. For example, chronic obstructive pulmonary disease (COPD) is associated with raised lung tissue levels of Cd, but not necessarily raised urinary Cd levels [31]. As COPD is more strongly associated with co–exposures to both inhaled dust and cigarette smoke rather than either alone [32] we have suggested that lung Cd adsorption by the inhaled dust results in highly localised lung Cd levels and triggers disease [33]. As we hypothesise that enhanced adsorption of Cd onto intra–pulmonary inhaled substrates will stimulate granulomatous nodule formation, this may result in a reduced systemic absorption and therefore reduced blood and urinary level of Cd despite high local lung levels. Given that dust inhalation is common in RA populations [34], it is essential that the occupational history in relation to dust exposure is accounted for when considering urinary Cd levels as a marker of Cd exposure.

Accordingly, we hypothesised that RA nodular disease represents a distinct subset of RA with elevated RF, ACPA and appreciably raised urinary Cd levels (>95th centile as determined for UK populations) specifically in those RA patients unexposed to occupational dust exposure.

6.3 Methods

6.3.1 Subjects

Two cohorts of RA patients were studied. Cohort 1 (n=303) consisted of all RA males and females attending routine rheumatology clinics at the Royal Cornwall Hospital, Cornwall, UK from August 2017 to February 2018 to determine prevalence of nodular RA, as part of Departmental Audit. Cohort 2 consisted of prospectively gathered nodular RA males and females (n=92), matched for age (+/– 2 years), sex, and smoking history (+/–2 pack years), to patients previously collected as part of project IRAS ID 194833, approved by South West Regional Ethical Committee (UK) as previously described [34] (n=133). Specifically more males were recruited to this arm of the study as our unit were actively investigating the relationship between occupational exposures and the development of male RA. Cohort 2 (n=225) was stratified for nodular disease and smoking with comparison made for urinary cadmium. 3 cases were excluded as extreme outliers with cutoff value of >3 μmol/mol creatinine used to bias towards the null hypothesis.

Both cohorts were amalgamated (Cohort 3) with duplicate records excluded (n=24). RF and ACPA levels were compared between all RA patients (n=504) stratified for nodular disease and smoking.

18 patients were excluded from ACPA analysis due to incomplete data (n=6 RF negative, n=3 RF weak positive, n=9 RF strong positive), Figure 15.

Data was anonymised at source. Age, age of RA onset, disease duration, smoking history prior to disease development, RF levels at disease onset and subsequent ACPA levels were recorded as part of routine clinical practice. Patient occupational histories were recorded as part of ongoing clinical audit using methodology previously described [34]. All patients fulfilled 2010 ACR/EULAR RA criteria at diagnosis [11], Table 10.

Figure 15. Flow diagram of patients included for analysis with reasons for exclusion

Table 10. Demographic data for each cohort

	Cohort 1 n= 303	**Cohort 2 n=225**	**Cohort 3 n=504**
Age at diagnosis (years, median (IQR))	54 (43–63)	54 (45–61)	54 (44–63)
Male sex (%)	99/303 (32.7%)	129/225 (57.3%)	221/504 (43.8%)
Female sex (%)	204/303 (67.3%)	96/225 (42.7%)	283/504 (56.2%)
Disease duration (years)	9 (3–16)	11.5 (6–22)	10 (4–18)
Median RF at diagnosis (IQR)	38.4 (1–169.5)	70.4 (19.4–215.1)	51 (14.9–181.5)
Prevalence RF + (%)	219/303 (72.2%)	184/225 (81.8%)	381/504 (75.6%)
Median ACPA at diagnosis (IQR)	161 (1–260)**	186.5 (25.6–366.5)	180 (1–319.8)
Prevalence ACPA + (%)	211/285 (74.0%)**	176/225 (78.2%)	349/486 (71.8%)
Smoking prevalence: smoker or ex–smoker (<20 years) at diagnosis	151/303 (49.8%)	147/225 (65.3%)	288/504 (57.1%)
Median pack years smoked (IQR)	30 (15–40)	11 (0–26)	17 (0–27.8)
*Removed from combined cohort as record duplicated n=24			
**Incomplete ACPA data (RF– n=6, RF weak + n=3, RF strong + n=9)			

6.3.2 Autoantibody measurement

RF was measured with Tina–quant Rheumatoid Factors II Test System (Roche Diagnostics Corporation), with a value of <14 IU/mL considered as negative as per manufacturer guidelines. ACPA was measured by Second Generation E170 Anti–CCP analysis (Roche Modular Analytics), with a negative value of <17 U/mL as per manufacturer guidelines.

6.3.3 Social deprivation analysis

Social deprivation analysis was undertaken through the UK government validated Index of Multiple Deprivation (IMD), a deprivation rank score of 32 844 neighbourhoods weighted by income, health/disability, education, housing/service access, crime and living environment, converted to equal deciles [35].

6.3.4 Statistical analysis

Chi square tests and multivariate logistic regression analysis was used to examine relationships between smoking, nodularity and urinary Cd levels, adjusted for gender. The Mann–Whitney U test was used for non–parametric comparisons of continuous data of differing sample group sizes, with values are expressed as a median (IQR) or number (%). All data were analysed using commercially available software (Microsoft Excel (Microsoft Corp) and Number Cruncher Statistical System for Windows (NCSS60)).

6.3.5 Urinary Cadmium analysis

Patients supplied first pass morning mid–stream urine samples into 13ml sterile polystyrene urine sample tubes (Thermo–Fisher, Newport, UK). Samples were posted within 72 hours to the Supra–Regional Assay Service Trace Elements Laboratory, Guildford, Surrey. Urinary cadmium was analysed by inductively coupled plasma mass spectrometry (ICP–MS), using a fully validated method previously described [30]. An appreciably raised urinary Cd was defined as a urinary Cd level above the 95th centile as determined for UK populations

unexposed to occupational Cd, equating to 0.65 mmol/mol creatinine (females), and 0.42 mmol/mol creatinine (males). The median levels were reported as 0.19 mmol/mol creatinine and 0.16 mmol/mol creatinine respectively for females and males [30].

6.3.6 Smoking history

Pack years smoked was recorded prior to the diagnosis of RA. One pack year is deemed equivalent to smoking 20 cigarettes daily for a year. A smoker was defined as an individual that had smoked in a period up to 20 years before RA diagnosis and > 5 pack years total. A non–smoker was defined as an individual who had either never smoked or had smoked in the distant past (>20 years prior to the diagnosis of RA) and < 5 pack years total.

6.3.7 Subcutaneous rheumatoid nodules

Individuals were examined in clinic to determine the presence of characteristic rheumatoid nodules on the hands, forearm, elbow and feet. Patients who had undergone surgical removal of nodules and histological examination had confirmed the diagnosis, were considered to be a rheumatoid nodular patient irrespective of the presence of rheumatoid nodularity at the time of the examination. No radiological evidence of pulmonary rheumatoid nodules was collected.

6.4 Results

6.4.1 Demographics

The median age, age of RA onset, disease duration, smoking history prior to disease development, RF levels at disease onset and subsequent ACPA levels are recorded in Table 1 for cohorts 1–3. There was an increased prevalence of males in cohort 2 as a result of our particular interest in risk factors associated with male RA development [34]. The prevalence data in cohort 1 in terms of age and gender are broadly in line with other UK RA populations [3].

6.4.2 Risk factors for rheumatoid nodularity (cohort 2)

Current smoking was significantly associated with rheumatoid nodules (OR 2.74 (CI 1.29–4.72)), p=0.006. Former smokers were not at an increased risk of rheumatoid nodules, OR 1.14 (CI 0.56–2.28). However, when account for pack years smoked was undertaken the principal association was between pack years smoked and rheumatoid nodularity (data not shown).
Dust exposure alone was not associated with rheumatoid nodularity, OR 1.11 (CI 0.64–1.94) when corrected for pack years smoked (data not shown).

6.4.3 Gender and Cd levels

Both males and females displayed higher median Cd levels than unexposed, non–disease UK median data [30]. In men (n=128) the median Cd levels were 0.38 (IQR 0.16–0.42) and in women (n=95) the median Cd levels were 0.57 (IQR 0.19–0.65), p< 0.00001.

6.4.4 Nodularity, gender and Cd levels

Overall, nodular RA patients (n=92) had median Cd levels of 0.54 (IQR 0.31–0.82) mmol/mol creatinine compared to non–nodular (n=131) RA patients of 0.43 (IQR 0.28–0.67) mmol/mol creatinine with no significant difference (p=0.13) in median Cd levels observed.

In female nodular RA (n=38) the median Cd level was 0.64 (IQR 0.43–0.91) compared to a median Cd level of 0.5 (IQR 0.35–0.74) mmol/mol creatinine in female non–nodular RA (n=57), p=0.06, Figure 2. In contrast, in male nodular RA (n=54) the median Cd level of 0.385 (IQR 0.25–0.68) mmol/mol creatinine was very similar to the Cd levels of 0.37 (IQR 0.26–0.57) mmol/mol creatinine in non–nodular male RA (n=74), p=0.6, Figure 16.

Figure 16. Significantly elevated urinary cadmium (mmol/mol creatinine) in female nodular patients, but not in males *p= 0.06, **p= 0.6

Subsequent analysis of occupations as previously described [34] revealed that the majority of the men (90/128, 70.3%) were dust exposed. Given that very few females had such exposures (8/95, 8.4%), we analysed Cd levels in relation to dust exposure and the risk of rheumatoid nodularity given that female nodular patients had the highest Cd levels of any group studied (almost twice as high as male nodular smoking RA patients).

In non–dust exposed individuals an appreciably raised Cd level was significantly associated with nodular RA, OR 2.26 (95% CI 1.08–4.73), p=0.03. In dust exposed individuals an appreciably raised Cd level was not associated with nodular RA, OR 0.78 (95% CI 0.35–1.76), p=0.557.

In non–smoking, non–nodular patients 1/19 (5.26%) had appreciably raised Cd levels compared to 3/10 (30%) of the non–smoker nodular patients. Amongst smoking non–nodular patients 39/79 (50.6%) had appreciably raised Cd levels compared to smoking nodular patients 36/67 (46%).

6.4.5 Nodularity and RA–associated autoantibodies

Nodular RA smokers had significantly raised RF levels of compared to RA smokers without nodular disease (median RF 171.5 (IQR 48–394) vs median RF 31.7 (10.3–170.3), p<0.00001), Figure 17.

Figure 17. Significantly elevated median RF (IQR) levels in nodular RA smokers and non–smokers *p<0.00001, **p=<0.00001

RF positivity was significantly more prevalent in nodular RA smokers compared to RA smokers without nodular disease (84/89 (94%) vs. 141/199 (71%), OR=6.9 (2.66–17.91), p<0.00001).

ACPA levels were also significantly raised in nodular smokers compared to non–nodular smokers (median ACPA 250 (IQR 145–426) vs 116 (1–257.5), p<0.0001) as were ACPA positivity rates (83/89 (93%) vs 123/191 (64%), OR=7.65 (3.17–18.4), p<0.0001), Figure 18.

Figure 18. Significantly elevated median ACPA (IQR) levels in nodular RA smokers and non–smokers *p=0.02, **p<0.0001

RF levels were significantly elevated in nodular never smokers, median RF 118 (62.6–267.3) vs. 30.5 (7–95.3) for never smokers without nodular disease, p<0.00001, Figure 3. ACPA levels were similarly significantly higher in nodular never smokers, median ACPA 245 (69.3–385.1) vs. 147.5 (1–280.3) for never smokers without nodular disease, p=0.02, Figure 4.

No significant differences were observed in RF (p=0.25), or ACPA (p=0.67) between nodular never smokers and nodular smokers.

6.5 Discussion

6.5.1 Nodular RA environmental exposures

Rheumatoid nodules have been of great interest to rheumatology researchers for over a hundred years. Histologically, rheumatoid nodules are granulomas consisting of a fibrous tissue shell arranged around a centre of fibrinoid necrosis [36]. Granulomas have been reported to occur in individuals in association with a number of metals including aluminium, barium, beryllium, cobalt, gold, titanium and zirconium [37]. In addition, Cd has also been observed to induce the formation of granulomas when nebulised into the lung in animal models [26–28]. Autophagy has emerged as an important process by which antigen presenting cells generate and present citrullinated proteins/peptides [38]. This process is induced in vivo by Cd and zinc rather than other metals such as cobalt, iron, lead, mercury and selenium [39]. Given that Cd as opposed to zinc is significantly raised in smokers as opposed to never smokers [40] and the strong relationship between smoking and rheumatoid nodules as highlighted above, we have hypothesised that Cd exposure is associated with nodular RA.

In this study we observed that an appreciably raised urinary Cd level in non–dust exposed RA patients was significantly associated with the presence of peripheral rheumatoid nodules. We suggest that inhaled Cd and the subsequent

accumulation of this metal in the lung triggers a granulomatous lung reaction as has been described with other inhaled metals such as beryllium [41]. As there is a striking concordance between pulmonary and peripheral rheumatoid nodule formation we suggest that smoking induces rheumatoid peripheral nodules as a consequence of Cd exposure.

6.5.2 Peripheral and pulmonary nodule differences

The central area of the rheumatoid nodule is necrotic and contains fibrinogen and in the peripheral part of the necrotic area is fibronectin [42]. Citrullination is noted to occur within the rheumatoid nodule as a study noted positive citrulline staining in the majority of 26 cases (70%) [43]. At the junction of the necrotic centre and the outer fibrous shell is a palisade of activated macrophages and fibroblasts. In the outer layer clustered around blood vessels are T lymphocytes of both CD4 and CD8 subtype and these tend to accumulate around vessels in the area immediately outside the palisade. In close proximity to T cells, dendritic cells have been observed [42]. Therefore there is the potential for antigen presentation in the peripheral nodule. However, peripheral rheumatoid nodules lack of organised lymphoid tissue containing plasma cells. Despite the abundance of citrullinated antigens such as fibrin and fibronectin, autoantibody generation within the peripheral rheumatoid nodule does not appear to be feasible primarily for this reason.

In distinct contrast pulmonary rheumatoid nodules have been observed to consist of lymphoid aggregates containing both B lymphocytes and T lymphocytes and germinal centres containing follicular dendritic cells in addition to the typical histological findings of the peripheral rheumatoid nodule [23]. Supporting the hypothesis that pulmonary nodules generate RA autoantibodies to trigger RA development (rather than arise as a result of RF and ACPA generated by the peripheral RA disease process), is the finding by Caplan [18] that pulmonary rheumatoid nodules often precede the development of RA and occurred commonly in the absence of RF prior to the development of RA [19]. The appearance of pulmonary rheumatoid nodules in the miners described by Caplan appears to be the strongest risk factor ever described in the literature for RA development with an approximate 364–fold increased risk (51% developing RA

as compared to a prevalence of RA in the same era in males living in non–mining communities of 0.14%) [44].

6.5.3 How this hypothesis changes understanding on the pathophysiology of nodular RA

Current dogma suggests that rheumatoid nodules develop as a result of the RA disease process generating RF and ACPA, and that immune complexes deposit in the subcutaneous tissues possibly enhanced by local pressure point areas at the elbow, facilitating a local inflammatory process with macrophage activation [reviewed in 24]. There has been great interest in the understanding of peripheral rheumatoid nodule formation as it has been suggested that the disease process that occurs in the rheumatoid nodule shares great similarities with the inflammatory process in the synovial tissue described in RA [45].

We have hypothesised that pulmonary rheumatoid nodules generate RA associated autoantibodies. In this study we observed that nodular RA patients had markedly elevated levels of RA autoantibodies compared to non–nodular RA patients and this remained the case when nodular patients were stratified for smoking. Smoking is the most important environmental risk factor for the development of raised RF levels in RA and is related to pack years smoked [46]. However the pack years smoked were very similar between smoking nodular and non–nodular RA patients and therefore do not explain the marked difference reported here. Remarkably non–nodular RA smokers had very similar RF levels to RA non–smokers without nodules. This finding is the first to be reported in the literature and suggests that smoking per se does not increase RF levels. We hypothesise that smoking may generate pulmonary rheumatoid nodule formation which in turn generate the rheumatoid associated autoantibodies and the subsequent development of peripheral rheumatoid nodules.

6.5.4 Limitations

A limitation of this study was to use the presence of peripheral rheumatoid nodules as a surrogate for the presence of pulmonary rheumatoid nodules. It is conceivable that in some patients with peripheral rheumatoid nodules that the

nodules developed as a result of RA associated autoantibody production in the joint as a result of longstanding RA. Further studies investigating the levels of RF and ACPA in newly diagnosed RA patients with or without peripheral rheumatoid nodules may consider undertaking CT chest scans to determine the presence of pulmonary rheumatoid nodules and correlate with levels of RA associated autoantibodies in order to predict disease progression and response to treatment.

The relationship between measurable bodily Cd levels and RA is complicated. For example we have recently demonstrated a markedly increased prevalence of inhalational occupational exposures in 546/726 (75%) men with RA [34]. These exposures included potent Cd adsorbers such as kaolin, silica, bitumen tar and wood dust. Furthermore we have suggested that lung Cd adsorption by the inhaled dust results in high lung levels and triggers disease [33]. Accordingly an enhanced Cd lung adsorption will result in a reduced systemic absorption and therefore reduced urinary level of Cd despite high lung levels which would enhance the risk of pulmonary rheumatoid nodule formation.

Interestingly in this study we found that urinary Cd levels were significantly lower in dust exposed nodular RA patients compared to non–dust exposed nodular and non–nodular RA patients. This highlights the importance of lung Cd adsorption and possibly suggests that measuring bodily Cd levels in terms of urine or blood levels may overlook high levels of Cd in the lung which may have the potential to initiate RA via the generation of rheumatoid nodule formation. We suggest that studies investigating the relationship between RA pathogenesis and Cd levels take into account the occupational history as well as the smoking history of the patient as blood or urine levels in Cd exposed individuals are likely to be reduced as a result Cd adsorption in co–exposed lungs. This is particularly relevant to men with RA as the majority of these individuals have been exposed to occupational dusts [34]. It is noteworthy that a large Korean epidemiological study of RA prevalence revealed a strong association between Cd blood levels in women, but not men [47].

6.6 Conclusions

There does appear to have been a change in the natural history of RA over the

last 40–50 years in terms of expression of nodular disease. It would be easy to ascribe this change to better treatment strategies and more efficacious medications for RA. However, these changes would not affect the type of RA at presentation. For example a study of 102 early RA patients in London, UK in the mid– 1970s observed that 12/102 (12%) individuals had subcutaneous nodules at presentation rising to 32/102 (31%) at 3 years [48]. In contrast a study in Norfolk, UK (1990–1993) observed that 37/486 (7.6%) of RA patients had subcutaneous nodules at presentation and at 3 years follow up this had fallen to 31/486 (6.4%) [10]. This stark change appears to have occurred before the advent of biologics therapies and before the routine first line use of methotrexate. We suggest that the natural history of nodular RA may reflect the marked drop in Cd exposure in UK society. The industrial revolution was associated with a 15–fold increase in anthropogenic emissions of Cd in Western Europe [49] and since the 1960s there has been a 2.5–fold decrease in emissions of Cd [50]. Additionally the marked reduction in the prevalence of smoking is also likely to reduced individuals exposure to Cd [51]. This is not the first study to observe an association between raised bodily Cd levels [47, 52–54], however this study is the first to describe a particular RA phenotype that most strongly associates with an increased Cd exposure. Additionally, this hypothesis has generated the first evidence of an environmental risk factor, other than cigarette smoking, for the development of nodular RA.

As described above the rheumatoid nodule is a potentially important source of citrullinated proteins such as fibrin and fibronectin which can be presented to the immune system by resident antigen presenting cells such as macrophages, fibroblasts and dendritic cells. Pulmonary rheumatoid nodules also have the potential to generate RA associated autoantibodies. Given that a recent study observed intracellular citrullination of proteins in lung cells exposed to Cd [55], we hypothesise that Cd inhalation orchestrates the development of pulmonary rheumatoid nodules, intracellular citrullination and the subsequent formation of RA associated autoantibodies leads to immune complex formation and activation of various immune cells that trigger the development of nodular RA. However, Cd exposure may also predispose to non–nodular RA. In this study we noted appreciably raised Cd levels in non–nodular patients irrespective of their smoking history and it is conceivable that Cd accumulation in the bone marrow may result

in altered mesenchymal stem cell viability as described in an animal model [56]. Certainly mesenchymal stem cells have the potential to have a significant role in the pathogenesis of RA [57].

It is noteworthy that 27 years ago that Fischer in *Medical hypotheses* hypothesised that tobacco smoking was a risk factor for RA [58]. We have extended Fischer's hypothesis further and suggest that specifically, it is Cd derived mainly from cigarette smoke that triggers RA disease development. We suggest that one potential mechanism by which Cd triggers RA is as a result of lung granuloma formation with a subsequent autoantibody response triggering seropositive RA.

The pilot data from the limited study presented here supports this hypothesis. We would welcome wider study incorporating local tissue cadmium analysis to study lung granulomata in RA and pre–RA states, and the use of diagnostic imaging in assessing pulmonary nodularity prevalence and impact on RA disease development.

6.7 Chapter 6 references

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Chapter 7: Further discussion and conclusions

The collection of published papers that comprise Chapters 2-6 of this dissertation are now related back to the original hypotheses detailed in Chapter 1, highlighted by the published case report therein. New findings, strengths, weaknesses, unanswered questions, and directions for future research are discussed.

7.1 Principal findings on original hypotheses

Hypothesis 1: RA is precipitated by inhaled environmental exposures, and patients with multiple inhalation exposures are more likely to have elevated autoantibody levels to both rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA).

Specific RA cases exposed to vapours, gas dust and fumes have been analysed and published [1]. A literature search into inhalation exposures in RA with reference to occupation has been undertaken and published [2], with discussion and reflection into potential pathophysiological mechanisms by which inhalational exposures may trigger RA. Data have been collected, analysed and published on the smoking habits, occupational exposures and rheumatoid autoantibody levels of an entire cohort of male RA patients under follow up at the Royal Cornwall Hospital, UK [3]. Overwhelming over-representation of vapour, gas dust and fume (VGDF) exposure (546/726, 75%) and smoking (561/726, 77%), was observed.

Significantly higher rheumatoid autoantibody levels were found in RA patients who have multiple inhalational exposures [3]. A positive correlation was observed between VGDF exposure and increased RF levels. Combining this with cigarette smoking resulted in significantly higher RF levels, particularly with multiple exposures. No association of ACPA seropositivity rates or titres with VGDF exposure was observed.

Hypothesis 2: There is a common autoantigen in many RA patients to explain autoantibody clustering of both RF and ACPA.

Chapters 3 and 4 [4,5] explored the possibility that RF and ACPA co-exist. A study to analyse the relationship between RF, smoking, and HLA-DRB1 alleles coding

a “shared epitope” (SE) in relation to ACPA positivity in RA patients was conceived, completed and published [4]. It was found that ACPA positivity in RA strongly associates with increasing RF titre independent of smoking; and this relationship is dependent on carriage of SE alleles. No relationship was found between ACPA and smoking in RF negative patients.

With the primacy of RF demonstrated above and given that immunoglobulin G (IgG) is the sole RF antigen, a hypothesis that post-translational modifications to IgG may explain clustering of RA associated autoantibodies such as ACPA was generated. Subsequent laboratory analysis suggested that post-translational modification processes such as citrullination and carbamylation, occurring at the Fc binding region of IgG heavy chain fragments, may be a potential universal antigen in RA.

Hypothesis 3: The presence of nodular RA is associated with raised rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA) levels irrespective of the smoking history.

Chapter 3 [4] was originally conceived as an interim analysis of evidence gathered; and revealed a striking pattern that was at odds with the current dogma on the relationship between smoking and autoantibody generation. It was discovered that ACPA-postive RA is primarily determined by RF titre and the shared epitope, rather than smoking in its own right. Following this, analysis was completed on RA patients with rheumatoid nodules stratified for smoking. Nodular RA patients demonstrated higher rheumatoid autoantibody levels than non smoking comparable cohorts.

Further analysis of the relationship between subcutaneous nodules**,** rheumatoid autoantibodies and smoking was undertaken. Nodular RA patients who were smokers had significantly higher RF levels than age, sex, and deprivation matched non-nodular RA controls when stratified for cigarette smoking (Chapter 6) [6]. This may be due to rheumatoid autoantibody production from rheumatoid pulmonary nodules in the lung (discussed below).

Hypothesis 4: RA smokers exposed to VGDF develop pulmonary nodules, adsorbing heavy metal toxins into lung granulomata rather than into the systemic

circulation.

Comparing nodular and non-nodular RA patients, significantly increased RF and ACPA levels were observed irrespective of the smoking history of the patient. Existing dogma would suggest that raised levels of RF predispose to the development of subcutaneous rheumatoid nodules in RA patients. However, my literature review [2] pertaining to the development of pulmonary rheumatoid nodules in Welsh coal miners (Caplan's syndrome) indicated that pulmonary nodules can commonly occur in individuals without RA and were not associated with RF in many of these individuals. This important historical antecedent strongly suggested that pulmonary rheumatoid nodules are not triggered by the RA disease process nor RF.

Interestingly in those individuals with pulmonary rheumatoid nodules described by Caplan, approximately 50% had a diagnosis of seropositive RA and peripheral rheumatoid nodules were highly prevalent [7]. This raises the possibility that pulmonary rheumatoid nodules triggered RA by local RF production in the lung and subsequently predisposed the men to subcutaneous nodules. I have hypothesised that subcutaneous nodular RA smokers have a high prevalence of pulmonary rheumatoid nodules and as such have higher RF levels than non-nodular RA smokers. The RF levels in nodular RA smokers were remarkably higher than non-nodular RA smokers and interestingly the non-nodular RA smokers/non-smokers had very similar RF levels. I suggest the main mechanism by which RA smokers generate raised RF levels is via pulmonary rheumatoid nodule formation, given that pulmonary rheumatoid nodules contain germinal centres capable of autoantibody production [6]. Comparing nodular and non-nodular RA patients, significantly higher urinary cadmium levels were seen in nodular RA patients who did not have exposure to vapour, gas dust or fumes. Despite comparable smoking histories, individuals with nodular RA who were VGDF exposed had significantly lower urinary Cd levels than individuals who were not VGDF exposed [6]. This intriguing finding strengthens the concept that adsorption onto inhaled dust particles may take place, potentially rendering urinary cadmium an ineffective measure of general Cd exposure; higher urinary cadmium levels are associated with the formation of peripheral rheumatoid nodules in RA patients unexposed to vapour, gas, dust and fumes (VGDF).

This has implications for the VGDF and cigarette smoke co-exposed RA patient. I have subsequently hypothesised that cadmium-laden dust complexes become “bound up” in localised granulomata forming rheumatoid pulmonary nodules which stimulate local rheumatoid autoantibody production and break immune tolerance. This hypothesis highlights the importance of taking into consideration the occupational history of an individual when considering the association between disease development and urinary Cd levels. It has recently been highlighted that measuring blood and urinary Cd levels may overlook the association between Cd exposure and diseases such as emphysema by lung retention [8], specifically triggering lung disease, suggesting that further studies investigating the relationship between Cd exposure and emphysema should specifically measure Cd lung tissue levels.

Similarly, given that RA is initiated in the lung, studies investigating the association between RA and Cd exposure need to consider stratifying individuals for exposure to dusts known to adsorb Cd. A recent Korean study supports a strong association between blood Cd levels (19-fold increased prevalence in those in highest vs. the lowest quartile), observed in women [9]. However, in this study no association was observed in men. It would be interesting to know the prevalence of VGDF exposure in the men and how this related to blood Cd levels in this study population.

7.2 New findings

7.2.1 Inhalation exposures increase RA autoantibody levels

Analysing the entire male RA cohort under follow up at the Royal Cornwall Hospital, it was found that VGDF exposure was associated with increased RF levels [3]. Combination exposure to smoking and VDGF resulted in higher RF levels, particularly with multiple exposures occurring in the workplace. These compelling findings demonstrate the importance of combinations of inhalational exposures in RF generation, and the consequent impact on RA disease development, more than observed in an individual exposure. This is the first study to focus on occupational exposures combined with smoking as risk factors for autoantibody development in male RA; and is the first study in RA to use combined analysis of vapour, gas dust and fume inhalation.

Over representation of certain trades has allowed for further subgroup analysis within this study. The granularity of the data and the ability to explore historical exposures via telephone questionnaire follow up of what a job entailed was a strength of this study. For example, of 155/726 (21%) wood dust exposed cases, 44/155 (28%) identified wood dust as their primary occupational exposure as carpenters, approximately seven times higher than expected given UK employment census data [10]. However, limitations in exposure classification, both in terms of definition and assessment or grade of exposure have been acknowledged [2,3]

My previously published data on this cohort have revealed significantly higher RF titres and ACPA seropositivity rates amongst woodworkers compared to matched controls [11]. Similarly, amongst rock miners, 54/98 (55%) had exposure to kaolin (china clay) dust, approximately 12 times higher than then expected from occupational census data for the population studied. Again, previously published data have revealed significantly higher RF and ACPA seropositivity rates and higher RF titres that matched controls [12]. Furthermore, significantly more kaolin exposed workers demonstrated nodular disease than controls, independent of smoking [12].

7.2.2 Autoantibody associations

Surprisingly, a literature search revealed no studies investigating the relationship between the presence and levels of a positive RF, cigarette smoking, carriage of the SE alleles, and the frequency of ACPA in RA. In analysing the relationship between RF titre, smoking and HLA-DRB1 SE alleles in relation to ACPA positivity, I discovered that ACPA positivity in RA strongly associates with increasing RF titre independent of smoking, with the relationship dependent on carriage of SE alleles. No relationship was observed between ACPA and smoking in RF negative patients [4]. Investigating the clustering RF and ACPA in both smokers and never smokers for the first time, this study addressed the co-existence of rheumatoid autoantibodies in those strongly positive for RF irrespective of smoking. These findings deepen the understanding from established studies identifying smoking and carriage of the SE alleles as risk factors for ACPA positive RA [13].

Previously, it was suggested that carriage of the SE was significantly associated with ACPA positive/RF negative RA rather than RF positive/ACPA negative RA. It was suggested that the primary process in RA involved anti-citrulline immunity influenced by an interaction between smoking and carriage of the SE. However, the tiny proportion of ACPA positive/RF negative cases (13%) compared to the majority of ACPA positive/RF positive cases (87%) [13], suggested an association here as well. It is perhaps surprising that an association has not been analysed sooner.

I would suggest that given the overall strong association between the carriage of SE alleles and ACPA positivity, it follows that the SE is principally associated with ACPA positive/RF positive RA. Consequently, the findings here [4] can be reconciled with previous research [13]; and can refocus the current understanding of autoantibody clustering in RA. Similarly, the previously held observation that smoking interacts with the SE to generate ACPA positivity overall in RA [13] is factually correct; my findings suggest that a more precise interpretation of this interaction is between smoking, the SE, and both RA autoantibodies. Furthermore, my findings have stimulated analysis in other groups looking at autoantibodies in RA such as the Karolinska Institute, Sweden, and planning to test my hypotheses in the wider “EIRA” case-control study population.

In researching the common clustering of autoantibodies in Hypothesis 2, this thesis has added significantly to understanding how autoantibodies associate in RA; and identified particular inhalation exposures that predispose. This thesis has stimulated global interest in this area, discussed below (7.5.5.2).

7.2.3 IgG as a common autoantigen

An important finding of this dissertation, and significant addition to academic thought, is the revelation of IgG as a potential common antigen in RA. It is suggested that a post translationally modified, misfolded heavy chain fragments of immunoglobulin G (IgG) could be dominant antigen in RA [14]. Post translational modification of the Fc binding region of IgG may generate an antibody response specific to RA, generating both a strongly positive “clustered” RF and ACPA response.

Recent findings of citrullinated IgG in synovial tissue biopsies of RA patients support this, with IgG as a target of peptidylarginine deiminase (PAD) 2 and PAD4 activity [15]. Bispecific antibodies against cyclic citrullinated peptides and IgG in RA have been demonstrated, suggesting that citrullinated IgG may generate both a RF and ACPA response [16]. Citrullinated heavy chain fragments of IgG have been demonstrated in RA synovial fluid [17], amongst other citrullinated peptides.

Whilst a plethora of proteins of a comparable size have the potential to be citrullinated and/or carbamylated, RF only has one autoantigen; the heavy chain fragment of IgG. This can be generated by B cells in the lungs of smokers; a process facilitated by SE alleles with the B cell acting as both an antigen presenting cell and the source of the antigen [5]. Though described in the lung, both rheumatoid joints and other mucosal surfaces could be the host the same process of B cell activation through the development of tertiary lymphoid tissue.

The explanation of a common autoantigen has been demonstrated for the first time as part of this research [5], with the finding that citrullinated and carbamylated IgG heavy chain fragments are present in the sera of RA patients and in a pre-RA bronchiectasis patient. Whilst citrullinated IgG was observed in healthy subjects and RA patients, specific citrullination and carbamylation of the RF binding site was only observed in patients with seropositive RA (4/8) and in a patient with bronchiectasis who progressed to develop seropositive RA (1/4). The role of post-translational modification to the RF binding site region of IgG in the development of RA may prove to be significant and is worthy of further study; especially given the changing receptor polarity of the Fc region that occurs when it becomes post-translationally modified, affecting ligand interaction [18] and potentially stimulating tertiary lymphoid tissue development, which sets it apart from other citrullinated autoantigens.

This aspect explored in my thesis is limited by the small sample size and its cross-sectional nature: my dissertation research only represents a moment in time in the sera of patients and healthy controls. However, an antibody response is a legacy of past antigen exposure, persisting over time. The development of an antibody against the post-translationally modified Fc region of IgG may be of value as a specific diagnostic marker in the early detection of RA.

7.2.4 Adsorption

Investigating my hypotheses in this thesis, I uncovered many interesting findings from historical analysis of literature, cutting edge analysis of proteomics, and the basic epidemiology of the cohort that I was studying. I wanted to find a unifying theory, an explanation for why VGDF and smoking co-exposures leads to more severe RA. From the literature searches and detailed patient histories conducted as part of this dissertation, I have presented information to support a novel hypothesis of heavy metal adsorption onto inhaled dust particles driving RA pathophysiology.

Utilising the historical significance of Caplan's syndrome seen in coal workers in the 1950s [7], I was struck by the prevalence in kaolin workers being highest of any occupation ever reported [19], given the over-representation of current and former kaolin workers found in the Cornish RA population [12].

I have suggested that the kaolinite mineral capacity for adsorption of heavy metals may explain the scale and pattern of Caplan's syndrome prevalence seen in global mining populations; and further explain the pronounced interaction seen in sequential dust exposure and heavy metal laden cigarette smoke, particularly with reference to cadmium. I believe this novel process of adsorption to be an overlooked factor in RA disease development, explaining the interaction of inhalational exposures and cigarette smoking seen in RA [3,4].

As discussed in Chapter 2 [2], the adsorption process is not exclusive to Kaolin, although the chemical structure of Kaolin renders it exquisitely capable in this regard. The RA affected kaolin mining population of Cornwall's unique exposures have highlighted what may be a widely applicable mechanism of disease development. Whilst the socio-economic determinants of health discussed in Chapter 1 drive a lot of the co-exposure burden seen in the Cornish RA population, the snapshot provided by the surviving workers of the Kaolin industry may prove invaluable in the history of RA understanding.

7.3 Strengths and weaknesses

7.3.1 Patient involvement

Patient involvement was central in this research and dissertation. The narrative of the patient case study detailed in Chapter 1 [1] was a typical example, providing the starting point from which the overall hypothesis and aims came from, developed from detailed histories of individual patient experiences of RA in Cornwall, UK. The initial hypothesis was presented at a local meeting of the National Rheumatoid Arthritis Society, attended by over 100 members, the majority of whom were RA patients. Subsequent interest following the meeting led to further discussion as to the development of a protocol that would be acceptable to patients in terms of its design and acceptability. From initial ideas, this was refined and presented to the committee of the Cornwall Arthritis Trust, a local charity supporting arthritis patients. Specific feedback around consent form wording, questionnaire design, and follow up arrangements enabled patients who may not have been able to complete or return written questionnaires to access the research.

Patient involvement in this process was invaluable for how best to manage and undertake the research in a way that was minimally intrusive for patients in their regular clinical care. This process was greatly beneficial in writing the Health Research Authority (HRA) protocol in Appendix 1. Dissemination of results relating to this research continues to take place within the Rheumatology Department, Royal Cornwall Hospital, via poster presentation.

The impact of such detailed patient involvement throughout the research process is demonstrated in the response rates seen; study design with face-to-face interviewing and telephone follow up was designed to minimise non-response. At termination, only 8/726 (1%) of the entire male RA cohort under follow up were non-responders.

Non-response, particularly in postal studies, is strongly correlated with low levels of formal education. This is particularly relevant to the investigation of RA as there is an established literature linking RA and low levels of formal education [20-22]. In the UK, RA patients have a high prevalence of functional illiteracy with 2 British studies observing an identical rate of 15% [23,24]. Perhaps unsurprisingly, a USA study observed that literacy was positively related to educational attainment.

College graduates recorded higher mean literacy scores than high school graduates, which were higher than “dropouts” [25]. A divergence in occupations related to literacy scores was noted. Agriculture, construction, manufacturing, and mining had the lowest scores [25].

Unsurprisingly again, such occupations are all associated with male RA [2]. 95% of occupations held by individuals studied here required no formal academic qualifications beyond secondary education. It was therefore not possible to compare low and high levels of formal education for RA autoantibody prevalence in our male RA cohort. I suggest that it is imperative that further studies investigating the impact of socioeconomic status or formal education levels on male RA susceptibility, severity, comorbidity, and mortality consider an individual’s occupation as an important confounding factor.

7.3.2 Literature search

From the literature search in Chapter 2 [2], evidence was found for a variety of occupations demonstrated to be associated with an increased risk for RA. This is the first such review to evaluate published clinical reports related to occupations associated with RA development. As such is a worthwhile addition to the area of occupations and RA. However, heterogeneity between study populations, data collection methods, and occupational classification systems precluded systematic literature review. The existing evidence was primarily generated through Swedish case referent and linkage studies, and involved exposure to inhalational particles, in the form of dust, fumes or both. It is acknowledged that a future systematic database review in this important area is still required; and should be a priority for researchers in this area as further evidence emerges.

The major limitation of the existing data lies in the extent to which it can be applied elsewhere; Swedish data may not adequately represent the occupational experiences of individuals in other countries. Global health and safety legislation, working patterns and smoking habits vary considerably. In addition to the reverse social class gradient amongst Swedish smokers discussed in Chapter 2 (making VGDF and cigarette smoking co-exposure less likely), Sweden has a long history of recognition of the potential occupational risks to its population, with consequent state intervention, since the Occupation Hazard Act of 1889 [26]. Occupational

health and safety in Sweden is regulated by state legislation, with the Occupational Safety and Health Act of 1949 and the Working Environment Act of 1977 predating equivalent health and safety legislation elsewhere in the world [26]. There is also a history of concordance with policy between the Swedish Employers Confederation (SAF) and the Swedish Trade Union Confederation (LO), being reported until the early 1990s [27].

I would argue that transposing the findings in this advanced western democracy, with a strong history of public health legislation and implementation to protect its population, would have limited applicability elsewhere. Risks highlighted in the Swedish population are likely to be underestimated, and further risks may well be missed.

7.3.3 Occupational exposure classification

This research does not contain a job exposure matrix to classify individual occupations. It was not intended to sub-stratify into occupational classifications, though the granularity of data collected does allow this; and the coding of occupations is ongoing as detailed in Chapter 5 [3]. Although the lack of a matrix may be interpreted as a weakness, the key argument against this is the finding that the most important determinant of autoantibody levels is the combination of inhalational exposures rather than the specific occupation itself.

Much of the literature investigating occupational risk and RA takes the form of case control/case referent or linkage studies from large dataset analysis (reviewed in [2]). Studies investigating occupation and RA risk may only consider the last occupation undertaken by the respondent [28], or the main occupation recorded in the database studied. It is not uncommon for individuals with low literacy levels to move from one casual job to another; accruing different occupational exposures over time and the last recorded job may not reflect these exposures. In poorer populations, trade sub-specialisation and health and safety legislation adherence may be less evident. Throughout their working careers, unskilled workers will encounter multiple occupational exposures. Construction labourers, for example, may be co-exposed to silica dust, inorganic non-silica dusts, wood dust, and diesel fumes.

Furthermore, an individual's occupation "title" does not always reflect the inhalational exposures the individual may have been exposed to, as well as missing factors such as hobbies, environmental residence, and primary and/or passive cigarette smoke. Job exposure matrices give a broad indication of what exposures may have occurred if one is to assume heterogeneity of tasks. However, subspecialisation with jobs is missed in such tools; e.g. in the case of a gardener, a job matrix would not identify the individual who specialises in building retaining walls (with consequent regular cement dust exposure), or the individual who primarily repairs garden machinery and is regularly exposed to welding fumes and oils. Therefore, we suggest that studies investigating individual occupational risk and RA via single job title analysis may be confounded by multiple inhalational insults, even when correcting for smoking. In an analysis of silica vs. non-silica dust exposed construction workers, Blanc et al. [29] excluded 40,645/240,983 cases who were exposed to wood dust, gas or fumes to reduce confounding. I would argue that this is precisely the type of multiple exposure that would benefit from further analysis to investigate RA risk.

Additionally, database referent studies choose to include patients under the age of 70 [30]. Many occupational diseases, such as asbestos exposure [31], have a long latency period between the inhaled occupational insult and disease development. Though we found a significantly earlier age of onset in our most severely co-exposed cohort, 104/726 (14%) cases studied were diagnosed over the age of 70. The risk of smoking and RA is evident up to 15 years after smoking cessation [32]. As polarisation of RA risk estimates occurs when a latency period is used in occupational exposures [30], using an age cutoff of 70 years of age does not allow an adequate latency period between exposure and disease development; although one acknowledges the impacts that such latency may create in the development of other smoking-associated cardiovascular and respiratory comorbidities.

I chose to minimise the potential limitations detailed above by making use of recent studies investigating the relationships between occupational exposures and chronic obstructive airways disease (COPD) [33], which utilised self-report of any occupational inhalational exposures together and considered these exposures as an entity that can trigger COPD. "Grouping" reported exposure to

VGDF in this way demonstrated an independent, modest association with COPD development [33]. Using a similar methodology in RA patients in my single centre study revealed the fascinating results described in Chapter 5 [3]. More detailed analysis of specific occupations and exposures for this and future studies in a wider population will be important to further investigate individual risks and test further hypotheses into pathophysiological mechanisms.

7.3.4 Cornish population-based specifics

Overall, this thesis suffers from a relative lack of comparable RA cases that are unexposed to VGDF and smoking, limiting the ability to provide statistical significance via an adequate control group to analyse specific VGDF exposures. The relative homogeneity of educational levels and social depravation indices between the patient groups in Chapters 3,5 and 6 [3,4,6] may explain why no significant differences were found here; although this may reflect the nature of how inhalational exposures manifest in a male RA cohort.

7.4 Unanswered questions

7.4.1 Exposure prevalence and treatment implications

The overwhelming majority of RA men in my analysis of an entire University hospital male RA cohort had exposure to vapours, gas, dust or fumes, and had been smokers before diagnosis [3]. Men who had such exposures combined had significantly higher RF titres, and were diagnosed at a significantly younger age compared to unexposed never-smoking RA cases. Given the prevalence of these exposures in this specific RA male cohort, it would be interesting to see if prevalence was representative of wider RA populations elsewhere. However, many men in Cornwall are also exposed to VDGF and cigarette smoke, and do not have RA. Further study of a case-control nature in similarly exposed non-RA individuals to determine disease susceptibility, particularly with regard to known genetic risk factors such as the number of HLA-DRB “shared epitope” copies.

A wider unexposed RA study population and a similarly matched non-RA cohort would both be useful for further analysis. Given that males who had such exposures combined had significantly higher RF titres and were diagnosed at a significantly younger age, further multi-centre study is required to confirm findings and further research pathophysiological mechanisms suggested, as the environmental exposures driving autoantibody generation seen here will affect response to treatment. Cigarette smoking reduces the clinical response to RA treatment with both methotrexate and tumour necrosis factor (TNF) inhibitors [34,35]; and higher levels of RF in RA patients with established disease receiving TNF inhibitors are much less likely to be in remission at 12 months follow up [36]. I suggest that RA patients exposed to VGDF and smoking will have a more pronounced blunting of response to conventional treatment due to higher RF levels.

The evidence from this research suggests that environmental and occupational exposures drive autoantibody generation. I have hypothesised that cadmium may lead to post translational modification of IgG and antibodies against IgG (RF), from pulmonary rheumatoid nodule formation. It is conceivable that this process may lead to anti-TNF autoantibody development. Given that higher levels of RF

in RA patients with established disease receiving tumour necrosis factor inhibitors to treat their disease are much less likely to be in remission at 12 months follow up [36], further research to assess the effect of such exposures in relation to treatment response is needed. In particular, CT chest radiography to look for pulmonary nodules and localised tissue Cd levels may help to guide treatment in poor responders.

7.4.2 Local cadmium levels stimulating autoantibody development

My research has strengthened the evidence that multiple inhalational insults are associated with higher autoantibody levels in rheumatoid arthritis [3,4,6]. However, my hypotheses around the adsorption of heavy metals such as cadmium in the lung remain unproven. Adsorption may explain the pronounced interactions of multiple inhalational exposures and cigarette smoking [2-4,6]. However, the direct analysis of lung tissue to analyse local cadmium levels has not been undertaken, due to the difficulty in obtaining sample tissue.

I found that nodular RA smokers who were not exposed to VGDF had significantly elevated urinary cadmium levels compared to RA smokers without nodular disease. However, amongst never smokers, urinary cadmium levels were similar irrespective of nodularity. Detailed discussion around the potential pathophysiological mechanisms by which cadmium adsorption may drive nodular disease has been undertaken [6]; and urinary cadmium levels reflective of total body burden may not reflect localised levels in lung or tissue granulomata. Direct histological analysis of lung tissue to investigate this would be an interesting avenue; this could be pursued by post mortem of RA patients, exposure-based animal models, or by biotechnology-based bronchial “chip” analysis of sequential exposures to further the understanding of the pronounced interaction seen in sequential dust exposure and cadmium-laden cigarette smoke.

7.4.3 Nodular disease

Chapter 6 [6] analysed the complex relationship between inhalational exposures, rheumatoid autoantibodies and nodular disease. I have demonstrated that nodular RA significantly associates with RF and ACPA, irrespective of smoking.

Furthermore, there appears to be relationship between urinary Cd levels and nodular RA only in nodular RA patients who have not had occupational VGDF exposure; this is an interesting finding and an addition to the academic thought in this area, given the findings of previous chapters regarding autoantibody levels and clustering [3-5]. VGDF-exposed nodular patients demonstrated significantly lower urinary cadmium levels compared with matched RA controls (without visible nodular disease), despite an increased cumulative exposure to cigarettes. At first glance this appears to refute the logical hypothesis that higher cadmium levels associate with formation of rheumatoid nodules in RA patients. This appears to be true only if their VGDF exposure is accounted for.

From testing the hypotheses in this thesis, I developed the adsorption hypothesis which suggests that VGDF exposure may “trap” cadmium in the lung [33], whereby cadmium derived from cigarette smoke is adsorbed onto previously inhaled fine particulate matter, stimulating B-cell mediated local production of citrullinated proteins manifesting as rheumatoid autoantibodies. Crucially, the incorporation of cadmium-dust adsorption complexes into granulomatous tissue would not show elevated levels of toxins (such as cadmium) in the systemic circulation, and would not be reflected in total body measures such as urinary cadmium.

It has recently been suggested that a focus on the total body burden of cadmium, estimated through urinalysis, may not reflect the local burden of cadmium as there is tissue-specific retention, particularly with lung levels [8]. As inhalation exposure affects lung tissue, assessing the local accumulation of cadmium in the lungs appears more relevant. Recent laboratory analysis has proven that cadmium oxide nanoparticle exposure facilitates protein citrullination in lung epithelial cells [37]. Furthermore, cadmium chloride combined with post-combustion ultrafine carbon black particle matter demonstrated a synergistic effect on citrullination, strengthening the adsorption hypothesis [37].

To prove this hypothesis, I would suggest that future studies analyse lung tissue directly. Whilst radiological imaging such as high resolution CT can detect pulmonary nodules, the direct analysis of such nodules could analyse both cadmium levels and the immunological “machinery” capable of producing the type of immune response suggested.

In this regard, analysing nodular disease provides a useful surrogate in the overarching hypothesis of this research. As smoking only associated with raised RF levels in nodular disease, I would argue that pulmonary rheumatoid nodule formation from inhaled VGDF particles, subsequently adsorbing cadmium from other environmental sources such as cigarette smoke, both generates RF and ACPA locally and stimulates RA disease development.

Whilst this is not the only pathophysiological mechanism at work, in breaking immune tolerance to trigger disease in the spectrum of presentations that fulfil the classification criteria of clinical RA, the predominance of the patterns of exposure described here (particularly in male RA) suggest that cadmium adsorption stimulating autoantibody development is of paramount importance. Further work is needed into the underlying pathophysiological mechanisms driving nodular disease to identify distinct disease patterns, particularly given the finding that RA never smokers had a significantly younger age of onset when nodular disease was apparent.

7.4.4 Respiratory mortality

The centrality of repeated insults to the lung is apparent in this research; and the subsequent analysis of mortality undertaken in the male RA cohort detailed in Chapter 5 has revealed a high respiratory implication in mortality. Of the 56/726 (8%) deaths during the study period, 35/56 (62%) deaths were respiratory related. In particular, 21/56 (37%) deaths were recorded with COPD, bronchiectasis, or emphysema as a contributory to the cause of death [33]. This strikingly high respiratory mortality may be explained by the inhalational exposures found in the cohort; as described, 561/726 (77%) had been smokers and 546/726 (75%) had occupational exposure to vapour, gas, dust or fumes [33]. This aspect of my thesis has developed a very high level of scientific interest, and may yet be revealed as the highest impact finding.

My review in Chapter 2 has highlighted the similarities in the interactive risks for COPD and male RA [2], with VGDF exposure combined with smoking showing an increased risk of COPD. Interactive risks for male anti–citrullinated protein antibody positive (ACPA) RA have also been highlighted [4]. An increased risk is seen when silica and cigarette exposure (>20 pack years) are combined (OR 14.9; 95% CI 5.32–37.84), far higher than ever smoking alone (OR 2.53; 95% CI 1.72 to 3.72) or silica exposure alone (OR 1.67; 95% CI 1.13– 2.48) [4].

It would be interesting to explore the extent to which RA and COPD are linked by shared environmental and occupational exposures and the common processes of post-translational protein modification which drive lung citrullination. Wider datasets exist in which large-scale mathematical “mapping“ of the nature of two distinct disease entities to deepen understanding of the relationship between RA and COPD. Although the precise role of B cells in mediating lung inflammation is unknown, B-cell mediated citrullination of proteins in lung tissue has been observed in mice exposed to organic dust [38]. Further research into these processes is suggested.

Although this may be the area of greatest interest arising from my thesis, given the applicability outside of the disease of RA and the impact of publications generated [33], it must be noted RA is becoming less evident in the UK population. In addition to evidence of reduced severity [39,40] predating the

routine use of disease modifying drugs as detailed in Chapter 6 [6], falling incidence and prevalence are noted [41].

Reasons given for this include the fall in the incidence of cigarette smoking, though the authors point out that geographic variation is seen, with variation in the prevalence of RA possibly be related to socio-economic deprivation that may co-associate with primary and secondary smoking [41]. This would be particularly pertinent to the RA population of Cornwall, given the high prevalence of both smoking and social deprivation, as discussed in Chapter 1 and revealed in the evidence gathered in Chapters 3,5 and 6 [1,3-6].

I have suggested that the natural history of nodular RA may reflect the marked drop in Cd exposure in UK society. The industrial revolution was associated with a 15–fold increase in anthropogenic emissions of Cd in Western Europe [42]; and since the 1960s, there has been a 2.5–fold decrease in emissions of Cd [43]. The findings in the socio-economically deprived, co-exposed RA population may not have wider applicability to wider UK trends, but show that acknowledgement of geographical variation is vital to plan public health services. Furthermore, the lessons of exposure that this largely historical set of conditions bestow can be applied to similarly exposed populations throughout the globe, particularly in deprived, rapidly developing economies.

Finally, one cannot help but be drawn back to the findings of Haldane in the machine rock drillers of Redruth on 1900-1902 [44]. His findings of excess mortality due the combination of TB (causing bronchiectasis) and dust exposure were a fine precursor to the findings detailed here.

7.5 Additional directions for future research

7.5.1 Histological analysis of lung tissue to prove inhalational heavy metal adsorption.

On the relationship between cigarette smoke and VGDF inhalation as a pathophysiological mechanism of RA initiation through the process of heavy metal adsorption and pulmonary nodule development; an animal model prone to rheumatoid nodule formation could be utilised to determine if the combination of cigarette smoke and dust nebulised in to the lung triggers pulmonary and subcutaneous rheumatoid nodule formation. The lung concentrations of heavy metals could be tested and compared with blood and urinary levels to test the Cd/VGDF adsorption hypothesis further.

Central to this research is the proposed mechanism of adsorption of trace elements such as cadmium *in vitro* onto previously inhaled substrates. This process drives the increased interactive RA risk seen in sequential inhalational exposures, enhancing ACPA generation in the lung via inducible bronchial lymphoid tissue (iBALT) and RF generation via upregulation of IgG-heat shock protein 70 complexes stimulating production of immunoglobulin A (IgA) and immunoglobulin M (IgM) rheumatoid factor. However, no direct analysis of lung tissue has been undertaken. Given the recent finding that lung epithelium is a site for citrullination when exposed to cadmium nanoparticles [37], it would be useful to develop an animal or advanced biotechnology “chip” model, by which the histological analysis of lung tissue could be undertaken when exposed to VGDF and cigarette smoke, to determine if sequential insults enhance this process.

ACPA generation in the lung via inducible bronchial lymphoid tissue (iBALT) and RF generation via upregulation of IgG-heat shock protein 70 complexes stimulate the production of IgA and IgM RF [5]. Further research is needed to investigate the potential for combination of inhalational insults to stimulate iBALT and rheumatoid pulmonary nodule formation in the lung, with the consequent impact on immunoglobulin G misfolding in the endoplasmic reticulum and the upregulation of autoantibody generation. This is particularly pertinent given that

B cell mediated lung citrullination has already been demonstrated in response to organic dust in an animal model [45].

Using the autoantibody analysis generated in my research, I have suggested that the main mechanism by which RA smokers generate raised RF levels is via pulmonary rheumatoid nodule formation, given that pulmonary rheumatoid nodules contain germinal centres capable of autoantibody production. It has been demonstrated in an animal model that lung granulomata develop as a consequence of exposure to nebulised Cd [45]. It would be of interest to determine if these lung granulomata are RF and/or ACPA secreting.

7.5.2 Autoantibody clustering in a wider population with non- RA controls

This study did not use non-RA controls to analyse rheumatoid autoantibody levels or urinary cadmium analysis. As such, comparison cannot be made to determine risks around raised elevated autoantibody levels and urinary cadmium in developing RA; and therefore, we cannot address if multiple work exposures to VGDFs increase the risk of developing R. Further studies are needed to address this important question, which would have to include accurate occupational exposure history to allow factor analysis, and could potentially analyse different combinations and timings of mixed exposures. Given that RF levels in the general, non-RA population increases the incidence of RA development by 20-fold when comparing the lowest vs highest titre of RF [46], it is essential that studies of RF in the general population and the risk of developing RA not only consider the smoking history, but also occupational exposures.

Work in this area is ongoing with the study population outlined in this research, with ethical approval for recruitment of non-RA controls having been approved (Appendix 1), to be gathered from primary care data in Cornwall, UK, for urinary cadmium analysis. Response to the wider findings on autoantibody patterns described here is expected. Wider RA population analysis with matched cases and controls will be of interest to confirm the findings that ACPA positivity in RA strongly associates with increasing RF titre independent of smoking; and that the relationship is dependent on carriage of SE alleles. Furthermore, the possible confirmation of the combination of VGDF exposure and smoking increasing RF

levels in a non-RA population, thereby increasing the risk of RA disease development with such exposures, would be fascinating.

7.5.3 Wider investigation of a common autoantigen

The findings in Chapter 4 [5] are of importance as they suggest that immunoglobulin G (IgG) is a potential universal antigen in RA, given that the association between the Fc binding site region of IgG and RF has long been recognised; and that a citrulline antibody response against IgG exists, with bispecific antibodies having been observed in RA. The specific post-translational changes noted at the Fc binding site region of Immunoglobulin G heavy chain fragments described in Chapter 4 [5] have only been described in a small pilot study.

However, the collection of serum made in and stored as part of this research (Appendix 2), means that this can be studied more thoroughly. Furthermore, a larger study, involving this collection and study elsewhere, is needed to confirm if the post-translational modifications to the Fc region of immunoglobulin G heavy chain fragments are present in the sera of individuals who have developed RA to determine if the presence of a specific antigen and ligand is unique to RA.

7.5.4 Wider public health implications

From a wider public health perspective, the results of this research would suggest that minimisation of inhaled occupational insults would help to minimise the potential development of RA. In January 2017, the UK Industrial Injuries Advisory Council undertook a review of the literature on cadmium and autoimmune diseases [47], including RA, as a direct result of a publication by myself prior to this thesis [48]. The Council concluded that the evidence base on Cd as a putative hazard was at a preliminary stage, though conceded that it was possible that cadmium can cause RA in at least some circumstances. The Council concluded that it would continue to keep this emerging literature under review, particularly with pertinence to a doubling or more of RA risk, as the usual threshold for prescription within the Industrial Injuries Disablement Benefit (IIDB) Scheme.

We have subsequently found that RA patients displayed higher median urinary Cd levels than unexposed, non–disease UK median data [6]. In men (n=128) the median Cd levels were 0.38 (IQR 0.16–0.42) and in women (n=95) the median Cd levels were 0.57 (IQR 0.19–0.65) ($p < 0.00001$). Furthermore, in non–dust exposed individuals, an appreciably raised Cd level (>95th centile for UK unexposed population) [49] was significantly associated with nodular RA, OR 2.26 (95% CI 1.08–4.73) ($p=0.03$) [6].

I would agree that further research is needed to provide evidence on the risk that Cd poses for the development of RA , to enable the UK Government, via the Industrial Injuries Advisory Council, to recommend prescription under the IIDB scheme. This is particularly true for those patients co-exposed to VGDF and cigarette smoke in whom the relationship is not clear Current regulations such as the Control of Substances Hazardous to Health 2002 (COSHH) [50] exist to protect workers from hazardous substances such as cadmium and its compounds, that have the potential to cause harm if inhaled. The COSHH requires the employer to carry out a risk assessment, to establish the hazards associated with the substances being used, and to put processes in place to control those risks.

However, this misses the potential for combinations of exposures, such as discussed here, to cause disease, especially over the life course: it is vital that further work is carried out to investigate the potential of dramatically increased intrapulmonary levels of Cd in those who are exposed to VGDF and who are exposed to high levels of Cd exposure from cigarette smoke.

The COSHH regulations require employers to prevent exposure to toxins such as cadmium by substituting them with a safer substance, or by totally enclosing the process. Where this is not possible, COSHH requires exposure to be controlled to as low a level as reasonably practicable and for it to be adequately controlled by the use of appropriate work processes, systems, engineering controls and measures including local exhaust ventilation systems to control exposure at source. I would argue that such measures need to be considered in exposure to adsorbing VGDF substrates in addition to smoking reduction programmes to make a wider public health impact.

7.5.5 Next steps

7.5.5.1 Non-RA controls

Data collection is currently underway on 32,000 patients from primary care in Cornwall, to identify age, sex and IMD matched controls to the already collected RA patients in this study. This has been approved and extended by the Health Research Authority and primary care practices involved, with a primary care research nurse in place to collect data as per Appendix 1. An approach has been made to Exeter University to fund further study. The advent of a new General Practice contract in April 2019, with explicit reference to Primary Care Networks (PCNs) becoming the “functional unit” of general practice on the UK, opens up exciting opportunities to better design the interface between primary and secondary care for patients with rheumatoid arthritis. Being at the forefront of research in this area allows for optimal service redesign potential, both for the data gathering and analysis, but perhaps more importantly, to use the research findings to deliver tailored healthcare to the population of Mid Cornwall from where this research arose.

7.5.5.2 Analysis in other populations

The publications in this thesis have driven global interest into the role of occupational exposures and autoantibody clustering in RA, evidenced by the wide online readership numbers seen for the published chapters, particularly Chapter 3. The findings within Chapters 3 and 4 have forced a re-examination of the complex relationships between smoking, HLA genes and serology in wider literature [51], using the Swedish EIRA cohorts discussed earlier. Helping to shape this paper through peer review, I also suggested to the Editor of the journal in which it was published that this fundamental shift in understanding was worthy of an editorial article within the journal. This has also now been published [52]. Both of these pieces of citing research work help to establish my thesis in fundamentally changing perceptions at the cutting edge of rheumatology research.

Furthermore, Californian researchers examining 266/973 men in the Appalachian Mountains, USA, exposed to coal mining, found significantly higher RA self-reported prevalence (12%, odds ratio 4.4, 95% confidence interval 2.7-7.2), than in unexposed coal workers [53]. A further recent publication by the same research group found the increased risk of RA in men exposed to coal mining, when adjusted for covariates, to have a odds ratio of 3.6 (95% CI 2.1-6.2) [54]. Contact with this research group is ongoing for collaborative future work, given the common interests identified through Chapter 2.

Italian researchers have recently demonstrated that diesel fume exposure induces autophagy and citrullination in bronchial epithelium [55], strengthening the findings and discussion generated by Chapters 5 and 6.

Interest in cadmium exposure has led to publications through collaboration with other wider cohorts globally [56]. Further collaborative efforts in wider populations are planned, pending publication. (personal communication to Dr. D. Hutchinson, publication pending). Professor Veena Antony of the University of Alabama, Birmingham, is looking in to kaolin and cigarette smoke co-exposure in an animal model.

I look forward to collaborating in these global efforts to increase research in this area, both as a peer reviewer and investigator, given that this PhD thesis has stimulated such interest. The kind donation of serum and blood from RA patients studied as part of this thesis into the Exeter Clinical Research Facility, will allow further collaborations into autoantibody and genetic typing to continue.

7.6 References

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Appendix 1. Health Research Authority Protocol, IRAS ID 194833: Dust, Cadmium and Rheumatoid Arthritis.

Full Title

An investigation into the dust exposures of rheumatoid arthritis patients in Cornwall and correlation to cadmium content

Short Title

Dust, cadmium and rheumatoid arthritis

- **This protocol has regard for the HRA guidance and order of content;**

FULL/LONG TITLE OF THE TRIAL

An investigation into the dust exposures of rheumatoid arthritis patients in Cornwall and correlation to cadmium content

SHORT STUDY TITLE / ACRONYM

Dust, cadmium and rheumatoid arthritis

SPONSOR: Royal Cornwall Hospitals NHS Trust

PROTOCOL VERSION: Version 1.0 11.04.16

Research Project 2016.RCHT.30 DM:

IRAS project ID 19483

1. SIGNATURE PAGE

The undersigned confirm that the following protocol has been agreed and accepted and that the Chief Investigator agrees to conduct the trial in compliance with the approved protocol and will adhere to the principles outlined in the Medicines for Human Use (Clinical Trials) Regulations 2004 (SI 2004/1031), amended regulations (SI 2006/1928) and any subsequent amendments of the clinical trial regulations, GCP guidelines, the Sponsor's SOPs, and other regulatory requirements as amended.

I agree to ensure that the confidential information contained in this document will not be used for any other purpose other than the evaluation or conduct of the clinical investigation without the prior written consent of the Sponsor

I also confirm that I will make the findings of the study publically available through publication or other dissemination tools without any unnecessary delay and that an honest accurate and transparent account of the study will be given; and that any discrepancies from the study as planned in this protocol will be explained.

For and on behalf of the Study Sponsor:

Signature: Date:
...../...../.....
.....

Name (please print):

.....

Position:
.....

Chief Investigator:

Signature: Date:
..../...../.....

Name: (please print):

.....

2.

3. KEY STUDY CONTACTS

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Funder(s)	Cornwall Arthritis Trust, C/o Department of Rheumatology, Royal Cornwall Hospital Trust, Truro, Cornwall TR1 3LQ email
Key Protocol Contributors	As above

4. STUDY SUMMARY

This study seeks to answer the hypothesis that inhalation of dusts and fumes in the work place is associated with RA independent of smoking and that this association is confounded by the simultaneous exposure to inhaled cadmium within the dusts/fumes at the work place. Unveiling a novel mechanism of cadmium stimulating RA development in dust exposed patents would link many known independent risk factors for RA could have implications for early identification of exposed workers for interventions to minimise risk of future disease development.

1. To identify if occupational dust exposure is a risk factor for development of rheumatoid arthritis amongst Cornish males.

2. To investigate if occupational dust exposure correlates with a raised urinary cadmium level.

3. To identify if urinary cadmium level is an independent marker for disease activity (DAS 28, X-ray erosions) when controlling for smoking, social deprivation score, age, smoking and occupation.

4. Cadmium levels and anti-citrullinated calreticulin antibody positivity in RA (lab based).

Trial Title	An investigation into the dust exposures of rheumatoid arthritis patients in Cornwall and correlation to cadmium content	
Internal ref. no. (or short title)	Dust, Cadmium and Rheumatoid Arthritis	
Clinical Phase	Not applicable	
Trial Design	Single centre case control	
Trial Participants	All males with rheumatoid arthritis follow up in Cornwall 2016, age sex and social deprivation matched to controls from primary care	
Planned Sample Size	Total cohort of RA males in Cornwall: 680. Dust exposure cohort expected to be approx.60% of this= 408. Statistical model suggests a smaller sample size to gain significant results. However, data collection on individual occupation groups would be pertinent to analyse for differences	
Treatment duration	Not Applicable	
Follow up duration	24 Months	
Planned Trial Period	Planned March2016 -Feb 2017	
	Objectives	Outcome Measures
Primary	Is occupational dust exposure a risk factor for rheumatoid arthritis amongst Cornish males, and is this confounded by cadmium contained within the dust?	Positive C-reactive protein measurements, elevated urinary cadmium levels and positive anti-calreticulin antibody serum testing,

		correlated with answers from Questionnaires
Secondary	1. To investigate if occupational dust exposure correlates with a raised urinary cadmium level. 2. To identify if urinary cadmium level is an independent marker for disease activity (comparing to commonly used and validated clinical scores that are used to map disease activity such as the DAS-28 CRP measure) when controlling for smoking, social deprivation score, age, smoking and occupation. 3. *Cadmium levels and anti-citrullinated calreticulin antibody positivity in RA*	Elevated urinary cadmium levels Questionnaire response Positive anti-calreticulin antibody serum testing
Investigational Medicinal Product(s)	Not Applicable	
Formulation, Dose, Route of Administration	Not Applicable	

FUNDING AND SUPPORT IN KIND

FUNDER(S)	**FINANCIAL AND NON FINANCIALSUPPORT GIVEN**
Cornwall Arthritis Trust	£20000 financial support

Protocol contributors

Dr. D Murphy: Design, conduct, data analysis and interpretation, manuscript writing, dissemination of results.

Dr. D. Hutchinson: principal investigator and academic supervisor to Dr. D. Murphy, data guardian.

Dr. P Eggleton: Academic supervisor to Dr. D. Murphy, specific responsibility for laboratory work on anti-calreticulin antibody.

Dr. C. Crawford: Sponsor's representative for governance purposes and protocol checking.

KEY WORDS: Occupation, rheumatoid arthritis, cadmium, citrullination, heavy metals, dust

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6. STUDY FLOW CHART

STUDY PROTOCOL

An investigation into the dust exposures of rheumatoid arthritis patients in Cornwall and correlation to cadmium content

7. 1 BACKGROUND

Evidence for occupational exposures relating to rheumatoid arthritis is accumulating. Specific occupations have been associated with seropositive rheumatoid arthritis (RA),[1,2,3,4] a mix of technical and mechanical occupations including mining, construction work and textile production. The potential exists for particle inhalation via dust, fumes or aerosolisation in the workplace. Both silica and non-silica based inorganic dust inhalation are independent risk factors for RA amongst male construction workers.[4] In addition to silica, other nanoparticles inhaled within the dust include cadmium (Cd). Cadmium is found in cement dust, wood dust, and coal dust and is associated with the finishing of textiles as a pigment and stabilising agent. Further RA risk factors include lower socio-economic class, a low level of formal education, exposure to tobacco smoke and residing close to main roads.[5] Intriguingly, these risk factors and occupational risk categories also associate with high levels of cadmium exposure.[6] These environmental triggers combined with pre-existing genetic factors (such as the HLA-DRB1 shared epitope (SE)), drive development of citrullinated antigens resulting in anti-citrullinated protein antibodies (ACPAs), which may further drive RA pathology.

Wood dust exposure has long been linked with adverse health effects such as dermatitis, mucosal changes, neoplasia and a range of pulmonary conditions.[7] Pulmonary fibrosis has been noted in controlled animal studies,[8] as have inflammatory changes such as lymphocytic aggregation and increase in pulmonary connective tissue components.[9] A review of non-malignant respiratory diseases found an association between fresh wood dust exposure and asthma, coughing, bronchitis, and impairment of lung function.[10] This is of particular relevance as established literature suggests that the initiation of seropositive RA occurs in the lung.[11] Interestingly, occupational exposure to cadmium associates with chronic obstructive pulmonary disease independent of cigarette smoking.[12]

Association between malignant disease both of the lung[13] and the nasopharynx[14] has been demonstrated. A disproportionally high lifetime risk of mesothelioma development of 5.9% has been shown in UK carpenters. Again, cadmium is an independent risk factor in the development of lung maligancy.[15]

Further industrial exposure to cadmium dust may occur in those extracting raw materials. Miners may well be particularly at risk, and a re-examination of the pathophysiology of Caplan's syndrome may well be needed. First described in 1953 amongst Welsh coal miners, Caplan's syndrome classically occurs in patients with both rheumatoid arthritis and pneumoconiosis related to occupational dust (coal, asbestos, silica) exposure.[16] First demonstrated on chest radiographs, it is characterised by development of multiple pulmonary nodules 0.5-5cm throughout the lung field. High rates of rheumatoid factor seropositivity and prevalence of rheumatoid arthritis amongst coal miners with classical chest x-ray changes of Caplan syndrome were demonstrated.[17] Histologically similar to rheumatoid nodules, Caplan’s nodules may have a layer of dust surrounding a central necrotic area. Dust-containing inflammatory cells such as macrophages and polymorphonuclear granulocytes surround the dust layer,[18] distinguishable from silicotic nodules or progressive massive fibrosis.

Within coal, kaolinite is a known contaminant across the South Wales coal fields in which Caplan’s patients worked, particularly in the anthracite-producing areas such as the Rhondda. A fascinating correlation is the strong association between kaolinite mineral contamination of coal and cadmium content.[19] The cadmium content of coal varies greatly, from 0.01-0.19 mg/kg.[20] South Wales has areas of soil contaminated with cadmium of the order 3.8-11mg/kg, thought to be due to mobilisation from contaminated coalfields and historical zinc ore smelting.[21] The worldwide toxic burden of cadmium from coal burning has been noted in arctic ice cores.[22]

In terms of roadside dust, cadmium levels have been shown to markedly decrease within 20 metres from the roadside.[9] Cadmium is a component of petrol

and diesel fuel and was previously used as a curing agent in tires, a component of brake pads, alloyed with copper in the production of car radiators and in car paints. Additionally, asphalt concrete road surfacing contains appreciable amounts of cadmium.[10] We suggest that cadmium-laden ultrafine dust will occur in close proximity to busy roads as a result of both vehicular component and road surface wear, and vehicular fuel emissions. Working in such an environment would expose to this dust.

Textile dust in the workplace has also been shown to increase the risk of developing RA.[3] A review article of trace metal pollution in soils affected by industrial processes highlighted that the textile industry was associated with the discharge of very high levels of cadmium.[23] Indian[24] and Bangladeshi[25] soils around textile plants demonstrated cadmium concentrations of 83.6 to 164 mg kg^{-1}, 28-56 times higher than European Union recommended levels.[26] Cadmium based pigments are utilised in the dyeing and finishing of technical textiles and include colour index (CI) pigment yellow 35, orange 20 red 108.[27]

Cadmium is a heavy metal, mobilised from geological matrices since the industrial revolution into biologically accessible forms, leading to a range of health effects in humans as described.[28] 5-35% of inhaled cadmium may be absorbed into the blood depending on particle size, site of deposition within the lung, and chemical form. Glomerular filtration and re-absorption at the proximal tubule results in free cadmium in the serum, with concomitant glomerular damage occurring at toxic levels. Cadmium concentration in the kidney is reflected in urinary excretion, with urinary testing reflecting long-term cadmium levels as elimination half-life is up to 20 years.[29] However, health effects are seen at sub-nephrotoxic levels, and it has been argued that urinary levels may not accurately reflect end-organ damage, particularly in the lung.[9]

Further cadmium sources that the occupationally exposed construction trades encounter include the substrates they cut,[30] their cutting blades,[31] and their portable tool power sources (cadmium nickel batteries).[32] It has recently been hypothesised that exposure to cadmium (cigarette smoke, occupational exposure

and residing close to main roads and in the northeast of the US) is implicated in RA development.[6] Cadmium has the potential to cause citrullination within the lung as a calcium channel activator, significantly raising intracellular calcium levels.[33] Interestingly, a number of nanoparticles (including silica and carbon, to which many of the occupational groups identified will be co-exposed), can induce lung citrullination via activation of cellular calcium channels with a subsequent rise in intracellular calcium and activation of peptidyl arginine deaminase.[34]

A recent animal model demonstrated the potential for cadmium to drive an inflammatory arthritic process. Cadmium administered to Wistar rats exacerbated collagen induced disease development. Higher dose oral cadmium administration demonstrated immunohistochemical expression of pro-inflammatory cytokines, confirmed with histopathological analysis.[35]

Exposure to cadmium links the longevity of risk conferred by smoking[36] (the most important aetiological factor in RA development),[37] to many of the other known contemporary risk factors for RA, including occupational exposures. It has a plausible biochemical mode of action, and efficacy to drive inflammatory arthritis demonstrated in animal studies. The carpenters with RA that we have investigated thus far have all had urinary cadmium levels higher than one would expect from never smokers. From the established effects of cadmium on the lung, it has been suggested that variability in metabolic pathways relevant to absorption, storage, injury, and repair render some individuals particularly susceptible to cadmium even at low levels of chronic exposure, particularly if inhaled.[9]

8. 2 RATIONALE

We intend to examine the occupational histories of male RA patients in Cornwall, examining the records for potential industrial exposures to dust and cadmium. It is hypothesised that we may discover that cadmium is an important confounding factor with respect to RA development in those with dust exposure. We hypothesise this for the following reasons:

- In conjunction with researchers at Trinity College, Dublin, we have demonstrated that cadmium is an independent citrullinating agent, and

that the resulting citrullinated proteins are distinct from those citrullinated by silica (unpublished).

- Unlike carbon nanoparticles or silica, cadmium can enter enter the circulation, and a cumulative dose-exposure response is seen in bone marrow.
- Cadmium is associated with a pro inflammatory state.[35]

2.1 Assessment and management of risk

The results of the investigations will be given to the participant, respective hospital consultant and general practitioner. In the event that a case or control demonstrates a urinary cadmium within the toxic range, chelation therapy will be offered. Should an occupationally exposed group or subgroup demonstrate statistically significant higher levels of urinary cadmium on interim analysis, appropriate steps will be taken to inform public health authorities of the risk uncovered.

In order to obtain informed consent, potential participants will be provided with information about the study. Following time for contemplation and understanding, this will be discussed in face to face consultation with a member of the rheumatology team. If the potential participant agrees to take part, written consent will be obtained.

The risk of data confidentiality breaches will be minimised as data collected will be stored on a spreadsheet within the rheumatology file area on the Trust's password protected computer system. Specific access has to be granted for access to this, via request from the "owner" of that area (Dr Alison Endean) to IT services. Transfer of data from here once dataset complete will be permitted once all patient identifiable information is removed. Control Cases to be recruited from St. Austell Healthcare group patient population. Hence, all patient identifiable information will be kept on the host organisation's host areas. Password and training to be provided by the host organisation to members of the investigating team when needed (pre-agreed).

9. 3 OBJECTIVES AND OUTCOME MEASURES/ENDPOINTS

This study will answer the hypothesis that inhalation of Cd enriched dusts and fumes in “at risk” workplaces is associated with RA independent of smoking and that this association is confounded by the simultaneous exposure to inhaled cadmium within the dusts/fumes at the work place. Unveiling a novel mechanism of cadmium, a known citrullinating agent, stimulating RA development in dust-exposed patents would link many known independent risk factors for RA could have implications for early identification of exposed workers for interventions to minimise risk of future disease development. The background information provided highlights current evidence around dust exposure in the workplace and development of RA, and for the potential role of cadmium as a confounding factor within this.

By analysing the association between dust exposure, cadmium, and RA disease activity, with controls matched for age, gender and social deprivation, and smoking habits corrected for by linear regression analysis, we hope to further research into the pathogenesis of RA.

Furthermore, establishing a comprehensive database of occupational histories and smoking habits of RA patients, with details on disease activity, severity, and response to therapy, we hope to produce a source of information for future studies to improve diagnosis and management occupationally exposed patients with RA.

3.1 Primary objective

To determine if Cd enriched dust exposure can trigger the development of RA autoantibody markers to drive the pathology of RA**.**

3.2 Secondary objectives

- To identify if occupational dust exposure is a risk factor for development of rheumatoid arthritis amongst Cornish males.
- To investigate if occupational dust exposure correlates with a raised urinary cadmium level.
- To identify if urinary cadmium level is an independent marker for disease activity (DAS 28, X-ray erosions) when controlling for smoking, social deprivation score, age, smoking and occupation.
- Cadmium levels and anti-citrullinated calreticulin antibody positivity in RA (lab based).

3.3 Outcome measures/endpoints

Anti-citrullinated protein antibody (ACPA) measurement

ACPA status will be assessed by using an anti-cyclic citrullinated peptide (anti-CCP) second generation fully-automated fluoroenzyme immunoassay measurement. Determination of IgG antibodies against CCP to be measured in patient serum, with response value is directly proportional to the specific IgG present in the specimen.

Definition of results as per ACR/EULAR RA criteria:

- negative: within normal range
- low positive: > normal range, < 3 x upper normal range
- High positive: > 3 x upper normal range
- Very high positive: > upper limit of analytical range

DAS-28 CRP measurement

The DAS-28 CRP is a validated measure of disease activity in RA, providing a number on a scale from 0-10:[40]

- DAS-28 CRP > 5.1 indicates high disease activity
- DAS-28 CRP ≥ 3.2 to ≤5.1indicates moderate disease activity
- DAS-28 CRP < 3.2 indicates low disease activity
- DAS-28 CRP < 2.6 indicates RA remission

The following values are required for calculation:

- The number of swollen joints out of 28 (SJC28), of proximal interphalangeal joints (PIP) 1-5, metacarpo-phalangeal joints (MCP) 1-5, wrist, elbow, shoulder, knee, on the right and left.
- The number of tender joints out of 28 (TJ28), of proximal interphalangeal joints (PIP) 1-5, metacarpo-phalangeal joints (MCP) 1-5, wrist, elbow, shoulder, knee, on the right and left.
- The CRP (measured in mg/L).
- Patient's general health (GH) measured on a Visual Analogue Scale (VAS) of 100mm.

The following (usually pre-programmed computed) algorithm is then completed:

DAS28-CRP = (0.56 x √[TJC28] + (0.28 x √[[SJC28]) + (0.36 x ln(CRP+1) + (0.014 x GH) + 0.96

C-reactive protein (CRP) measurement

Serological marker of inflammation will be measured in all disease group patients. Quantification via Modular P autoanalyzer (Roche/Hitachi) immunoturbidimetric assay using reagents from Roche Diagnostics GmbH, (Mannheim, Germany). CRP sample reacts specifically with anti-human CRP antibodies coated on the latex particles to yield insoluble aggregates. The turbidimetric absorbance of aggregates is proportional to the CRP concentration in the sample. The normal range considered to be below 5mg/l.

Urinary cadmium measurement

Selected patients and controls to be provided with sterile urine sample pots to collect first pass morning urine samples. To be processed by Royal Cornwall Hospital biochemistry laboratory, Analysis to be conducted by:
SAS Trace Elements Laboratory, Surrey Research Park, 15 Frederick Sanger Road, Guildford, Surrey, GU2 7YD

Laboratory analysis: Anti-citrullinated calreticulin antibody positivity in RA

There is evidence to suggest that citrullinated calreticulin binds to the share epitope of individuals with HLA-DRB1-SE alleles and promotes proinflammatory events in bystander cells leading to RA pathology.[41,42] Moreover it has been found that increased calreticulin and anti-calreticulin antibodies are found in patients with RA.[43,44] However the presence of anti-citrullinated calreticulin antibodies have not been measured. The generation of such antibodies would indicate a) the existence of citrullinated calreticulin in patients sera that is known to bind to the HLADRB1 shared epitope and b) may indicate the generation of a 'protective' antibody against citrullinated calreticulin that inhibits its interaction with the shared epitope.

10.

11. 4 TRIAL DESIGN

4.1 Subject selection

All RA disease cohort patients under secondary care follow up at RCHT. To be recruited from the results of a questionnaire completed during an internal audit. Healthy controls to be recruited from participating GP surgeries, matched for age +/-2 years, sex (male), and social deprivation.

4.2 Deprivation matching

Patients to be matched using individual postcode data to controls from a postcode within same decile from English Index of Multiple Deprivation (EMD) 2015 data, derived from 2011 census.[39]

4.3 Questionnaire design, physician assessment and data storage

Cases: All cases of rheumatoid arthritis in males under current follow up identified through departmental records. Dust exposed occupational groups have been identified by postal return questionnaire, registered as formal audit at Royal Cornwall Hospitals Trust. All RA patients identified as potential participants by audit questionnaire on occupations and smoking history registered by Dr D Murphy 20/5/2015. Data collation has been ongoing since, with the potential for further research highlighted to patients.

Recruitment into case control study to take place via clinical appointments, Dr Hutchinson and Dr. Murphy, March 2016 to December 2016 for RA patients.

Controls: age, and sex matched from GP records at participating practices, with postcodes referred to through government deprivation data to match for Indices of Multiple Deprivation. Dust exposed occupational groups have been identified via postal return questionnaire. These will be followed up by further questionnaire, telephone consultation, or clinical appointment.

This will all be done by the direct care giving team at the Rheumatology Dept, Royal Cornwall Hospital, with assistance from trained medical students. Data recorded on Microsoft Excel spreadsheet for subsequent analysis. Personal data will be stored in a spreadsheet format in the rheumatology shared computer drive. This is a password protected area on a secure server. Access granted via specific request to IT services. Only able to be accessed via NHS computer system, computers kept in a locked room only accessible to members of the clinical care team.

Once the data set is complete, any data leaving the trust will be anonymised for export outside of NHS setting, with all patient identifiable information removed. Data will be stored at the Rheumatology dept, standard access and security

arrangements for research data as detailed above: secure server, password protected under custodian Dr. D. Hutchinson, which will be stored for 5 years.

Anti-calreticulin antibody serum testing will be processed by Dr. Dan Murphy under supervision of Dr. Paul Eggleton at University of Exeter Medical School Laboratory. Patient identifiable information will be converted to unique patient identifier prior to transport and subsequent and analysis.

Patient identifiable information will be converted to a unique patient identifier prior to transport and subsequent analysis, to be related back to individual patient record by direct care team.

Clinician review will be undertaken at a follow up clinical appointment to ensure up to date clinical measures (ACPA, DAS-28 CRP), demographic data and smoking histories are present, and inclusion criteria are met.

12. 5 STUDY SETTING

A single centre, case control study comparing RA males with occupational dust exposure, RA alone, with control groups of non-RA with occupational dust exposure, non-RA unexposed.

Cases selected from follow-up RA population at The Royal Cornwall Hospital Trust, Truro, Cornwall, UK (RCHT): RCHT serves a population of 400,000. There are approximately 2250 RA patients under regular follow up, 680 who are male.

Controls to be selected from existing GP records from practices in Cornwall, UK. Controls positively selected on basis for matching age, sex, and social deprivation indices of cases. St Austell group practices have given written permission to search their records to match controls and subsequently approach patients to be potential participants.

Lab work will be carried out at UEMS Research Laboratories (St. Luke's Campus and RILD).

13. 6 ELIGIBILITY CRITERIA

The patient population comprise male RA patients currently being followed up at the Royal Cornwall Hospital during the study period. Matching controls will be positively identified from participating GP practices.

6.1 Inclusion criteria

Generic inclusion criteria (all groups):

- Age range 18-95
- Gender: Male participants

Inclusion criteria (RA, dust exposure):

- Diagnosis of RA based on approved classification criteria[38]
- Exposure to occupational dust via working in occupations as detailed by Hutchinson[6] for >1 year

Inclusion criteria (RA alone):

- Diagnosis of RA[38]
- Absence occupational dust exposure on occupational questionnaire

Inclusion criteria (Dust exposed, non-RA):

- Exposure to occupational dust via working in occupations as detailed by Hutchinson[6] for >1 year
- Absence of inflammatory arthritis or connective tissue disorder in past medical history

Inclusion criteria (No dust exposure, non-RA):

- No exposure to occupational dust via working in occupations as detailed by Hutchinson[6] for >1 year over lifetime
- Absence of inflammatory arthritis or connective tissue disorder in past medical history

6.2 Exclusion criteria

It is expected that participants may have significant comorbidity. However, adults lacking capacity to consent. And those unable or unwilling to comply with the protocol will be excluded.

14. 7 TRIAL PROCEDURES

7.1 Planned recruitment start date

Recruitment Expected to Start May 2016.

15.

7.2 Recruitment

Participants for all groups identified through existing departmental and GP records, utilizing search criteria for males with rheumatoid arthritis via clinical coding for cases, cross-checked with laboratory records for anti-CCP testing since 2007 in Cornwall to identify potential missed cases not currently coded but under follow up.

Participants will be informed of the study on clinical review with the rheumatology team with information of study provided on clinical review. Cases will then be approached with a telephone call for further information and expression of willingness to take part. If willing, further clinical review to be arranged for consent form signing and sample taking. Written information will be provided on the day of consenting again to ensure participants have read and understood the information.

For controls, to identify via age, sex and social deprivation index score dependent searches of existing data within GP practice. Controls will then be approached with a telephone call for further information and expression of willingness to take part. If willing, information will be sent out by post or email. Further clinical review to be arranged for consent form signing and sample taking. Written information will be provided on the day of consenting again to ensure participants have read and understood the information.

All participants will be approached via telephone call from clinical team for potential involvement in research prior to written information being provided. Timings of intervals for each potential participant will vary, but all are expected to be >24 hours between each step. Provision of information on the day will be used as a contingency in case information has not reached the patient, and/or information has been lost or forgotten.

General information posters on occupation and rheumatoid arthritis are present in the rheumatology department, but this is not an overt recruitment tool and is for information only.

Only a member of the patient's existing clinical care team, working with medical students, will have access to patient records without explicit consent in order to identify potential participants, check whether they meet the inclusion criteria or make the initial approach to cases. Dr. Murphy will make the initial approach to potential controls following identification from records at participating GP practices.

7.3 Consent

Informed consent will be obtained prior to the participant undergoing any activities that are specifically for the purposes of the study.

Following provision of information as described above, an understanding check will be performed on clinical review by member of direct clinical team. Consent to be taken by Dr. Murphy or Dr. Hutchinson, or designated members of the direct clinical team of the rheumatology department following training and protocol guidance under direct supervision.

The process of consent will provide:

- presentation of written material (information leaflet and consent document following approval by REC)

- the opportunity for potential participants to ask questions in face to face clinical meeting
- discussion between the potential and an individual knowledgeable about the research about the nature and objectives of the study and possible risks associated with their participation
- assessment of capacity. For consent to be ethical and valid in law, participants must be capable of giving consent for themselves. A capable person will:
    - understand the purpose and nature of the research
    - understand what the research involves, its benefits (or lack of benefits), risks and burdens
    - understand the alternatives to taking part
    - be able to retain the information long enough to make an effective decision.
    - be able to make a free choice
    - be capable of making this particular decision at the time it needs to be made (though their capacity may fluctuate, and they may be capable of making some decisions but not others depending on their complexity)
    - where participants are capable of consenting for themselves but are particularly susceptible to coercion, it is important to explain how their interests will be protected

7.4 Study Assessments

Study participant will be invited to a routine clinic appointment where they will be provide a one off urine sample. A one off anti CCP blood test will be added to the list of routine blood tests if it has not been conducted previously. Participants will be asked questions regarding their occupation and dust exposure for correlation with the blood and urine results.

7.5 Long term follow-up assessments

Participants will be followed up according to their routine clinical follow up. Any positive blood tests in the controls will be follow up in clinic and a treatment offered accordingly.

7.6 Withdrawal criteria

Participants are free to withdraw from participation at any time.

7.7 Storage and analysis of samples

Urine and blood samples will be stored within the microbiology lab or dedicated refrigerators with the Research department until there are sufficient samples to batch send for analysis. The samples will be disposed of according to Laboratory's waste product's procedure.

7.8 End of study

The study is scheduled to end at completion of analysis expected February 2017.

16.

17. 8 STATISTICS AND DATA ANALYSIS

Sample size calculations have been performed to determine sample sizes for specific questions related to the research. However, it is our intention to collect urinary cadmium samples from all of our RA population. Such data will be collected as little data currently exists in this area, and sample size calculations are based on assumptions that may not prove to be valid in clinical practice amongst the population studied. Widening the sample size will allow for a more robust dataset to be gathered. Furthermore, we expect to find interesting differences within certain occupational subgroups. A wider sampling size will

allow for more thorough subgroup analysis. For these reasons, we intend to sample our entire male RA follow-up population that meet the inclusion criteria.

8.1 Sample size calculation

Occupations in RA case-control study:

RA males matched for age, gender, social deprivation score

Defined groups:

- RA males with occupational dust exposure
- RA alone
- Control groups of non-RA with occupational dust exposure
- Non-RA unexposed

Null Hypothesis: there is no association between occupational dust exposure and RA development.

Prevalence of dust exposed occupational categories etc., in the background male population of Cornwall is thought to be in the region of 28%, summarised in Table 1:[45]

Table 1: Census data of male occupations in Cornwall

Occupational group	**Male %**
Mining	1.1
Manufacturing	11.3
Construction	15.6
Total	28

Prevalence of dust exposed occupational categories in the male RA population of Cornwall studied is thought to be in the region of 57% (pilot data, n=385)

Sample size calculated as test of 1 proportion (p), 1 sample, 2 sided equality: is the proportion(p) equal to the reference value (p0)? Null hypothesis (H0): p=p0, Alternative hypothesis (H1): p≠p0

$n=p(1-p)[(z1-\alpha/2+z1-\beta)/p-p0]^2$

$1-\beta=\Phi(z-z1-\alpha/2)+\Phi(-z-z1-\alpha/2),z=p-p0p(1-p)n$

where

- n is sample size
- p0 is the comparison value
- Φ is the standard normal distribution function
- Φ−1 is the standard normal quantile function
- α is Type I error
- β is Type II error (1−β is power)

sample size =26

If the hypothesis is correct we expect the dust exposed groups to demonstrate a stronger association of RA development. Using this proportionality method, we estimate that with 26 cases and 26 controls we have a 90% power (β type II error = 0.1) to detect a statistically significant increase in proportion of RA prevalence in dust-exposed workers. (2-sided test, p=0.05 α type I error = 0.05).

Does occupational dust exposure correlate with a raised urinary cadmium level? Case control study with controls for smoking, social deprivation score, age, and occupation.

If the null hypothesis is true, (i.e. there is no association between dust exposure and urinary cadmium levels), we would not expect to find any significant

differences in urinary cadmium levels between to dust and non-dust exposed groups.

Personal communication has indicated that urinary cadmium levels in RA dust exposed workers are 3-4 fold higher than the normal range found in healthy individuals. This correlates with the 3-4 fold higher levels of cadmium found in hair samples of RA patients by Afridi et al, irrespective of smoking.[46] Morton et at al[47] found a median urinary cadmium concentration of 0.15µmol/mol creatinine (n=82, 95th centile 0.42µmol/mol creatinine) in healthy males. From this, one would expect to find urinary cadmium values of 0.45-0.6µmol/mol creatinine in our dust exposed cohorts if our hypothesis is correct. For each group we would expect:

RA and dust exposure = urinary cadmium 0.45

RA alone = urinary cadmium 0.15

Non RA, Dust exposure alone = urinary cadmium 0.45

Non RA, unexposed = urinary cadmium 0.15

No published data has been found to further quantify the reliability of this estimate in larger groups with respect to urinary levels in RA. Further subdivision may be possible to quantify differences based on seropositive (ACPA positive) vs seronegative RA depending on dataset.

Whilst imperfect, attempting to utilise the average urinary cadmium concentration given[47] as a mean average, with 95 % CI and SE of 2x1.96, we can estimate a SD of:

SD= 9.055 x (0.42-0.15)/3.92

SD= 0.63

Therefore to calculate the difference of 2 “means” as an average of 2 expected datasets:

$nA=\kappa nB$

and

$nB=(1+1/\kappa)(\sigma[z1-\alpha/2+z1-\beta/\mu A-\mu B])^2$

$1-\beta=\Phi(z-z1-\alpha/2)+\Phi(-z-z1-\alpha/2), z=(\mu A-\mu B)/\ \sigma\sqrt{}(1/nA)+(1/nB)$

where

$\kappa=nA/nB$ is the matching ratio

σ is standard deviation

Φ is the standard Normal distribution function

α is Type I error (0.05)

β is Type II error, meaning $1-\beta$ is power (0.8)

and

Group A mean= 0.15

Group B mean= 0.45

Standard deviation= 0.62

Sampling ratio= 1:1

Sample size= 67

If our hypothesis is correct we expect the dust exposed groups to demonstrate a higher level of urinary cadmium. Using this estimate of interpreted, expected average 2 value comparison with 2 sided equality, we estimate that with 67 cases and 67 controls we have an 80% power (β type II error = 0.2) to detect a statistically significant increase in proportion of RA prevalence in dust-exposed workers. (2-sided test, p=0.05 α type I error = 0.05).

Is urinary cadmium level an independent marker for disease activity? (DAS 28, X-ray erosions) when controlling for smoking, social deprivation score, age, smoking and occupation.

Further analysis of dataset is required to ascertain levels of disease activity in dust exposed and unexposed groups of RA males

Urinary cadmium levels and anti-citrullinated calreticulin antibody positivity in RA (lab based)

Our previous published studies indicate that cohorts of 50 or more patients provide sufficient power. We will be consulting with statisticians with experience in calculating power of such studies to determine sample size for calreticulin testing.

8.2 Sampling technique

Cases selected using purposive sampling of male RA patients under follow up at Royal Cornwall Hospital, Truro, 2016-2017.

Controls selected from GP records matched for age, sex, social deprivation

Rationale: to enable most robust dataset possible for analysis of occupations, potential for subgroup analysis. Controls selected from same geographical area as cases.

Random sampling from cases for subset analysis of anti-calreticulin antibody levels.

9 DATA HANDLING

9.1 Data collection tools and source document identification

An occupational questionnaire has been used within an audit provides the baseline information for identifying potential participants. The answers provided for this audit will form the basis for comparison with the sample results. Potential participants who were not part of the audit and also the controls will be required to complete the questionnaire.

9.3 Data handling and record keeping

Personal data will be stored in a spreadsheet format in the rheumatology shared computer drive. This is a password protected area on a secure server. Access granted via specific request to IT services to members of the investigatiing team on an individual basis. Only able to be accessed via NHS computer system, computers kept in a locked room only accessible to members of the clinical care team. No patient identifiable data will leave the Trust

9.4 Access to Data

Data will be stored by Rheumatology dept, standard access and security arrangements for research data as detailed above: secure server, password protected under custodian Dr. D. Hutchinson, stored for 5 years.

Access will be limited to the minimum number of individuals necessary for quality control, audit, and analysis. A sponsor's representative may require access to all data for audit and governance purposes

9.2 Archiving

The study documents will be archived for 5 years.

10 MONITORING, AUDIT & INSPECTION

The study is subject to internal audit.

18. 11 ETHICAL AND REGULATORY CONSIDERATIONS

11.1 Research Ethics Committee (REC) review& reports

Ethical approval is to be obtained and appended in final review. Study has been designed to protect the dignity and confidentiality of participants by the contact, acquisition, storage and dissemination of finding s as detailed in the proposal.

This protocol has been prepared in line with relevant legislation for submission to HRA for approval.

In patient terms:

The possible disadvantages and risks of taking part

The blood sample we need for the study is the same process as any blood test normally undertaken. To minimise discomfort, with consent, we will use some of the blood taken for normal monitoring blood tests. If no monitoring blood tests are undertaken, taking part in the study may mean we would need to take a blood test. We know that people who have special proteins (ACPAs) are more likely to have rheumatoid arthritis. Therefore if there is dust exposure and no rheumatoid arthritis, and we find the special proteins via a blood test taken at a later date, it does not mean the participant has rheumatoid arthritis or will get rheumatoid arthritis. What it does tell us is that they could be at increased risk of rheumatoid arthritis in the future. Some people may not want to know if they could be at higher risk of developing rheumatoid arthritis and may not wish to take part.

If the amount of cadmium in your urine is very high to the point where your immediate health is at risk, we may call you for another appointment to offer medicine to lower your cadmium levels.

The possible benefits of taking part

We know that treating rheumatoid arthritis early is very important. By identifying people who are at increased risk of developing rheumatoid arthritis, we can then monitor these people more closely, so if they then develop rheumatoid arthritis we can start treatment earlier and try and stop people from suffering ill health.

11.2 Assessment and management of risk

A risk assessment has been undertaken to identify potential risks associated with the study and factors in place to reduce harm to participants. (See table of risks and benefits below)

Table 2 Identified risk and mitigation.

Identified risk	Management strategy
Participant demonstrates ACPA positivity and does not have a diagnosis of rheumatoid arthritis,	Participant will be reassured that a proportion of the normal population demonstrate such seropositivity, and it would be important to report any joint symptoms in the future. Their general practitioner will be informed of the result, and they may be offered a rheumatology appointment if they wish to discuss the results further.
Participant demonstrates a urinary cadmium within the toxic range.	chelation therapy with EDTA will be offered (method of removing cadmium from the body)
Occupationally exposed group or subgroup demonstrate statistically significant higher levels of urinary cadmium on interim analysis,	Appropriate steps will be taken to inform public health authorities of the risk uncovered, via contact to Public Health England South west tel 0300 303 8162
Participant alludes to chronic health problem unrelated to area of study, needing review	Signpost to General Practitioner for review and advice
Participant alludes to acute health problem unrelated to area of study, needing review	Signpost to relevant healthcare professional depending on nature of problem: may include GP, specialist nurse, referral to hospital inpatient teams or emergency department

Identified risk	Management strategy
Participant alludes to life-threatening health problem unrelated to area of study, needing review	Immediate lifesaving measures as appropriate measures such as advanced life support, referral to relevant team: emergency department, critical care outreach, psychiatric liaison services. In case of ongoing suicide risk, patient to be kept in a place of safety pending psychiatric review.
Participant demonstrates worsening control of rheumatoid arthritis, such as flaring of disease or medication side effect	Contact Dr. Murphy, who will arrange further clinical review and discussion of relevant treatment adjustments with Patient's consultant at earliest opportunity

11.3 Research Ethics Committee

Before the start of the study, approval will be sought from a REC (researchers should check if they are required to gain NHS REC or other REC approval) for the study protocol, informed consent forms and other relevant documents

Substantial amendments that require review by REC will not be implemented until the REC grants a favourable opinion for the study

All correspondence with the REC will be retained.

It is the Chief Investigator's responsibility to produce the annual reports as required.

The Chief Investigator will notify the REC of the end of the study.

An annual progress report (APR) will be submitted to the REC within 30 days of the anniversary date on which the favourable opinion was given, and annually until the study is declared ended.
If the study is ended prematurely, the Chief Investigator will notify the REC, including the reasons for the premature termination.
Within one year after the end of the study, the Chief Investigator will submit a final report with the results, including any publications/abstracts, to the REC.

11.4 Peer review

The study and protocol has undergone expert and proportionate peer review process prior to commencement. The initial peer review was conducted by academic supervisors (Dr Hutchinson & Dr. Eggleton). Subsequent review has been undertaken by the sponsoring NHS trust R&D team.

11. 5 Patient & Public Involvement

From the outset, this research has been designed in conjunction with the rheumatoid arthritis population of Cornwall. The ideas behind this research were presented at a local meeting of the National Rheumatoid Arthritis Society, attended by over 100 members, the majority of whom were RA patients. Subsequent interest following the meeting of many RA sufferers led to further informal discussion as to the development of a protocol that would be acceptable to patients in terms of its design and acceptability. From initial ideas, this was refined and presented to the committee of the Cornwall Arthritis Trust, a local charity supporting arthritis sufferers.

In agreeing to fund the research, the board of Cornwall Arthritis Trust provided invaluable support in acting as a "sounding board" for how best to manage and undertake the research in a way that was minimally intrusive for patients in their regular clinical care.

11.6 Protocol compliance

Protocol will be conducted in accordance with the sponsor's SOPs. Protocol non-compliance is not expected however, they can occur from time to time. A deviation log will be kept in the site file.

11.7 Data protection and patient confidentiality

All investigators and study site staff must comply with the requirements of the Data Protection Act 1998 with regards to the collection, storage, processing and disclosure of personal information and will uphold the Act's core principles.

11.7 Indemnity

The NHS indemnity scheme will apply for this study. No arrangements for compensation in the event of harm to participants where no legal liability arises.

11.8 Amendments

Investigating team will be responsible for the decision to amend the protocol and the sponsor will be responsible for deciding whether an amendment is substantial or non-substantial.

Substantive changes will be communicated to relevant stakeholders via the Chief Investigator. Amendment history will be tracked to identify the most recent protocol version, and it is expected that all substantial amendments will be submitted on the relevant notification template that can be accessed from IRAS, and sent in accordance with the instructions in IRAS as per guidance at: http://www.hra.nhs.uk/nhshsc-rd-uk-process-management-amendments/

11.9 Access to the final study dataset

All members of the investigating team will have access to the final dataset. As a single site investigation (with separate GP practice involvement for control matching) there is no recourse to restrict access for study investigators at any point during the study.

12 DISSEMINIATION POLICY

12.1 Dissemination policy

- Data arising will remain under the ownership of the investigating team
- On completion of the study, the data will be analysed and tabulated and a Final Study Report prepared
- The full study report is aimed to be published as an MD thesis
- Investigating team retain the rights to publish extracts of study data subject to peer review and with acknowledgement of other members of investigating team
- Cornwall Arthritis Trust to be acknowledged as funder on subsequent publications. Cornwall Arthritis Trust does not have any review or data access rights.
- In-department poster presentation and in-department displays of published material will be used to disseminate findings. Personal findings to be communicated with individual patients and GP

- The study protocol, full study report, anonymised participant level dataset, and statistical code for generating the results will be made publicly available via media as described above.

12.2 Authorship eligibility guidelines and any intended use of professional writers

The final study report will be under the authorship of Dr. D. Murphy, under the clinical and academic supervision of Dr. D Hutchinson and academic supervision of Dr. P. Eggleton.

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14. APPENDICIES

14.1 Appendix 1- CVs of investigating team

CURRICULUM VITAE

Name: Daniel Murphy	
Present appointment: *(Job title, department, and organisation.)*	
Trust Grade Doctor, Rheumatology Dept, Royal Cornwall Hospitals Trust	
Address: *(Full work address.)*	
Rheumatology Dept, Royal Cornwall Hospital, Treliske, Truro, TR1 3LJ	
Telephone number: , Direct line: 01872 253978 Dept fax: 01872 252845	**Email address:** Daniel.murphy8@nhs.net
Qualifications:	
1996-1999: Loughborough University, BSc Physical Education and Sports Science 2002-2003: College of St. Mark and St. John, Postgraduate Certificate in Education 2007-2012: Peninsula Medical School, BmBs Medicine	
Professional registration: *(Name of body, registration number and date of registration.)*	
General Medical Council number 7280893, August 2012	
Previous and other appointments: *(Include previous appointments in the last 5 years and other current appointments.)*	

Aug 2014-Feb 2016: General Practice Trainee, Royal Cornwall Hospital Aug 2012-Aug 2014: Foundation Doctor, Royal Cornwall Hospital
Research experience: *(Summary of research experience, including the extent of your involvement. Refer to any specific clinical or research experience relevant to the current application.)*
2015: Literature search and commentary analysis on occupational exposures in rheumatoid arthritis. 2014: Designed and led retrospective cohort analysis into cases of reptile associated salmonellosis in children aged 5 or under in Southwest UK. Findings published internationally in academic and mainstream media. 2012: Designed and led investigation into discharge summary preparation at Royal Cornwall Hospital as a medical student, leading to presentation at international conference and subsequent publication, changing practice.
Research training: *(Details of any relevant training in the design or conduct of research, for example in the Clinical Trials Regulations, Good Clinical Practice, consent or other training appropriate to non-clinical research. Give the date of the training.)*
1997-1998: Training given as research assistant in postgraduate investigations into amino acid supplementation in endurance cycling performance: double blind RCT protocol administration 2007-2012: Research methodology as part of undergraduate degree 2012: Statistical analysis training for methodology into discharge summary analysis investigation 2013: In-house training given during secondment to Public Health England for research purposes

Relevant publications: *(Give references to all publications in the last two years plus other publications relevant to the current application.)*	
Murphy D, Mathew A, James B, Hutchinson D. Could the inhalation of cadmium and other metals in addition to textile dust account for the observed increased risk of rheumatoid arthritis in textile workers? Ann Rheum Dis 2016; Published online 9th February 2016. DOI:*10.1136/annrheumdis-2016-209228* Murphy D, Hutchinson D. Cadmium, road dust and rheumatoid arthritis: an alternative hypothesis to general air pollution. J Inflamm 2015;12:58. DOI: 10.1186/s12950-015-0103-2 Murphy D, Sinha A, Hutchinson D. Wood dust: a trigger for rheumatoid arthritis? Am J Med 2015;128(12):e35. DOI: 10.1016/j.amjmed.2015.06.054 Murphy D, Oshin F. Reptile-associated salmonellosis in children aged under 5 years in South West England. Arch Dis Child. 201;100(4):364-5. doi: 10.1136/archdischild-2014-306134	
Signature:	**Date:**
D. Murphy	21 March 2016

CURRICULUM VITAE

<table>
<tr><td colspan="2">Name: David Hutchinson</td></tr>
<tr><td colspan="2"></td></tr>
<tr><td colspan="2">Present appointment: (Job title, department, and organisation.)</td></tr>
<tr><td colspan="2">Honorary Senior Lecturer in medicine, University of Exeter .

Consultant Rheumatologist Royal Cornwall Hospital Trust</td></tr>
<tr><td colspan="2">Address: (Full work address.)</td></tr>
<tr><td colspan="2">Rheumatology Department, Royal Cornwall Hospital Trust, Truro, TR1 3LJ

; Fax +44 (0)1872 252845 e-mail:</td></tr>
<tr><td>Telephone number: UK. Tel. + 44

(0)1872253978</td><td>Email address:

david.hutchinson5@nhs.net</td></tr>
<tr><td></td><td></td></tr>
<tr><td colspan="2">Qualifications:</td></tr>
<tr><td colspan="2">1986–1991: Leicester University. Leicester, England.

MB ChB

1994: MRCP

2003: MD

2005: FRCP</td></tr>
</table>

Professional registration: *(Name of body, registration number and date of registration.*
GMC 1991
Previous and other appointments: *(Include previous appointments in the last 5 years and other current appointments.)*
House Officer Appointments Aug 1991 – Aug 1992 Six months medicine – Pilgrim Hospital, Boston Six months surgery – Pilgrim Hospital, Boston Senior House Officer Appointments: Aug 1992 – Feb 1993 Six months general medicine – Gibraltar, Southern Spain Feb 1993 – Aug 1993 General medicine – Stafford District General Aug 1993 – Aug 1994 General medicine – North Staffordshire Hospitals: Cardiology, Neurology, Diabetes and Endocrine, Renal Aug 1994 – Oct 1995 General medicine – Derby General Hospital: Gastroenterology, Hepatology, Specialist Registrar Rotation rheumatology: Oct 1995 – Oct 1997 Rheumatology – Whiston and St Helens hospitals, Merseyside Oct 1997 – Oct 1998 Full-time Rheumatology – University Hospital Aintree Oct 1998 – Oct 2001 Rheumatology Lecturer, University of Liverpool, University Hospital Aintree

Oct 2001- present Rheumatology Consultant Royal Cornwall Hospital, Truro Cornwall, Honorary Senior Lecturer at the University of Exeter Medical School.

Research experience: *(Summary of research experience, including the extent of your involvement. Refer to any specific clinical or research experience relevant to the current application.)*

- **Overview of research experience:**

M.D THESIS: Cigarette smoking and rheumatoid arthritis by Hutchinson, David, M.D., UNIVERSITY OF LEICESTER (UNITED KINGDOM), 2003, 221 pages; U181154. (314 Citations up until Oct 2014)

Cigarette Smoking and Rheumatoid arthritis

This thesis was undertaken whilst I was a clinical lecturer at University Hospital Aintree,

Cigarette smoking is now acknowledged as the most important environmental risk factor for RA development.

The principle aim of my thesis was to test the hypothesis that heavy smoking is an aetiological factor in RA and generates a distinct subgroup of the disease definable in terms of clinical phenotype, particularly severity.

A second aim was to investigate possible molecular mechanisms linking smoking with RA and what I believed to be candidate mechanisms involving the glutathione S transferase Mu 1 (GST M1) gene and oxidative damage to the alpha 1 antitrypsin protein (alpha1 PI).

These studies involved a review of the literature regarding the link between RA and both smoking and alpha1 PI deficiency. I investigated the relationship between heavy cigarette smoking and hospital based, more severely affected RA

patients. Additionally the age of onset and smoking history was compared in familial and sporadic RA cases. Regarding smoking and severity of RA, a cohort of RA patients were studied to determine if smoking was an independent risk factor for severe RA and whether this effect was influenced by polymorphisms of the detoxifying genotype GST M1. Oxidative damage in RA to the alpha-1 PI protein was studied in relation to rheumatoid disease activity, GST M1 and cigarette smoking. The oxidative damage to alpha-1 PI was measured in terms of serum levels of Immunoglobulin A- alpha-1 PI (IgA-alpha-1 PI).

In summary, I demonstrated that heavy cigarette smoking is strongly associated with hospital based RA. Secondly that familial RA presents at an earlier age than sporadic RA in individuals smoking at disease onset only and that sporadic RA patients are significantly more likely to smoke at disease onset than familial RA patients. I have confirmed previous findings that raised serum IgA-alpha-1 PI levels are associated with erosive as opposed to non-erosive RA cases and demonstrated that principally these raised serum complexes occur as a result of cigarette smoking and increased disease activity.

It was also demonstrated that in RA patients attending hospital outpatients with longstanding disease, cigarette smoking is associated with a worse prognosis in terms of joint damage (Larsen score) and disability (HAQ score). Potentially underlying this association was the finding of a greatly increased prevalence and concentration of rheumatoid factor in smokers. A correlation between the number of years smoked and pack years smoked by the patient and rheumatoid factor levels was observed.

- **Post graduate Students -** I have supervised 1 MD student (Exeter).

Research training: *(Details of any relevant training in the design or conduct of research, for example in the Clinical Trials Regulations, Good Clinical Practice, consent or other training appropriate to non-clinical research. Give the date of the training.)*

I have spoken at national meetings regarding lung disease and rheumatoid arthritis.

Teaching and Research- Medical Students, Peninsula Medical School/University of Exeter Medical School. Lead consultant for rheumatology teaching at the Royal Cornwall Hospital Trust. 6 students per year as part of rheumatology Special Study Unit culminating in student presenting posters or oral presentations at national and international meetings. Member of the Arthritis Research UK USER committee over 3 years until 2015

Clinical Research Network lead for musculoskeletal disease SW Peninsula 2014 onwards

Relevant publications: *(Give references to all publications in the last two years plus other publications relevant to the current application.)*

- Quirke, A.-M., Perry, E., Cartwright, A., Kelly, C., De Soyza, A., Eggleton, P., Hutchinson, D. and Venables, P. J. (2015), Bronchiectasis Is a Model for Chronic Bacterial Infection Inducing Autoimmunity in Rheumatoid Arthritis. Arthritis & Rheumatology, 67: 2335–2342. doi: 10.1002/art.39226
- Perry, E., Eggleton, P., De Soyza, A., Hutchinson, D. and Kelly, C. (2015), Increased disease activity, severity and autoantibody positivity in rheumatoid

arthritis patients with co-existent bronchiectasis. International Journal of Rheumatic Diseases. doi: 10.1111/1756-185X.12702

- Hutchinson, D. (2015), Cadmium, one of the villains behind the curtain: has exposure to cadmium helped to pull the strings of seropositive rheumatoid arthritis pathogenesis all along?. International Journal of Rheumatic Diseases, 18: 570–573. doi: 10.1111/1756-185X.12673.
- Dan Murphy, Aishwarya Mathew, Benjamin James, David Hutchinson. (2016), Could the inhalation of cadmium and other metals in addition to textile dust inhalation account for the observed increased risk of rheumatoid arthritis in textile workers? Ann Rheum Dis doi:10.1136/annrheumdis-2016-209228
- Daniel Murphy, David Hutchinson. (2015), Cadmium, road dust and rheumatoid arthritis: an alternative hypothesis to general air pollution. Journal of Inflammation201512:58 DOI: 10.1186/s12950-015-0103-2.
- Daniel Murphy, Alisha Sinha, David Hutchinson. (2015), Wood Dust: A Trigger for Rheumatoid Arthritis? DOI: http://dx.doi.org/10.1016/j.amjmed.2015.06.054
- Behçets- A man with multiple venous thrombosis .D Hutchinson, R Moots. Postgraduate journal of medicine 1999;75:254-255
- Haloperidol old drug , new tricks. Z Al-Saffar, D Hutchinson, RN Thompson, SP Golding, JP McLaughlin and RJ Moots. Ann rheum dis 1999; 58:
- Devics Neuromyelitis optica A primary autoimmune condition. D Hutchinson, R Moots Rheumatology 2000 Feb;39(2):215-7.
- Implementation of antithrombotic management in atrial fibrillation. McNulty SJ, Hutchinson D, Hardy KJ. Postgrad Med J. 2000 Dec;76(902):783-6.

- RA, poverty and smoking. Hutchinson D, Moots R. Ann Rheum Dis.2000;59:396-397.
- Diagnostic evaluation of classification criteria for RA and reactive arthritis. Hutchinson D, Moots RJ. Ann Rheum Dis. 2000 May;59(5):396-7.
- Heavy cigarette smoking is strongly associated with rheumatoid arthritis (RA), particularly in patients without a family history of RA. Hutchinson D, Shepstone L, Moots R, Lear JT, Lynch MP. Ann Rheum Dis 2001;60:223-27.
- The influence of current cigarette smoking on the age of onset of rheumatoid arthritis (RA) in individuals with sporadic and familial RA. Hutchinson D, Lynch MP, Moots RJ, Thompson RN, Williams E. Rheumatology. 2001;40:1068-70.
- Heavy cigarette smoking and rheumatoid arthritis: rheumatoid factor production may not be the primary contributing mechanism. Hutchinson D, Moots RJ. Ann Rheum Dis 2001;60:1154-5.
- Coffee consumption, RF, and the risk of RA. Hutchinson D, Moots RJ. Ann Rheum Dis 2001;60:540-541.
- Are cases of rheumatoid arthritis in smokers and lifelong nonsmokers representative of different rheumatoid disease processes? Hutchinson D. Arthritis Rheum. 2001;44:2942-3.
- Cigarette smoking and severity of rheumatoid arthritis. D Hutchinson, RJ Moots. Rheumatology (Oxford). 2001;40:1426-7.
- Late onset Raynaud's phenomenon, hypertension, deteriorating renal function, and a fit in a middle aged woman. Hutchinson D, Moots RJ. : Postgrad Med J. 2001 Dec;77(914):E10.

- Sigmoid diverticular abscess perforation in 2 patients with rheumatoid arthritis treated with high dose corticosteroids. A cautionary tale. Hutchinson D, Lynch M. : J Rheumatol. 2001 Aug;28(8):1935-6.
- Smoking and disease severity in rheumatoid arthritis: association with polymorphism at the glutathione S-transferase M1 locus. Derek L Mattey, David Hutchinson, Peter T Dawes, Nicola B Nixon, Sheila Clarke, June Fisher, Ann Brownfield, Julie Alldersea, Anthony A Fryer, Richard C Strange. Arthritis Rheum. 2002;46:640-6.
- Serum complexes of immunoglobulin A-□1-proteinase inhibitor in rheumatoid arthritis: Association with current cigarette smoking and disease activity. David Hutchinson, Peter T Dawes, Nicola B Nixon, Derek L Mattey. Clin Exp Rheumatol 2002;20:387-391.
- Association between bronchiectasis and smoking in patients with rheumatoid arthritis. Kaushik VV, Hutchinson D, Desmond J, Lynch MP, Dawson JK Ann Rheum Dis. 2004 Aug;63(8):1001-2.
- Steroids, non-steroidal anti-inflammatory drugs, and sigmoid diverticular abscess perforation in rheumatic conditions. Mpofu S, Mpofu CM, Hutchinson D, Maier AE, Dodd SR, Moots RJ. Ann Rheum Dis. 2004 May;63(5):588-90.
- Painful hips in a shipyard worker. Hutchinson D, Mascarenhas R. : Rheumatology (Oxford). 2004 Apr;43(4):524.
- Primary synovial non-Hodgkin's lymphoma in association with ankylosing spondylitis. Khan SY, Hutchinson DG. Rheumatology (Oxford). 2004 Mar;43(3):391.

- Smoking and HLA-DR shared epitope alleles in rheumatoid arthritis: Mattey DL, Hutchinson D. Arthritis Rheum 2005 Nov;52(11):3675-6.
- Spontaneous pneumomediastinum secondary to refractory dermatomyositis successfully treated with rituximab. Lee MA, Hutchinson DG. Clin Rheumatol. 2010 Aug;29(8):945-6.
- HRCT-proven leflunomide pneumonitis in a patient with psoriatic arthritis and normal lung function tests and chest radiography. Lee MA, Hutchinson DG. Rheumatology (Oxford). 2010 Jun;49(6):1206-7.
- Clinical images: rice body arthritis. Hauser B, Hutchinson D. Arthritis Rheum. 2011 Apr;63(4):1158.
- RA autoantibodies as predictors of rheumatoid arthritis in non-cystic fibrosis bronchiectasis patients. Perry E, Stenton C, Kelly C, Eggleton P, Hutchinson D, De Soyza A. Eur Respir J. 2014 Oct;44:1082-1085.
- The lung in ACPA-positive rheumatoid arthritis: an initiating site of injury? Perry E,
- Kelly C, Eggleton P, De Soyza A, Hutchinson D. Rheumatology (Oxford).2014 May 15. pii: keu 195.
- Cadmium the missing link between smoking and increased rheumatoid disease activity? Cates M, Hutchinson D. Ann Rheum Dis - 2014 Sep 23. pii: annrheumdis-2014-206538. doi: 10.1136/annrheumdis-2014-206538.

Reviews

- Elizabeth Perry, Clive Kelly, Paul Eggleton, Anthony De-Soyza & David Hutchinson (2014) The Lung in ACPA-Positive Rheumatoid Arthritis: An Initiating Site of Injury? *Rheumatology (Oxford)* 53(11) 1940-50. IF 4.44.
- 10.1517/14728222.2016.1164695

Signature:	**Date:**
David Hutchinson	21 March 2016

CURRICULUM VITAE

<table>
<tr><td colspan="2">Name: Paul Eggleton</td></tr>
<tr><td colspan="2"></td></tr>
<tr><td colspan="2">Present appointment: (Job title, department, and organisation.)</td></tr>
<tr><td colspan="2">Immunology Senior Lecturer in Immunology, Medicine, University of Exeter</td></tr>
<tr><td colspan="2">Address: (Full work address.)</td></tr>
<tr><td colspan="2">Institute of Biomedical & Clinical Science, University of Exeter Medical School, St Luke’s Campus, Room G09, Heavitree Road, Exeter EX1 2LU Devon.
UK. Tel. + 44 (0)1392 722940 ; Fax +44 (0)1392 262926 e-mail:
p.eggleton@exeter.ac.uk</td></tr>
<tr><td>Telephone number:</td><td>Email address:</td></tr>
<tr><td></td><td></td></tr>
<tr><td colspan="2">Qualifications:</td></tr>
<tr><td colspan="2">1981–1983: Plymouth University. Plymouth, Devon, England.
B.Sc. Hons Biological Sciences – Upper second class (2.i).
1985–1987: University College London - Royal Free Hospital School of Medicine (RFHSM).
M.Phil. Medical Microbiology.
1987–1991: University College London - Royal College of Surgeons of England & RFHSM.</td></tr>
</table>

Ph.D. Biochemistry

Professional registration: *(Name of body, registration number and date of registration.)*

None

Previous and other appointments: *(Include previous appointments in the last 5 years and other current appointments.)*

University of Alberta, Edmonton, Alberta, Canada

August 2012 – ongoing. Dept. Biochemistry, Faculty of Medicine & Dentistry

Position**: Visiting Professor** (sabbatical).

Laboratory Host: Professor Marek Michalak. **Project A**: Investigate the role of calnexin in multiple sclerosis pathology. **Project B**: Develop new therapeutic strategies to boost host immunity against cancer.

Research experience: *(Summary of research experience, including the extent of your involvement. Refer to any specific clinical or research experience relevant to the current application.)*

a. Overview of research experience: My research interests are broad and varied, which has allowed me to work in many cross-disciplinary biomedical subject areas over the past 15-20 years in the fields of innate and adaptive immunity, cancer, inflammation, medical microbiology, neurology and hyperbaric medicine. I am a leading authority in the following areas:

b. Post graduate Students - I have supervised 5 D.Phil students (Oxon), 1 MSc student (Oxon), 9 PhD students (Exeter) and 1 MD student (Exeter).

- Role of chaperones in ER stress, autoimmunity and tumour vaccination.

- Inflammatory mechanisms of musculoskeletal and autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis).
- Diagnostic biomarkers of autoimmune disease.
- Mechanisms of Apoptosis/frustrated autophagy in colon cancer.
- Co-discoverer of the role of $CD20^{+ve}$ T-cells in autoimmune diseases and cancer
- Co-discoverer of components of the lectin (MBL) pathway of complement activation.
- Co-discoverer of many innate immune functions of C1q, collectins and ficolins.
- Co-discoverer of the extracellular immune roles of calreticulin (complement inactivation) in human disease
- Proteomic and genomic analysis of proteins in autoimmune diseases.
- Mechanisms of Hyperbaric Oxygen Therapy in wound repair

Research training: *(Details of any relevant training in the design or conduct of research, for example in the Clinical Trials Regulations, Good Clinical Practice, consent or other training appropriate to non-clinical research. Give the date of the training.)*

- I have designed and conducted research trials for 30 years, written MREC and IRAS ethics for multi-centres. Sat on the Exeter and Plymouth Medical School Research Degree Committees for 14 years (2002-2016) and lectured at national and international events to medical trainees and patients.
- Northcott Devon Medical Foundation Committee Member (2006-present). Scientific advisor for charitable Trust, funding local research within the

Universities and Hospitals of Devon, UK. Over the past 9 years, I have reviewed over 130 research grants and successfully lobbied for the support of pump-priming funding for approximately 50 research grants for researches at PCMD and UEMS.

- Regional clinical and patient training lectures
- Research for Patients Benefit Guest speaker at the Devon and Cornwall Lupus Group. Various venues (Exeter, Plymouth, Ashburton and Dartington), 2008, 2009, 2011, 2012 and 2015.
- '*Development of new cancer therapeutics'* 'Bridging the Gap' Immunology Workshop, Exeter University, 29th July 2013.
- Speaker: 'Breakdown of immune tolerance by post translational modification of host proteins' American College of Rheumatology/ARHP annual meeting, Boston, Nov 15-19th 2014.
- Chair and speaker: *'Chaperones and disease activity in Rheumatoid Arthritis'* 11th Intl. Calreticulin workshop. New York City, May 8-11th 2015.
- Speaker '*The generation of autoantibodies to C1q and their usefulness in diagnosing Lupus nephritis'* International Conference 'Antibodies-2015', Birmingham, UK, August 10-12 2015.
- Speaker '*Galectin-9 levels in bronchiectasis: a possible promoter of immunopathology in rheumatoid arthritis*. West and Wales Regional Lung Meeting 19th Jan 2016.
- Guest Speaker. The challenges of infection and immunity for medics in the 21st century'. Exeter Medics Society. March 2016.

- Guest Speaker ‘Bronchiectasis as a trigger of rheumatoid arthritis’ British Society of Rheumatology Training meeting. Birmingham, UK. April 2016.
- King Faisal Prize for Medicine committee Member to select the winner of the 2015 King Faisal International Prize for Medicine. Topic: ‘Intestinal Microflora and Human Health’. Riyadh, KSA and select the subject for the 2017 King Faisal International Prize for Medicine. Riyadh, KSA, March 2015.

Relevant publications: *(Give references to all publications in the last two years plus other publications relevant to the current application.)*

Books and book chapter 2014-2016

1 Eggleton P, Bremer E & Michalak M. (2016) Endoplasmic reticulum and its role in tumour immunity. Frontiers/Nature Journals e-book. Switzerland. 101 pages. ISBN-10: 978-2-88919-786-6

2 Eggleton Paul, Javed Moazzam, Pulavar David and Sheldon Gemma (April 2015) Immune Complexes. In: eLS. John Wiley & Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a0001118.pub2

3 P Eggleton, M Michalak & E Bremer Endoplasmic reticulum and its role in tumor immunity – Editorial Frontiers in Oncology e-book

4 P Eggleton, G R. Smerdon, J Holley, & N J. Gutowski (2016) Manipulation of Oxygen and Endoplasmic Reticulum Stress Factors as Possible Interventions for Treatment of Multiple Sclerosis: Evidence For And Against. (Ed: F Al-Khamis, A. A. Al-Sulaiman, P. Kaur and A. A. A. Asea) In: Multiple Sclerosis: Global Perspectives of a Silent Killer. Springer International Publishers (Netherlands). (To be published Spring 2016)

Research papers 2014-16

1 Wiersma VR, He Y, Sampionus DF, van Ginkel RJ, Gerssen J, Eggleton P, Bremer E & Helfrich W (2014) A CD47-blocking TRAIL fusion protein with duel pro-phagocytic and pro-apoptotic activity. *Brit J Haematol* 164(2): 304-7. IF 4.9.

2 Brent J. Ryan & Paul Eggleton (2014) Detection and characterization of autoantibodies against modified self-proteins in SLE sera after exposure to reactive oxygen and nitrogen species. *Methods Mol Biol* 1134: 163-71. IF 1.3.

3 Isabel Cottrell, Asma Khan, Sidra Maqsood, Jemma Thornes & Paul Eggleton (2014) *Meta-analysis as a diagnostic tool for predicting disease onset and/or activity in systemic lupus erythematosus. Methods Mol Biol* 1134: 249-59. IF 1.3.

4 Paul Eggleton, Obioha Ukoumunne, Isabel Cottrell, Asma Khan, Sidra Maqsood, Jemma Thornes, E. Perry & David Isenberg (2014) Autoantibodies against C1q as a diagnostic measure of lupus nephritis: systematic review and meta-analysis. *J Clin Cell Immunol* 5(2): 1-14. IF 5.6.

5 Elizabeth Perry, Chris Stenton, Clive Kelly, Paul Eggleton, David Hutchinson, Anthony De Soyza (2014) A combination of positive rheumatoid factor and anti-cyclic citrullinated antibodies as a predictor of non-cystic fibrosis bronchiectasis patients developing rheumatoid arthritis. *Eur Resp J*. 44:1082-5. IF 7.1.

6 Holley JE, Bremer E, Kendall AC, de Bruyn M, Helfrich W, Tarr JM, Newcombe Jia, Gutowski NJ, & Eggleton P. (2014) CD20+ inflammatory T-

cells are present in blood and brain of multiple sclerosis patients and can be selectively targeted for apoptotic elimination. *Multiple Sclerosis and Related Disorders* 3:5 650-658.

7 M de Bruyn, VR Wiersma, W Helfrich, P Eggleton & E Bremer. (2015) The ever-expanding immunomodulatory role of calreticulin in cancer immunity. *Frontiers in Oncology*. 5:35. doi: 10.3389/fonc.2015.00035

8 M de Bruyn, VR Wiersma, DF Samplonius, HG Klip, W Helfrich HW Nijman, P Eggleton & E Bremer. (2015) CD20+ T cells have a predominantly Tc1 effector memory phenotype and are expanded in the ascites of patients with ovarian cancer. *Oncoimmunology* 4(4):e999536 *DOI:10.1080/2162402X.2014.999536* IF 6.28.

9 Quirke AM, Perry E, Kelly C, de-Soyza A, Eggleton P, Cartwright A, Hutchinson D, Venables P. (2015) Bronchiectasis: a model for chronic bacterial infection inducing autoimmunity in rheumatoid arthritis. *Arthritis and Rheumatology* 2015 May 27. doi: 10.1002/art.39226. IF 7.8.

10 VR. Wiersma, M de Bruyn,*, Y Wei, R J. van Ginkel, M Hirashima, T Niki, N Nishi, D F. Samplonius, J Gerssen, H W. Nijman, P Eggleton, W Helfrich, & E Bremer. (2015*)* Galectin-9 induces frustrated autophagy and cell death in K-Ras mutant colon carcinoma. *Autophagy Jun 18:0.* IF 12.0.

11 VR Wiersma, D Hendrike, E, Dudek, W-A Wang, Y He, P van Bommel, R van Ginkel, D Samplonus, J Gerssen, H Nijman, W Helfrich, M Michalak, P Eggleton & E Bremer. (2015) Calreticulin regulates anti-cancer innate immunity and activates T-cell responses. *Cancer Immunology Research (Under review)*.

12 E Perry, P Eggleton, A De-Soyza & D Hutchinson & C Kelly. (2015) Increased disease activity, severity and autoantibody positivity in rheumatoid arthritis patients with co-existent bronchiectasis. *Intl J Rheumatic Diseases* doi: 10.1111/1756-185X.12702. IF 1.7.

Reviews

1 Elizabeth Perry, Clive Kelly, Paul Eggleton, Anthony De-Soyza & David Hutchinson (2014) The Lung in ACPA-Positive Rheumatoid Arthritis: An Initiating Site of Injury? *Rheumatology (Oxford)* 53(11) 1940-50. IF 4.44.

2 VR Wiersma, M Michalak, TM Abdulla, E Bremer & P Eggleton. (2015) Mechanisms of translocation of ER chaperones to the cell surface and immunomodulatory roles in cancer and autoimmunity. *Frontiers in Oncology* 5(7); 1-14 doi:10.3389/fonc.2015.00007.

3 Eggleton P, Bishop A and Smerdon G (2015) Safety and efficacy of hyperbaric oxygen therapy in chronic wound management: current evidence. *Chronic Wound Care Management & Research* 2:81-93

4 Leslie Gold, David Williams, Jody Groenendyk, Marek Michalak, P Eggleton (2015) Unfolding the complexities of ER chaperones in health and disease: Report on the XI international calreticulin workshop *Cell Stress and Chaperones* 20:875-883 IF 3.16.

5 P Eggleton, E Bremer, Elzbieta Dudek and M Michalak. (2016) Calreticulin: a therapeutic target in cancer? *Expert Opinions on Therapeutic Targets*. IF 5.14. DOI: 10.1517/14728222.2016.1164695

Signature:	**Date:**
	21 March 2016

Appendix 2 - Text of participant information sheet. See separate document for version controlling on headed paper.

Dust, Cadmium and Rheumatoid Arthritis.

Participant Information Sheet

RESEARCH INVITATION

We would like to invite you to take part in our research study. Before you decide if you would like to participate we want to explain why this research is being done and what it would involve for you.

One of our team will be available to go through the information sheet with you at next clinic appointment and answer any questions you have. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Take time to decide whether or not you wish to take part.

If you would like to contact us before coming in to clinic to discuss the research in more detail our contact details are listed below:

Dr. D Murphy

Dr. D Hutchinson

Rheumatology Department

Royal Cornwall Hospital

Truro

Cornwall

TR1 3LJ

Tel. 01872 254690

Email: daniel.murphy8@nhs.net

In this information sheet part 1 tells you the purpose of this study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study.

Please feel free to ask us at any stage if there is anything that is not clear.

PART 1

What is the purpose of this study?

Emerging research suggests that people exposed to dust in their workplace may be at higher risk of developing rheumatoid arthritis (RA). This has been shown in groups of people exposed to silica, textiles, and mineral oils amongst other trades. We have noticed that in Cornwall, particularly amongst men, we have strikingly high rates of dust-exposed trades with rheumatoid arthritis. To date, no-one has put forward a reason to link these different occupations, or suggest how the dust may be triggering rheumatoid arthritis.

Lots of other risk factors have been identified with developing rheumatoid arthritis. Interestingly, these also associate with high levels of cadmium exposure.

The potential exists for cadmium (Cd) inhalation via dust, fumes or fine spray in the workplace. Cadmium is found in cement dust, wood dust, and coal dust and is associated with the finishing of textiles as a pigment and stabilising agent. These environmental triggers combined with pre-existing genetic factors result in the development of special proteins, called anti-citrullinated protein antibodies (ACPAs), which then drive RA pathology.

By measuring the urine cadmium levels of people exposed to dust, we can see if they are higher than we would expect. If we find raised levels in certain

occupations, we may invite you back for a blood test to measure the special proteins. By measuring the special proteins (ACPAs) in the blood, we can see if the dust exposed people are at higher risk of developing rheumatoid arthritis.

Why does this study matter to me?

This has never been proven before and would mean that we could link cadmium exposure to the development of these special proteins (ACPAs) in people with dust exposure to see if they are at risk of developing rheumatoid arthritis. We can then monitor these people more closely to allow us to diagnose rheumatoid arthritis and dust exposure earlier, so we can start treatment earlier and try and stop people from suffering ill health.

By measuring the cadmium levels, we can see if there is a link between inhaled dust, cadmium levels and RA development in Cornish workers. This will help us to understand the way in which RA develops in some people, and allow us to better identify which individuals are more at risk of future disease development than others. Better understanding of the roles played by dust and cadmium will also help us to develop new treatment options to treat rheumatoid arthritis and possibly in the future, to be able to stop some people from developing these conditions at all.

Why have I been invited?

We are inviting all men in Cornwall with rheumatoid arthritis into the study, and men who are similar age but do not have rheumatoid arthritis. You have been chosen as you are between 18 and 95 years-old, have either rheumatoid arthritis or fit into one of the groups we need to study which are detailed below:

Rheumatoid arthritis on its own

Rheumatoid arthritis and dust exposure

Dust exposure on its own

No dust exposure or rheumatoid arthritis

Do I have to take part?

It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

What will happen if I agree to take part?

The study in total should take no more than 15 minutes of your time and where possible will be performed alongside your normal clinic appointment to save you from having to make an extra journey. The points below detail what will happen if you would like to participate in the study.

1. You will be offered the opportunity to meet with one of the research team at your next clinic appointment to talk over the research in more detail and answer any questions you have.

2. We will complete a short questionnaire to make sure we can include you in the study (you may have already done this as part of our departmental audit).

3. If you are happy to participate in the research, we will ask you to sign the consent form which we have attached to this information sheet so you have time to read it before seeing us.

4.

a. If you are due routine blood tests we will use 10-20mls of this blood to test for the special proteins (ACPA's).

b. If you are not due routine blood tests we will take an extra blood test (10-20mls only, no more than a normal blood test). We will send the blood test away to test for special proteins (ACPA's).

c. If you do not currently have a diagnosis of rheumatoid arthritis but are taking part in the study, we will not routinely take a blood sample from you, but may invite you for a blood test at a later date

5. We will give you a sample pot to collect some urine. This should be from the first urine you pass after waking up in the morning.

6. We normally expect results to take around 3 months to process. We will write to you, your consultant and your GP (General Practitioner) explaining the results of your tests. Should you wish to discuss your results in more detail, we will include details in the letter of how to arrange this.

What are the possible disadvantages and risks of taking part?

The blood sample we need for the study is the same process as any blood test you would normally have, some people don't like to have extra blood tests and so where possible with your consent, we will use some of the blood taken for your normal monitoring blood tests. If you are not having any monitoring blood tests, taking part in the study would mean we would need to take a blood test. We know that people who have special proteins (ACPAs) are more likely to have rheumatoid arthritis. Therefore if you have dust exposure and don't have rheumatoid arthritis, and we find the special proteins via a blood test taken at a later date, it does not mean you have rheumatoid arthritis or that you will get rheumatoid arthritis. What it does tell us is that you could be at increased risk of rheumatoid arthritis in the future. Some people do not want to know if they could be at higher risk of developing rheumatoid arthritis and so may not wish to take part.

If the amount of cadmium in your urine is very high to the point where your immediate health is at risk, we may call you for another appointment to offer medicine to lower your cadmium levels.

What are the possible benefits of taking part?

We know that treating rheumatoid arthritis early is very important. By identifying people who are at increased risk of developing rheumatoid arthritis, we can then monitor these people more closely, so if they then develop rheumatoid arthritis we can start treatment earlier and try and stop people from suffering ill health.

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2. If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

PART 2

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this. The Patient Advice and Liaison Service (PALS) can be contacted on local team telephone number 01872 252793, or email rcht.pals@nhs.net.

In the event that something does go wrong and you are harmed during research and this is due to someone‘s negligence, then you may have grounds for a legal action for compensation against your NHS Trust who is performing the research but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Will my taking part in this study be kept confidential?

If you join the study, some parts of your medical records and the data collected for the study will be looked at by authorised persons from the research team. They may also be looked at by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant.

What will happen to any samples I give?

Blood and urine samples taken for the study will be used only for this study and any monitoring bloods that your doctor has asked for from your clinic appointment. The samples will be looked at by only the research team and authorised laboratory team, any excess will be destroyed after 24 months.

What will happen to the results of the study?

We will publish the results in the public domain in scientific journals and literature in order to increase international understanding of rheumatoid arthritis and dust exposure. You will not be identifiable in any way from the results.

Who is organising and funding the research?

The research is being funded by Cornwall Arthritis Trust, working with doctors in the Rheumatology Department and employed by the Royal Cornwall Hospitals Trust. The research team for this work is led by Dr D Murphy and includes Dr D Hutchinson, consultant rheumatologist Royal Cornwall Hospital.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study is to be reviewed and given favourable opinion by the local ethics committee before commencing.

Many thanks for taking the time to consider participation in our study. I enclose a consent form for you to read, do not worry about completing the consent form. This is something we can do at your clinic appointment.

11.3 Appendix 3- Consent form. See separate document for version controlling on headed paper.

Participant Details: (please use hospital sticker if available)

Name: _____

NHS Number: _____

Hospital Number: _____

Date of birth: _____

Title of Project: Dust, Cadmium and Rheumatoid Arthritis.

Researchers: Dr Dan Murphy, Dr David Hutchinson (Royal Cornwall Hospital)

1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I understand that sections of my medical notes may be looked at by authorised members of the research team. I give my permission for these individuals to have access to my records.

4. I accept that I will donate 20 mls of blood for the study, and acknowledge that data from this will kept anonymous.

5. I am happy for my urine to be tested for cadmium levels.

6. I acknowledge that the samples I donate will be stored until analysed and for a maximum of 24 months and then disposed of without loss of confidentiality

7. I agree to take part in the above study

8. I would be happy to be contacted by the research team to be invited to participate in future research studies.

Signature _____

Print name_____ Date _____

11.4 Appendix 4- GP information form. See separate document for version controlling on headed paper.

Dust, Cadmium and Rheumatoid Arthritis.

General Practitioner Information Sheet Version 0.1

We are writing to inform you that the following patient is participating in the above study:

If you would like to contact us to discuss this research in more detail please contact:

Dr. D Murphy

Rheumatology Department

Royal Cornwall Hospital

Truro

Cornwall

TR1 3LJ

Tel. 01872 254690

Email: daniel.murphy8@nhs.net

What is the purpose of this study?

Emerging research suggests that people exposed to dust in their workplace may be at higher risk of developing rheumatoid arthritis (RA). This has been shown in groups of people exposed to silica, textiles, and mineral oils amongst other trades. We have noticed that in Cornwall, particularly amongst men, we have strikingly high rates of dust-exposed trades with rheumatoid arthritis. To date, no-one has put forward a reason to link these different occupations, or suggest how the dust may be triggering rheumatoid arthritis.

Known RA risk factors include lower socio-economic class, a low level of formal education, exposure to tobacco smoke and residing close to main roads. Intriguingly, these risk factors and occupational risk categories also associate with high levels of cadmium exposure.

The potential exists for cadmium (Cd) nanoparticle inhalation via dust, fumes or aerosolisation in the workplace. Cadmium is found in cement dust, wood dust, and coal dust and is associated with the finishing of textiles as a pigment and stabilising agent. These environmental triggers combined with pre-existing genetic factors (such as the HLA-DRB1 shared epitope (SE)), drive development

of citrullinated antigens resulting in anti-citrullinated protein antibodies (ACPAs), which then drive RA pathology.

What does the study involve?

Our study is a single centre epidemiological, observational, case control study comparing four well defined cohorts: RA and dust exposure, RA alone, dust exposure alone and healthy unexposed individuals. To test our hypothesis we will collect samples of venous blood to measure ACPAs and urinary cadmium in each cohort. By comparing the groups we will be able to see if more people with dust exposure have high levels of urinary cadmium, and if this correlates with RA.

What are the implications of the study to my patient?

The study will be performed where possible during the patient's routine follow-up appointment. A simple data collection form will be completed, written consent and venous blood sample taken for testing.

We know that people who have ACPAs are more likely to have or to develop rheumatoid arthritis. Any patients who tests positive for ACPA in the dust exposure alone group or the healthy individuals control group will be informed of the result and given an opportunity to come to a rheumatology clinic to discuss the result further. We will also send notification to yourself so that a high index of suspicion for rheumatoid arthritis should they develop joint symptoms in the future.

Appendix 3 – Study Time lines

Ethical and HRA approval (awaited, Apr 2016)

Submission of protocol and IRAS form

Following local R&D approval

Commence research (awaited, Apr 2016)

Approach potential study group via telephone call

Provision of written information, invitation to clinical appointment

Approach matched controls (Pending approval, expected May 2016)

Identify through GP records, approach via telephone call

Provision of written information, invitation to clinical appointment

Consent and sampling (May-Dec 2016)

Formal clinical appointment for answering questions, consent and data gathering. Provision of urine sampling pot

Urine sample batching (May-Dec 2016)

Urine samples to be batched and sent to lab as per protocol (cadmium)

Blood samples batched for lab processing as per protocol (calreticulin)

Sample matching (May-Dec 2016)

Samples to be matched back to cases

PII removed for data analysis

Data analysis (expected Dec 2016- Mar 2017)

Appropriate statistical methodology to be checked by independent statsitician

Results (expected Dec 2016- Mar 2017)

Communication of personal results with GP and patient

Final study report (expected Apr 2017)

Under authorship of Dr. D Murphy, Academic supervision: Dr. D Hutchinson,

Dr. P Eggleton

Dissemination of results via peer reviewed publication, potential MD thesis

Appendix 4 – Occupation Quesitonnaire

Appendix 5 – Amendment History

Amendment No.	Protocol version no.	Date issued	Author(s) of changes	Details of changes made
1	0.2	11.4.16	DM	Proofreading following R&D input
2	1.0	19.4.16	NM	Transferred to correct template

List details of all protocol amendments here whenever a new version of the protocol is produced.

Protocol amendments must be submitted to the Sponsor for approval prior to submission to the REC committee.

Appendix 2. National Institute for Health Research, Clinical Research Facility (Exeter). Application for a new collection: CRF 249.

Cadmium and rheumatoid arthritis: could cadmium exposure break immune tolerance by stimulating post-translational modification and subsequent autoantibody development in genetically susceptible individuals?

Introduction

Subcutaneous nodule development is a common extra-articular manifestation of RA, found 20–25% of cases [1]. Rheumatoid nodules are associated with more severe disease progression and joint damage. The exact mechanisms involved in the pathogenesis of rheumatoid nodules remain uncertain. It has been suggested that rheumatoid factor (RF), smoking, and HLA-DRB1/TNF gene interaction have linked but separate roles in the development of rheumatoid nodules [2]. Nodular RA associates with both RF positivity and cigarette smoking in patients with RA [3-7]. Production of RF also associates with cigarette smoking in patients with RA and healthy individuals [5-10].

Potential environmental triggers

Nodules also occur in patients with RA who have never smoked. Other environmental factors have been suggested as a possible trigger [2]. Known risk factors for RA development include lower socio-economic class, a low level of formal education, exposure to tobacco smoke and residing close to main roads [11]. Intriguingly, these risk factors also associate with high levels of cadmium exposure [12]. We propose that cadmium inhalation is a plausible trigger for nodular RA development. Emerging literature suggests that RA is associated with

a number of occupations associated with cadmium exposure [12]. These include underground mining work (odds ratio (OR) 8.47 (95% CI 2.59 to 27.66), bricklaying and working with concrete (OR: 2.6, 95% CI: 1.3–4.9) , working with electrics and electronics (OR: 1.8, 95% CI: 1.0–3) , workers exposed to mineral oils (relative risk (RR): 1.4, 95% CI: 1.0–2.02) , workers exposed to hydraulic oils (RR: 1.7, 95% CI: 1.1–2.6), asphalters (OR: 14.0, 95% CI 1.2–799.0) and conductors, freight and transport workers (OR: 4.7, 95% CI 1.4–16.3). Further studies demonstrate a link with smelting and working in metal foundries (OR 2.8, 95% confidence interval (CI): 1.0–7.4)[13].

Rheumatoid autoantibodies

Nodular RA development associates with high titres of rheumatoid autoantibodies. Routine testing for rheumatoid autoantibodies consists of rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPA).

Rheumatoid factor is an autoantibody directed against the CH2 and CH3 areas of the Fc portion of human IgG. The specificity of detection of RF is about 79%, with sensitivity of about 60% [14].

ACPA testing has been derived from cross-reactivity testing of endogenous antibodies to a synthetically derived form of citrullinated filaggrin acting as antigen. Citrullination is the process by which arginine residues are replaced by citrulline. Sensitivity and specificity are 65-70% and 96-98% respectively [15]. De novo, this process is referred to as post-translational modification. However, the exact antigen to which the RA patient develops antibodies remains unknown.

In analysing citrullinated peptides found in the rheumatoid patient as potential autoantigens, researchers have looked at fibrin [16], fibrinogen [17], vimentin [18] and alpha-enolase [19]. Citrullinated alpha-enolase has been detected in the synovial tissue of patients with rheumatoid arthritis [20], and high levels have been found within rheumatoid nodules along with neurone-specific enolase [personal communication].

Additionally, antibodies against proteins that have undergone post-translational modification by carbamylation (whereby lysine residues are replaced by homocitrulline) have been described in RA patients' serum [21]. Homocitrulline is present in rheumatoid nodules alongside citrulline [22], and antibodies against carbamylated proteins were found to predict joint damage.

Our own work has led to a recently published hypothesis that a post-translationally modified heavy chain fragment of IgG, that has undergone both citrullination and carbamylation, would be the most obvious linking autoantigen to explain the presence of rheumatoid factor, anticitrullinated peptide antibodies and anti-carbamylated protein antibodies in the RA patient [23], given its relative abundance, size and ability to undergo post-translation modification.

Genetic susceptibility

In analysing HLA DRB1 genotypes in an RA population, it has been shown that DRB1*0401 homozygotes are associated with nodular disease development, independent of smoking and rheumatoid factor seropositivity [2].

Conclusion

We wish to store samples of urine and serum on both nodular and non-nodular RA patients to enable further testing of specific rheumatoid autoantibodies, and specific HLA types for future use given our evolving interest in this area.

We anticipate further applications to use the samples in collaboration with other researchers pending completion of our initial work on urinary cadmium testing via case-control of nodular vs. non-nodular RA patients.

References.

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Appendix 3. Data recording questionnaire and capture forms

Request Form for "Dust, Cadmium and Rheumatoid Arthritis" trial samples (CRF 261)

***** For further instructions, please refer to Sample Management Work Instruction *****

DETAILS:	REFERRER
ID numbers:	**Name:**
	Job title:
	Signed:
Gender:	**Date sample taken:**
Male Female	
Patient's Date of Birth:	**Time sample taken:**
	(24h clock, e.g. 13:20)
	Contact Tel: _____

Additional Notes/Instructions:

FOR LAB USE ONLY

2x Purple EDTA tubes	
1x Gold Top Becton Dickinson SST tube	
3x Urine tubes	

2x EDTA to Exeter as whole blood for DNA analysis

1x Urine to Guildford SAS Trace Elements Lab for Cadmium

1x Urine to store in -80 Freezer

1x SST – spin and separate serum into three labelled micro-tubes for storage in -80 freezer

*****CLINICAL TRIAL SAMPLE *****

Dust, cadmium and rheumatoid arthritis. IRAS ID: 194833
Donor registration information and data capture form- primary care controls

Samples donated

Sample type	Date	Collaborating clinician

Smoking history 1 pack year = 1 year at 20/day, equivalence calculator at: http://www.smokingpackyears.com/

Ever (E), Never (N), Current (C)- if E or C, if E or C please document pack years below	
Pack years up to date of contact	
Years since stopped smoking	

Occupational history (industry and job title, significant exposures)

Current/ last occupation	
Other occupations involving dust or fume exposure greater than one year (continue below if necessary)	

Medical history (Type of condition and duration)

Lung disease	
Inflammatory arthritis (NOT osteoarthritis)	

Appendix 4. Publications associated with this research

Murphy D, Sinha-Royle E, Bellis K, Harrington C, Hutchinson D. Nodular rheumatoid arthritis (RA): A distinct disease subtype, initiated by cadmium inhalation inducing pulmonary nodule formation and subsequent RA-associated autoantibody generation. Med Hypotheses 2019;122:48-55.

Murphy D, Bellis K, Hutchinson D. Occupational dust and cigarette smoke co-exposure results in local adsorption of toxic elements such as cadmium, linking rheumatoid arthritis to COPD via lung citrullination. Lancet Resp Med 2018;S2213-2600(18)30188-7.

Murphy D, Bellis K, Hutchinson D. Vapour, gas, dust and fume occupational exposures in male rheumatoid arthritis patients resident in Cornwall (UK) and their association with rheumatoid factor and anti-cyclic protein antibodies: a retrospective clinical study. BMJ Open 2018;8:e021754.

Murphy D, Marshall R, Harrington C, Taylor A, Hutchinson D (2017) Rheumatoid Pulmonary Nodules and Significantly Elevated Urinary Cadmium in a Kaolin (China Clay) Worker: Could Cadmium Adsorption onto Occupationally Inhaled Dust Explain Caplan's Syndrome?. J Rheum Dis Treat. 2017;3:057.

Murphy D, Bellis K, Hutchinson D AB0306 Caplan's syndrome, cadmium and china clay: could occupational kaolin inhalation enhance cadmium exposure to explain the sixty year conundrum of caplan's syndrome first reported in coal miners? Ann Rheum Dis. 2017;76:1155-1156.

Murphy D, Mattey D, Hutchinson D. Anti-citrullinated protein antibody positive rheumatoid arthritis is primarily determined by rheumatoid factor titre and the shared epitope rather than smoking per se. PloS one. 2017;12:e0180655.

Murphy D, Hutchinson D. Is Male Rheumatoid Arthritis an Occupational Disease? A Review. Open Rheumatol J. 2017;11:88–105.

Hutchinson D, Clarke A, Heesom K, Murphy D, Eggleton P. Carbamylation/citrullination of IgGFc in bronchiectasis, established RA with bronchiectasis and RA smokers: a potential risk factor for disease. ERJ Open Res 2017;3:0018-2017.

Bellis K, Whallett M, Murphy D, Hutchinson D. FRI0101 RA in male carpenters: occupational wood dust exposure increases ACPA and RF seropositivity and significantly raises RF titres. Ann Rheum Dis. 2017;76:518.

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Murphy D, Pay J, Benham R, James B, Hutchinson D. 2016. Comment on “Rheumatoid Arthritis in Agricultural Health Study Spouses: Associations with Pesticides and Other Farm Exposures.” Environ Health Perspect 124:A196.

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Murphy D, Hutchinson D. Cadmium, road dust and rheumatoid arthritis: an alternative hypothesis to general air pollution. J Inflamm. 2015;12:58.

Murphy D, Sinha A, Hutchinson D. Wood Dust: A Trigger for Rheumatoid Arthritis? Am J Med. 2015;128:e35.

Appendix 5. Overview of methods

Each chapter as a separate peer-reviewed publication contains a methods section specific to that publication. A more detailed description of the methods are collated here to link each chapter and describe a narrative arc into investigations around inhalational insults in RA.

Appendix 5.1 Chapter 1

Written consent for the clinical case report contained in Chapter 1 was obtained from the patient to use specific details and images from clinical history, and was recorded in the patient notes. The case in question was discussed with histopathological and laboratory colleagues as per author listings. Computed Tomography radiological images were obtained from the patient's digital record. Macroscopic images of kaolinosis lung were donated from the personal archive of Professor Robert Marshall, University of Exeter Medical School. Exeter, UK.

Urinary cadmium (UCd) analysis

For the case report detailed in Chapter 1 and for urinary cadmium (UCd) measured elsewhere in this dissertation, UCd was analysed by inductively coupled plasma mass spectrometry (ICP-MS), using a fully validated method by the Supra Regional Assay Service Trace Element Laboratory, Guildford, Surrey, UK [1].

Equipment consisted of iCap-Q (ThermoFisher Scientific, Hemel Hempstead, UK) collision cell ICP-MS instrument, polyethylene tubes (8 mL Midi Vials, PerkinElmer).

Reagents were reverse osmosis/deionised water (RO/DI) with >18.2 MΩ cm resistivity (Millipore, Elix/Synergy Water Purification System). Instrument wash solution was concentrated trace element grade nitric acid (50mls); 5% (v/v) Triton X-100 (2.5 mls); made up in 5 L ultra-pure RO/DI. Concentrated nitric acid, trace analysis grade, (Fisher Scientific). Nitric acid 1% (v/v): dilute 10 mL concentrated nitric acid to 1 L with ultra-pure RO/DI water. Cadmium standard solution, 1000 mg L-1 (BDH,Prolab).

Cadmium in urine was measured by ICP-MS in kinetic energy discrimination (KED) mode using helium as the collision cell gas. Samples were diluted 1 in 50 with a diluent containing germanium (Ge), rhodium (Rh) and iridium (Ir) as internal standards added to a final concentration of Ge 15 µg/L^{-1} and Rh, Ir 1.5 µg/L^{-1}. Samples were assayed against a calibration curve matrix matched with urine and containing the same internal standards as above.

Analytical Performance was calibrated as accuracy (bias) 1.5%; repeatability (std. dev.), 0.8 nmol/L; intermediate precision (%RSD), 6%; expanded uncertainty, 1.9 nmol/L; calibration standards 5–40 nmol/L.

Appendix 5.2 Chapter 2

From the anecdotal evidence of case reports such as found in Chapter 1, it was hypothesised that RA should be considered an occupational disease as a consequence of dust and fume inhalation in the workplace, as such exposures may stimulate immune tolerance breakdown**.**

A literature search was performed using Pubmed, Embase, and Cochrane databases and to cover all relative reports relating to RA and occupation. Titles and abstracts of identified articles were screened for eligibility and relevance to the *a priori* hypothesis detailed above. Duplicates and reports generated that were not relevant to the study aims were removed. Reference lists of relevant articles were searched for further evidence by hand around mechanisms of action and further hypothesis refinement. (Figure 1).

Figure 1. Literature retrieval flowchart

Occupational exposure

Initial searches were conducted using the following keywords: “occupation OR mining OR silica”, in combination with disease specific term “Rheumatoid arthritis”. Subsequent searches of disease and exposure detail were made using the terms “Rheumatoid factor OR anti-citrullinated protein antibody/ies”, AND “silica OR dust OR fumes OR metals OR cadmium OR asbestos OR mining”. A separate search was made using the combination terms “rheumatoid AND smoking AND occupation”. It became apparent that knowledge gaps were evident in the pathophysiological mechanisms by which inhalational insults may drive the process of immune tolerance breakdown. Such insults were found to be associated with the development of bronchial associated lymphoid tissue, and concurrent laboratory work/ hypothesis generation on the post-translational modification of IgG fragments suggested the implication of heat shock protein, specifically heat shock protein 70, as detailed in Chapters 2, 3.2, 5 and 6. Following hypothesis generation and in the process of refinement, further searches were made using the combination terms “Rheumatoid arthritis AND bronchial associated lymphoid tissue OR adsorption OR heat shock protein 70” (Figure 4).

Appraisal

For occupational exposures, studies were examined for information relevant to output fields of sex, country of study, follow up period and exposure type. Reported or calculable RA risk being statistically significant for increased RA risk (with lower 95% confidence interval greater than 1.0) was used for inclusion. Literature searching was undertaken by Dr. D Murphy under the supervision and guidance of Dr. D. Hutchinson. Relevant papers were discussed and agreed for inclusion or exclusion as appropriate.

Integration

For critical review purposes, statistically significant data pertaining to increased RA risk in male occupational exposures were integrated into a single table (Table 1, Chapter 2), for descriptive comparison. Data not reaching statistical significance were not included in the table, though elements of non-statistical significance of relevance

are included in the discussion. Heterogeneity in study populations, comparison groups and outcome measures precluded further pooled analysis or systematic review.

Chapter 3

In this paper, two cohorts of RA patients were studied to analyse the relationship of autoantibodies to the known inhalational insult of smoking. As a highly prevalent inhalational insult in other RA populations, it was felt important to have a firm understanding on the nature of the relationship between smoking and RA autoantibody generation.

Cohort 1 subjects

Cohort 1 consisted of males and females attending routine rheumatology clinics at the Royal Cornwall Hospital, Cornwall, UK from February 2015 to August 2016. The data were anonymised at source, collected as part of project IRAS ID 194833, approved by South West Regional Ethical Committee as detailed previously, and was undertaken as a form of interim analysis of data collected on male and female patients. This cohort was selected to achieve an intended total cohort size of six hundred gender matched patients for analysis. Patients reviewed in clinic with a new or existing diagnosis of RA during the study period were included for analysis, with 60 patients subsequently excluded (see below). 298 male and 300 female RA patients were included to provide a suitable cohort size for serological subgroup analysis, with approximately equal numbers of each gender selected to detect potential differences in smoking rates, age of onset, and serological status rather than to represent the overall male to female prevalence ratio.

Recruitment for this analysis ceased when the target cohort size was reached. Data were recorded by clinicians in a standard questionnaire via face-to-face interview (Appendix 3). Missing data were obtained by follow up telephone conversation. Age, age of RA onset, disease duration, smoking history prior to disease development, RF levels at disease onset and subsequent ACPA levels were recorded as part of routine clinical practice. Testing for SE status does not form part of routine clinical practice and was therefore unavailable. All patients fulfilled the 2010 American College of Rheumatology/ European League Against Rheumatism (ACR/EULAR) RA criteria at

diagnosis [2].

As per guidelines, a negative RF or ACPA was defined as a level within the normal range, a weakly positive RF or ACPA <3 times the upper limit of normal and a strongly positive RF or ACPA >3 times the upper limit of normal [2]. Six percent (18/318) of females and 12% (40/340) of males were excluded during the collection period due to >20 years between smoking and RA diagnosis. A further 2/340 males were excluded due to incomplete data on subsequent review. Social deprivation analysis was undertaken through the UK government validated Index of Multiple Deprivation (IMD) [3], a deprivation rank score of 32,844 neighbourhoods weighted by income, health/disability, education, housing/service access, crime and living environment, converted to equal deciles.

Cohort 1 autoantibody analysis

RF was measured with Tina-quant Rheumatoid Factors I1 Test System, by Roche Diagnostics Corporation. A value of < 14 IU/ml was considered as negative as per manufacturer guidelines. ACPA was measured by Roche Modular Analytics Second Generation E170 Anti-CCP analysis, with a negative value of <17 U/ml as per manufacturer guidelines.

Cohort 2 subjects

Given the striking findings of autoantibody analysis in cohort 1, a collaboration was sought to verify findings in a different, pre-existing cohort dataset. A second cohort (n=409) of RA patients consisted of 154 males and 255 females attending rheumatology clinics at the Haywood Rheumatology Centre in North Staffordshire, UK, in whom “shared epitope” (SE) status had been determined. Prevalent cases were recruited consecutively from a clinic established to monitor the effects of disease modifying anti-rheumatic drugs including hydroxychloroquine, sulfasalazine, D-penicillamine, gold and methotrexate. Sample collection occurred from 1994 to 2002 as part of a study to investigate the relationship between genetic factors and outcome in RA. Ethical approval was obtained from the North Staffordshire local research ethics committee and written informed consent was provided by all patients. All patients had established disease (median disease duration 9.2 years), fulfilling 1987 RA criteria at

time of diagnosis [4]. A full smoking history was obtained on 386/409 patients. Smokers were defined as smoking >1 cigarette (or equivalent)/day for >1 year, and categorised according to pack years smoked [5]. All patients had been genotyped for Human Leucocyte Antigen (HLA)-DRB1 [6].

Cohort 2 autoantibody analysis

In the Staffordshire cohort (cohort 2), IgM RF was measured by nephelometry at disease onset while ACPA was measured subsequently using a commercially available anti-CCP2 ELISA (Axis-Shield, Dundee, Scotland). RF levels were reported in International Units (IU). An RF level > 60 -180 IU/ml was considered weakly positive and a level >180 IU/ml was strongly positive. ACPA measurements above 5 units/ml were considered positive as per manufacturer guidelines.

Statistical analyses

Chi square tests and multivariate logistic regression analysis were used to examine the relationships between ACPA, IgM RF, HLA-DRB1 shared epitope and cigarette smoking. Analyses were adjusted for age, sex and disease duration where appropriate. Mann-Whitney U testing was performed to analyse differences in RF titre. Given the heterogeneity between different cohorts, no pooled analysis was made.

All analyses were carried out using the Number Cruncher Statistical System for Windows (NCSS60).

Chapter 4

Given the findings in chapter 3, formal conferences were held within the study group to discuss how autoantibody generation may be linked. It was felt that RF, as a heavy chain fragment of IgG, may be a target protein for post-translational modifications such as carbamylation and citrullination. If this form of modification were to happen at the Fc binding site, this could have a dramatic influence in terms of local polarity as a ligand and in conformational change of the binding area. A pilot study was devised to test this provisional hypothesis, with analysis taking place prior to sample collection under IRAS ID 194833. As such, samples were taken from a pre-existing sample set at the National Institute for Health Research (NIHR) Exeter Clinical Research Facility,

Exeter, UK. Directed literature search was undertaken by Dr Hutchinson and Dr. Murphy to develop and refine the hypothesis. Dr. Eggleton and Mr. Clarke designed the methodology and undertook laboratory studies at Exeter University Medical School, Exeter, UK, presenting data via videoconference. Mass spectrometry review was undertaken by Dr. Heesom at Bristol University, Bristol, UK.

Subjects

Sera from patients and controls were selected from the Bronchiectasis, Asthma, Control, Rheumatoid Arthritis (BRACRA) study: a prospective, multicentre, case–control, observational study, conducted to determine the relationship between bronchiectasis, RA, RF and anti-cyclic citrullinated peptide antibodies (anti-CCP as the terminology of the BRACRA study, ACPA as used in this study). ethically approved under Integrated Research Application System approval number 12324, Health Research Authority, London, UK. The original study was supported by a grant from Arthritis Research UK. Samples were collected with support of the National Institute for Health Research (NIHR) Clinical Research Network, and stored for future use with the assistance of the NIHR Clinical Research Facility, Exeter University Medical School, Exeter, UK, who were responsible for providing ethically approved healthy control serum samples. The overall study design (including assays used for the determination of RF and anti-CCP levels) and approval has been previously reported [7]. All the RA patients fulfilled the ACR/EULAR 2010 classification criteria for RA with definitions of a negative, low positive and high positive RF and anti-CCP as detailed within [2].

Study design

Serum samples from four bronchiectasis (BR) seroconverts (anti-CCP positive prior to developing RA), four RA smokers without overt lung disease, four bronchiectasis- RA (BRRA) never smokers and ten control subjects (five never smokers and five current/ever smokers) were studied.

Protein analysis

Equal serum protein loads as determined by nanodrop spectrometry were separated

on 8-16% SDS-PAGE gradient gels. A protein band identified by immunoblotting with anti-human citrulline antibody, contained numerous citrullinated proteins in the region of 37-50 kilodaltons (KDa). The bands were excised from Bio-safe™ Coomasie stained gradient gels and subjected to in-gel tryptic digestion using a DigestPro automated digestion unit (Intavis Ltd.). The resulting peptides were fractionated using an Ultimate 3000 nanoHPLC system. Tandem mass spectra were acquired using an LTQ- Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against the UniProt Human database (131351 entries) using the SEQUEST algorithm. Search criteria routinely included carbamidomethylation of cysteine (+57.0214) as a fixed modification and oxidation of methionine as a variable modification. In addition, citrullination (+0.984Da) at Arg, Asn and Gln and carbamylation (+43.006Da) at Lys, Met, Arg, Ser, Thr and Tyr, were included as variable modifications in two separate searches.

Only peptides where citrullination at Arg and carbamylation at Lys were ranked 1 in the respective searches (indicating that those residues were the most likely sites of modification) were considered.

This identified IgG heavy chain as one of the most abundant proteins identified as being citrullinated and carbamylated. The amino acid sequence of IgG was obtained from the Uniprot database and then submitted to a phyre2 search (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) to obtain models in 3D. The UCSF Chimera protein viewing software (http://www.rbvi.ucsf.edu/chimera/) was used to annotate the amino acid modifications of interest to highlight the citrullinated and carbamylated sites on each IgG molecule.

To confirm the presence of citrullination and carbamylation in the light and heavy chains of IgG in subjects, IgG was purified from individual sera by protein G affinity chromotography using an AKTA FLPC system (GE healthcare) employing unicorn 5.1 software. Protein aliquots of IgG were run on SDS-PAGE under reducing conditions to separate the 25 kDa light (IgGL) and 50 kDa heavy chain (IgGH), then immunoblotted and probed with primary anti-citrulline (ab100932) or anti-carbamyl-lysine (ab175132) specific antibodies. Post-translational modifications were detected

by probing the blot with 1:15000 dilutions of IR Dye800-labeled anti-rabbit secondary antibody (Li-Cor P/N 925-32213) followed by infrared imaging (Li-Cor Odyssey infra imaging system).

Chapter 5

The data in this study were collected by myself and trained members of the clinical team in the Rheumatology Department, Royal Cornwall Hospital, UK, as part of project IRAS ID 194833, approved by South West Regional Ethical Committee (UK). The cohort for this chapter consisted of males attending routine rheumatology clinics at the Royal Cornwall Hospital, Cornwall, UK from February 2015 to August 2016. Data were anonymised at source. Patients reviewed in clinic with a new or existing diagnosis of RA during the study period were included for analysis, initially invited to complete a written questionnaire. Data were recorded by clinicians in a standard questionnaire via face-to-face interview to determine current occupation, former occupation if retired, and previously held employments for >1 year. Missing data were obtained by follow up telephone administration of the same questionnaire (Appendix 3). Written consent was obtained for patients enrolled as part of the IRAS ID 194833 project investigating the role of cadmium inhalation and the development of RA. All job titles were recorded, and specific exposure to vapours, gas, dust or fume exposure (VGDF) was recorded, with occupations to be coded via Standard Occupational Classification 2010 (SOC2010) Volume 2 coding index (ONS SOC coding tool, Office for National Statistics, UK) [8], with codes generated relating to the International Standard Classification for occupations (ISCO). Coding does not form part of the analysis for this paper. Individuals were stratified for the number of VGDF exposed occupations held for >1 year (0, 1,≥2). Age, age of RA onset, disease duration, smoking history prior to disease development, RF levels at disease onset and subsequent ACPA levels were recorded as part of routine clinical practice. All patients fulfilled 2010 ACR/EULAR RA criteria at diagnosis [2].

Subjects

726 patients were included for initial analysis. Of these, 22/726 (3%) had incomplete smoking data and were excluded from linear regression analysis. 3/726 (0.4%) had no verifiable RF data and were excluded from RF analysis. 54/726 (7%) had no ACPA data and were excluded from ACPA analysis. 16/726 (2%) were excluded from occupational analysis; 5/16 died during the study period and therefore had incomplete occupational data, 3/16 did not want to disclose occupation in writing or on subsequent interview. 8/16 did not return initial questionnaires and were lost to follow up (Figure 2). Prevalence analysis of VGDF and smoking was analysed as a proportion of the entire identified cohort (n=726) to bias towards the null hypothesis.

Patient involvement

As previously stated, the initial concept of this research was developed from detailed histories of individual patient experiences of RA in Cornwall, UK. Initial hypotheses were presented at a local meeting of the National Rheumatoid Arthritis Society, attended by over 100 members, the majority of whom were RA patients. Subsequent interest following the meeting led to further discussion as to the development of a protocol that would be acceptable to patients in terms of its design and acceptability. From initial ideas, this was refined and presented to the committee of the Cornwall Arthritis Trust, a local charity supporting arthritis patients. Specific feedback was offered on wording of consent forms, questionnaire design and arrangements for follow up on patients who may not have been able to complete written questionnaires. Patient involvement in this process was invaluable for how best to manage and undertake the research in a way that was minimally intrusive for patients in their regular clinical care.

Dissemination of results to patients will take place via departmental displays post publication.

Autoantibody measurement

RF was measured with Tina-quant Rheumatoid Factors I1 Test System (Roche Diagnostics Corporation), with a value of < 14 IU/ml considered as negative as per manufacturer guidelines. ACPA was measured by Second Generation E170 Anti-CCP analysis (Roche Modular Analytics), with a negative value of <17 U/ml as per manufacturer guidelines.

Social deprivation analysis

Social deprivation analysis was undertaken through the UK government validated Index of Multiple Deprivation (IMD) [3], a deprivation rank score of 32,844 neighbourhoods weighted by income, health/disability, education, housing/service access, crime and living environment, converted to equal deciles.

Statistical analyses

Values are expressed as a median (interquartile range (IQR), or number (%). The Mann-Whitney U test was used for non-parametric comparisons of continuous data of differing sample group sizes. All data were analysed using commercially available software (Microsoft Excel (Microsoft Corp) and Number Cruncher Statistical System for Windows (NCSS60)).

Chapter 6

Given the fascinating findings uncovered on the prevalence of inhalational insults, their effect on autoantibody generation, and implications for disease severity, it was felt that intra-cohort analysis was needed to analyse possible differences between patients with nodular disease and those without. Initially determining the overall prevalence of nodular disease in the Cornish rheumatoid arthritis (RA) cross-sectional patient cohort, analysis was made to compare rheumatoid factor (RF) levels, anti-citrullinated protein antibody (ACPA) levels and urinary cadmium (Cd) levels amongst nodular and non-nodular patients.

Subjects

Two cohorts of RA patients were studied. Cohort 1 (n=303) consisted of all RA males and females attending routine rheumatology clinics at the Royal Cornwall Hospital, Cornwall, UK from August 2017 to February 2018 to determine prevalence of nodular RA, as part of Departmental Audit. Cohort 2 consisted of prospectively gathered nodular RA males and females (n=92), matched for age (+/– 2 years), sex, and smoking history (+/–2 pack years), to patients previously collected as part of project IRAS ID 194833, approved by South West Regional Ethical Committee (UK) as previously described [9] (n=133). Specifically more males were recruited to this arm of the study as our unit were actively investigating the relationship between occupational exposures and the development of male RA. Cohort 2 (n=225) was stratified for nodular disease and smoking with comparison made for urinary cadmium. 3 cases were excluded as extreme outliers with cutoff value of >3 μmol/mol creatinine used to bias towards the null hypothesis.

Both cohorts were amalgamated (Cohort 3) with duplicate records excluded (n=24). RF and ACPA levels were compared between all RA patients (n=504) stratified for nodular disease and smoking. 18 patients were excluded from ACPA analysis due to incomplete data (n=6 RF negative, n=3 RF weak positive, n=9 RF strong positive), Figure 3. Data was anonymised at source. Age, age of RA onset, disease duration, smoking history prior to disease development, RF levels at disease onset and subsequent ACPA levels were recorded as part of routine clinical practice. Patient occupational histories were recorded as part of ongoing clinical audit using methodology previously described [9]. All patients fulfilled 2010 ACR/EULAR RA criteria at diagnosis [2].

Autoantibody measurement

RF was measured with Tina–quant Rheumatoid Factors II Test System (Roche Diagnostics Corporation), with a value of <14 IU/mL considered as negative as per manufacturer guidelines. ACPA was measured by Second Generation E170 Anti–CCP analysis (Roche Modular Analytics), with a negative value of <17 U/mL as per manufacturer guidelines.

Social deprivation analysis

Social deprivation analysis was undertaken through the UK government validated Index of Multiple Deprivation (IMD), a deprivation rank score of 32 844 neighbourhoods weighted by income, health/disability, education, housing/service access, crime and living environment, converted to equal deciles [3].

Statistical analysis

Chi square tests and multivariate logistic regression analysis was used to examine relationships between smoking, nodularity and urinary Cd levels, adjusted for gender. The Mann–Whitney U test was used for non–parametric comparisons of continuous data of differing sample group sizes, with values are expressed as a median (IQR) or number (%). All data were analysed using commercially available software (Microsoft Excel (Microsoft Corp) and Number Cruncher Statistical System for Windows (NCSS60)).

Urinary Cadmium analysis

Patients supplied first pass morning mid–stream urine samples into 13ml sterile polystyrene urine sample tubes (Thermo–Fisher, Newport, UK). Samples were posted within 72 hours to the Supra–Regional Assay Service Trace Elements Laboratory, Guildford, Surrey. Urinary cadmium was analysed by inductively coupled plasma mass spectrometry (ICP–MS), using a fully validated method previously described [1]. An appreciably raised urinary Cd was defined as a urinary Cd level above the 95th centile

as determined for UK populations unexposed to occupational Cd, equating to 0.65 mmol/mol creatinine (females), and 0.42 mmol/mol creatinine (males). The median levels were reported as 0.19 mmol/mol creatinine and 0.16 mmol/mol creatinine respectively for females and males [1].

Smoking history

Pack years smoked was recorded prior to the diagnosis of RA. One pack year is deemed equivalent to smoking 20 cigarettes daily for a year. A smoker was defined as an individual that had smoked in a period up to 20 years before RA diagnosis and > 5 pack years total. A non–smoker was defined as an individual who had either never smoked or had smoked in the distant past (>20 years prior to the diagnosis of RA) and < 5 pack years total.

Subcutaneous rheumatoid nodules

Individuals were examined in clinic to determine the presence of characteristic rheumatoid nodules on the hands, forearm, elbow and feet. Patients who had undergone surgical removal of nodules and histological examination had confirmed the diagnosis, were considered to be a rheumatoid nodular patient irrespective of the presence of rheumatoid nodularity at the time of the examination. No radiological evidence of pulmonary rheumatoid nodules was collected.

Appendix 5 references

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