Major involvement of bacterial components in rheumatoid arthritis and its accompanying oxidative stress, systemic inflammation and hypercoagulability

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Impact statement
Rheumatoid arthritis (RA) is accompanied by long-term inflammation that is mediated by cytokines and cross-reactive (auto-)antigens. Here we suggest one explanation is the presence of a (dormant) microbiome in RA that sheds the highly potent inflammagen, lipopolysaccharide lipopolysaccharides (LPS) to catalyze inflammagenesis, including via β-amyloid formation. We discuss various co-existing features in RA, including iron dysregulation, hypercoagulability, anomalous morphologies of host erythrocytes, and microparticle formation. We review literature and provide coherent evidence that an aberrant blood microbiome in RA has a major involvement in the development, progression, and therefore over-all etiology of the disease.

Abstract
We review the evidence that infectious agents, including those that become dormant within the host, have a major role to play in much of the etiology of rheumatoid arthritis and the inflammation that is its hallmark. This occurs in particular because they can produce cross-reactive (auto-)antigens, as well as potent inflammagens such as lipopolysaccharide that can themselves catalyze further inflammagenesis, including via β-amyloid formation. A series of observables coexist in many chronic, inflammatory diseases as well as rheumatoid arthritis. They include iron dysregulation, hypercoagulability, anomalous morphologies of host erythrocytes, and microparticle formation. Iron dysregulation may be responsible for the periodic regrowth and resuscitation of the dormant bacteria, with concomitant inflam-magen production. The present systems biology analysis benefits from the philosophical idea of “coherence,” that reflects the principle that if a series of ostensibly unrelated findings are brought together into a self-consistent narrative, that narrative is thereby strengthened. As such, we provide a coherent and testable narrative for the major involvement of (often dormant) bacteria in rheumatoid arthritis.

Keywords: Rheumatoid arthritis, dormancy, iron dysregulation, atopobiosis, infectious agents, lipopolysaccharides, Proteus, inflammation, comorbidities

Introduction: Disease background
RA is a complex and heterogeneous disease, sometimes classified as a syndrome with shared clinical manifestations.1 It is the most common immune-related chronic, inflammatory, autoimmune disease and affects approximately 0.5–1% of the adult population worldwide. It occurs as 20–50 cases per 100,000 annually, most commonly in women over 40.2–5 Although this is not yet apparently a mainstream recognition, a frankly overwhelming amount of epidemiological and experimental evidence, that we shall review here, indicates a microbial origin for RA. The clinical features of RA involve the presence of systemic inflammation, with various imbalances between pro- and anti-inflammatory cytokine activities, which may lead to multisystem immune complications.4 In RA patients, serum or plasma levels of cytokines are considered to indicate the severity of disease4 and this pathophysiologic presence of pro-inflammatory cytokines is known to be involved in the degradation of bone and cartilage.4 Due to the complexity of the disease, treatment and disease tracking after diagnosis is very difficult. In this article, we shall review briefly current knowledge regarding the involvement of cytokines and other markers in RA, which are also the hallmarks of systemic inflammation in all other inflammatory conditions. We also discuss clot hypercoagulability and platelet and erythrocyte (RBC) changes, that is consequent upon this persistent systemic inflammation, and how microparticle formation (from both platelets and RBCs) is
a characteristic feature of RA. We then review a consider-
able literature (summarized in part in two books6,7) that
suggests that the presence of a variety of detritus produced
from walls and membranes of Gram-negative and other
bacteria (including wall-less forms) may play a fundamen-
tal role in RA development, as well as the accompanying
cardiovascular disease and systemic inflammation seen in
RA. We discuss how the exposure of genetically susceptible
individuals to environmental factors (1) that can act as trig-
gers (2), cause an immunological reaction, followed by an
autoimmune response (3), can result in RA (4). We review a
plethora of evidence, collectively referred to as Ebringer’s
theory (5), that points to the environmental trigger as micro-
bial (particularly from e.g. urinary tract infections) (6). We
then look at the role of LPS from these microbes (7) in caus-
ing an imbalance between pro- and anti-inflammatory cyto-
kines, followed by systemic inflammation, and the effect on
the cardiovascular and hematological health of the RA
patient (8) (see Figure 1). Finally, recognizing the lack of
easy and accessible biomarkers, we suggest that in a truly
patient (8) (see Figure 1). Finally, recognizing the lack of
cardiovascular and hematological health of the RA
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Epidemiology

An initial analysis of the potential causes of a disease is wise
to explain any unusual epidemiological features it may pos-
sess,9 following appropriate controls for their veracity.10
Clearly, within an overall prevalence of ca 0.5–1%, one fea-
ture of this disease is its considerable predilection for
women (69% of cases in a recent UK survey5) over men,
despite some reduction in female prevalence attributed to
the use of oral contraceptives.11,12 This is a striking differ-
ence of approximately two-or three-fold (see also litera-
ture2,12–15). Clearly, it might be linked to hormonal
differences, or to something on the X-chromosome, but
we know of no persuasive study that suggests what that
might be.

Twin studies “show concordance rates of 15% to 30%
between monozygotic twins and 5% among dizygotic
twins,14 suggesting that 50% to 60% of RA cases are due
to genetic factors.16,17” Other studies comparing monozy-
gotic twins alone show an occurrence in a second twin,
when a first twin manifests the disease, as just 12% in
Finland,18 15% for the UK,19 and 10% in Denmark.20 Thus,
environmental influences have a major influence.
Consequently, the leading hypothesis for RA (and indeed
for most other autoimmune disorders) is that RA is the
result of an environmental exposure or “trigger” in a gen-
etically more susceptible individual, that causes an
immunological reaction to the triggering antigen that hap-
pens to share one or more epitopes with a host protein (and
see Kell and Pretorius21), thus manifesting the autoimmune
responses. What might be the most common triggers? One
possibility to link the triggers with infection is to look at the
presence of flares. Flares are defined as a worsening of signs
and symptoms of sufficient intensity and duration to lead to
changes in therapy as per the Outcome Measures in
Rheumatology Clinical Trials (OMERACT) RA Flare
Definition Working Group, developed at OMERACT 9 in
2008.22 This working Group developed a standardized
method for description and measurement of “flare in RA”
to guide individual patient treatment.23

A very high proportion of sufferers were actually
exposed to an infection before their disease was diagnosed,
but sadly these kinds of data are not typically recorded
properly in the scientific literature. Consequently, and as
this becomes increasingly easy with electronic health
records, we do encourage clinical readers to make such ana-
lyses available. However, as with several related diseases
(e.g. literature21,24–26), our role as systems biologists is to put
gether a coherent, systems biology picture, and with all
the evidence that we shall review below, it is very clear
indeed that RA is driven by a microbial component. To
this end, one very major (and in our view clear) differenti-
ator between women and men is the equally more common,
anatomy-based prevalence in women over men of urinary
tract infection.29–31 This is the first “plank” in Ebringer’s
impressive series of arguments (most recently at 23)
that actually gives a satisfying and coherent account of at
least one microbial origin for RA, Proteus spp., that we now
review.
Ebringer's theory (with experiments) that *Proteus* infection from the urinary tract is a major cause of RA

Ebringer sets his work in a Popperian framework (for reviews of that see literature\(^{34–36}\)) but we consider that it is more conveniently set in a systems biology manner as a logical series or chain of intellectually linked events, and this is what we do here. Ebringer (we like especially Ebringer et al.\(^{32}\)) makes the following 10 claims (the many supporting references are in the paper):

1. HLA-DR4 lymphocytes injected into a rabbit evoke specific antibodies against *Proteus* cells (mainly the species *mirabilis* and *vulgaris*).
2. Antibodies to *Proteus* spp. are present in RA patients from 14 different countries.
3. Antibodies to *Proteus* bacteria in RA patients are disease-specific since no such antibodies are found in other conditions.
4. When RA patients have high titer of antibodies to *Proteus* such bacteria are found in urinary cultures.
5. Only *Proteus* bacteria and no other microbes evoke significantly elevated antibodies in RA patients (this is not 100% true, see below).
6. A “shared epitope” EQR(K)RAA shows “molecular mimicry” with the related sequence ESRRAL found in *Proteus* hemolysis.
7. *Proteus* urease contains a sequence IRRET which has “molecular mimicry” with the related LRREI found in collagen XI of hyaline cartilage.
8. Sera obtained from RA patients have cytopathic properties against sheep red cells coated with the cross-reacting EQR(K)RAA and LRREI self-antigen peptides.
9. *Proteus* sequences in hemolysin and urease as well as the self-antigens, HLA-DR1/4 and collagen XI, each contain an arginine doublet, thereby providing a substrate for peptidyl arginine deiminase (PAD) to give rise to citrulline, which is the main antigenic component of CCP, antibodies to which are found in early cases of RA.
10. Antibodies to *Proteus* come not only from sequences cross reacting to self-antigens but also from non-cross reacting sequences, thereby indicating that active RA patients have been exposed to infection by *Proteus*.

Taken together, these arguments show strongly that microbes, especially those derived from urinary tract infections, can act as triggers of autoimmunity, via established epitopes or antigens. We should point out, however, that many other studies (and it is highly doubtful that there could be a unitary cause) indicate antibody and PCR-based evidence for the presence or role of other microbes in RA. Both gut dysbioses and a changed oral microbiota have also been implicated. There is also evidence of a significant association between periodontitis and RA.\(^{37–43}\) Gut dysbioses are also frequently found in RA individuals\(^{44,45}\) and it was recently shown that dysbioses in RA patients may reflect an unusual abundance of certain rare bacterial lineages.\(^{46}\) Normalizing the gut microbiota was also suggested in assessing prognoses and in the treatment of RA.\(^{45,46}\) Some of these other microbes that are associated with RA are listed in Table 1 (and see also literature\(^{21,27,29}\)).

This was a binary (presence/absence) assessment of the microbial contribution, but microbes have many properties beyond presence and absence. In particular, a notable and missing feature of most of these studies (including those of Ebringer) involves (i) the physiological state of the organisms in question, and (ii) what causes them to manifest their activities periodically (for instance as the “flares” characteristic of RA). This we therefore discuss next.

**Dormancy, resuscitation, and iron dysregulation**

Clinical or infection microbiologists typically recognize bacteria as being in one of two physiological macrostates, either being “alive” (on the basis of their being capable of replications, e.g. to form a colony on a petri dish containing a suitable solid medium), or if not being so capable then being assumed “dead.” However, these are not the only two major physiological states, and indeed they are probably the least common in natural environments! Importantly, the definition of these states is operational: the appearance of a microbe’s physiology also depends on the experiment being used to test it and is not of itself an “innate property” of the organism.\(^{65–67}\) In environmental microbiology, most microbes are non-growing because they lack the nutrients and/or signaling molecules to replicate, but they are not (irreversibly) “dead.” They are best described as “dormant,” and the means by which they are brought back from an apparent state of non-aliveness to one in which they can be cultivated is conventionally known as “resuscitation.” We demonstrated this in a series of papers in laboratory cultures of various actinobacteria (e.g. literature\(^{65,68–73}\)), leading to the discovery of an autologous “wake-up” molecule, the “resuscitation promotion factor” or Rpf\(^{74–78}\) that was necessary for resuscitating dormant bacteria in the presence of weak nutrient broth. Note that assays must be done under conditions of dilution to

| Organism                  | Evidence                  | Selected references |
|---------------------------|---------------------------|---------------------|
| Campylobacter             | Microbiology              | 47                  |
| Chlamydia trachomatis     | Synovial tissues          | 48                  |
| Escherichia coli          | Antibodies                | 49–50               |
| Multiple organisms        | Review                    | 27,51–53            |
| Mycoplasmas               | PCR, westerns, antibodies | 54–57               |
| Porphyromonas gingivalis  | Antibodies, PCR, culture  | 38,58–63            |
| Staphylococcus aureus     | Microbiology of hip joint infections | 64 |
Calculated growth requirements
Unknown growth and/or isolation
medium requirements
Unknown growth and/or isolation
Medium requirements
Antibiotic sensitivity
Phenotype switching between cultureability and dormancy
Dormant bacteria
Non-culturable bacteria
Culturable bacteria
Subpopulations within a differentiated bacterial system
Figure 2  A bacterial system contains distinct subpopulations (1), that we classify as culturable, dormant and non-culturable (2). Specific attention is given to persister cells (3), and the inter-relationship (4) between the subpopulations and phenotypic switching between cultureability and dormancy (5). Throughout we follow a systems biology approach to suggest resuscitation due to various triggers like iron and noradrenaline (6). (A color version of this figure is available in the online journal.)

extinction,66,67 to avoid the possibility of simple regrowth of small numbers of cells that were always “alive” and never dormant. In clinical microbiology, the terms “persister” and “persistent” are commonly used to refer to a phenotypically non-growing (but non-dead) subset of microbes, typically those that have been treated with but tolerant to antibiotics (e.g. literature27,28,79–86). In general terms, these too are operationally dormant as defined above, though their relative physiological states (e.g. as judged by expression profiles) are not really established. We have recently summarized the evidence for a dormant blood microbiome including in red cells21,27,28 (and see also Damgaard et al.87) to complement other literature pertaining to white cells and tissues (e.g. literature88–92). Such dormant cells are, of course, well placed to create inflammation via a continuing production of inflammatory agents such as LPS and molecules with antigenic properties as described above. We note that the emergence of infection may also accompany extinction,66,67 to avoid the possibility of simple regrowth of small numbers of cells that were always “alive” and never dormant.

The question then arises as to what kinds of stimuli trigger the resuscitation. Two are well established. One is the stimulation of Gram-negative bacterial growth by the stress hormone noradrenaline (NA) and other auto-inducers.97–103 One of the roles of NA is to act as a siderophore,104,105 since it is normally available iron that limits microbial replication in vivo.106–111 a phenomenon that adds considerably to the undesirable and purely chemical effects of the second one, which is the presence and availability of unliganded iron that is liable to catalyze the highly damaging Fenton reaction.25,112 (see Figure 2 adapted from Kell et al.27).

Pathophysiologic markers of inflammation in RA

As is well established, inflammatory agents such as LPS lead to the induction of inflammatory cytokines, most commonly IL-6, IL-1β, and TNF-α.129,147,201,142,159 Cytokine-mediated pathways are central to the pathogenesis of RA.119 A MEDLINE, Google Scholar, Scopus, and Web of Science assessment revealed that a plethora of cytokines and markers of inflammation are involved in RA pathology, and importantly, these cytokines are not only localized to the synovial fluid, but are systemically present and detectable in serum samples (see Table 2). Furthermore, a changed systemic cytokine activity is typically associated with oxidative stress, and this is also true in RA individuals.202–205

Systemic inflammation, cardiovascular disease, and RA

All of the above-mentioned cytokines and related inflammatory mediators are known to be involved in both systemic inflammation and cardiovascular disease, and it is also known that patients with RA carry an excess risk for cardiovascular disease,119,206–210 i.e. that there is a comorbidity. Indeed, cells and cytokines implicated in RA pathogenesis are involved in the development and progression of atherosclerosis, (which is generally recognized as an inflammatory cardiovascular disease219). Analysis of RA patients selected from an RA clinic in South Africa (ethical clearance obtained) confirmed that indeed cardiovascular complications are an important part of the clinical profile of RA patients (see Table 3).

Cardiovascular comorbidities relate more than others to disease activity in RA, and particularly type 2 diabetes and hyperlipidemia were found to be associated with disease activity.211 Cardiovascular disease, and particularly diabetes, has also been linked to gut dysbioses and bacterial translocation.212–216 Recently, it was also suggested that a co-morbidity index should be used both at baseline, and as a continuous variable in analyses in RA patients,217 as some co-morbidities are causally associated with RA and many others are related to its treatment.218

Systemic inflammation is entirely consistent with the plethora of diseases and comorbidities that exhibit iron dysregulation,24,112 raised serum ferritin,219 hypercoagulation and hypofibrinolysis,25 and anomalous morphological changes in both erythrocytes and fibrin.230,231 We thus turn to hypercoagulation in RA.

Hypercoagulation, erythrocyte (RBC), and platelet involvement in RA as a result of systemic inflammation

Another hallmark of systemic inflammation (as well as cardiovascular pathology) is clot hypercoagulability, and a hypercoagulable state is also found in RA,222–231 together with a decreased clot lysis ability,25,225,226 possibly due to genuine amyloid formation.232 Systemic inflammation, oxidative stress, and hypercoagulability all affect erythrocytes (RBCs) and platelets. One of the changes due to the systemic inflammation and oxidative stress noticeable in RBCs and platelets is the production of cell-derived microparticles (MPs).219

Flow cytometry is the usual method to quantify MP233,234; unfortunately, the small size of these structures and lack of standardization in methodology complicates
Mostly, as MP contain surface and cytoplasmic contents of the parent cells and bear phosphatidylserine, antibodies to specific cell surface markers and annexin V can be used for identification or for tissue factor-dependent FXa generation assays. Their sizes can vary but are of the order of 50 to 800 nm and are therefore easily detected on and around their mother-cell by scanning electron microscopy.

In RA, circulating MPs exposing complement components or activator molecules are elevated, and their

### Table 2

| Cytokines and other markers of inflammation | Effect on RA                                                                 | Selected references |
|--------------------------------------------|------------------------------------------------------------------------------|--------------------|
| IL-1β                                      | Upregulated; strong stimulator of bone resorption. Linked to joint inflammation and cartilage and bone destruction in patients with RA. | 113–118            |
| IL-6                                       | IL-6 is involved in the regulation of immune responses, hematopoiesis and inflammation and is found in abundance in the synovial fluid and serum of patients with RA and the level correlates with the disease activity and joint destruction. Present in serum of RA patients. | 119,120,121,113,114,141 |
| IL-8                                       | It is a neutrophil-activating peptide and the degree of neutrophil turnover is linked to the anaerobic metabolism of the synovial cavity. Upregulated in RA; and IL-6/IL-1β co-stimulation increases IL-8 production. Present in serum of RA patients. | 129,130–135         |
| IL-12                                      | Linked to leukocyte migration, bone erosions and angiogenesis in RA. Present in serum of RA patients. | 114,136–141         |
| IL-15                                      | Upregulated and expressed in synovial fluid. Long-term retention of IL-15/IL-15R complexes on the surfaces of monocytes and dendritic cells. Present in serum of RA patients. | 142,143–146         |
| IL-16                                      | Present in human synovial fibroblasts. Present in serum of RA patients.     | 147,148–151         |
| IL-17                                      | Plays key roles in the propagation of joint inflammation, cartilage destruction, and bone erosion; IL-17 inhibits progenitor cells in RA cartilage; has regulatory roles in host defense and chronic inflammation which result in tissue damage and autoimmunity; shares downstream transcription factors with IL-1 and TNF-α; promote osteoclastogenesis. Present in serum of RA patients. | 147,152,147,113,114,123,132,153–158 |
| IL-18                                      | Detected in synovial fluid in RA patients. Present in serum of RA patients. | 159,137,160–165     |
| IL-23                                      | IL-23 is essential for the differentiation of Th17 lymphocytes, a subtype of T lymphocyte implicated in RA and promotes osteoclastogenesis Present in serum of RA patients. | 123,139,157,166–168 |
| IL-27                                      | IL-27 upregulated and produced by antigen-presenting cells. Linked to leukocyte migration, bone erosions and angiogenesis. Present in serum of RA patients. | 139,155,169–175     |
| IL-7, IL-10, IL-19, IL-20, IL-22, IL-32, IL-35 | All implicated in contributing to the pathogenesis of RA. | 138,139,176         |
| Toll-like receptor 2 (TLR-2) and TLR-4     | Expressed in inflamed RA synovium, and the expression of these receptors is associated with the presence of inflammatory cytokines. expressed by cells within the RA joint and a variety of endogenous TLR ligands are present within the inflamed joints of patients with RA. | 147,177–180         |
| TNF-α                                      | Promotes systemic inflammation and autoimmune pathology and is one of the cytokines that make up the acute phase reaction. Linked to joint inflammation and cartilage and bone destruction in patients with RA. Present in serum of RA patients. | 119,114,117,120,132,161–184 |
| interferon γ (IFN-γ)                       | Promotes autoimmune pathology and plays a role in immunity against intracellular pathogens; abundantly expressed in rheumatoid synovitis. Present in serum of RA patients. | 113,185–188         |
| Prostaglandin E2                           | Acts as mediator of immune inflammation. | 132,189,190         |
| Thromboxane-A2 and COX-2 [cyclooxygenase (COX)-2/thromboxane A2 (TxA2)] | Thromboxane-A2 produced by activated platelets and has prothrombotic properties, while COX-2 is responsible for the formation of thromboxane and prostaglandins. | 191–193              |
| Iron                                       | Increased levels in synovial fluid and changed serum ferritin levels         | 194–200             |
levels are correlated with disease activity.\textsuperscript{240} These MPs are also of great importance in cardiovascular diseases, and this may be one reason for the enhanced cardiovascular morbidity and mortality seen in RA.\textsuperscript{240} Furthermore, MPs may contribute to the local hypercoagulation and fibrin deposition in inflamed joints of patients with RA.\textsuperscript{241}

Particularly, MPs derived from platelets that are involved in various thrombotic events are elevated in RA patients, and these platelet-derived MPs may be an important role-player in RA\textsuperscript{240,242,243}; they may even be used as a biomarker reflecting systemic cell activation in RA.\textsuperscript{244,245} Platelet MPs in RA have also been found to be responsible for detrimental effects on endothelial cells, thus supporting their role as biomarkers of vascular damage.\textsuperscript{246} Although RBC MP formation is not very well described in RA in particular, we recently discussed possible mechanisms by which RBC MP formation may occur in RA.\textsuperscript{245} The nature and distribution of lipids in RBC bilayers are altered in RA, showing a decreased level of cholesterol and phospholipids when compared to healthy controls.\textsuperscript{247} Changed levels of cholesterol found in the RBC phospholipid bilayer can determine its capacity to express phosphatidylserine (PS) on the outer leaflet, independent of ATP-driven flip-pases.\textsuperscript{248} It is also well known that oxidative stress in RA influences RBC membrane integrity (see Table 4), and this supports the possibility that portions of the RBC membrane may bud off to form RBC MPs.\textsuperscript{174}

Pathologic changes in coagulation result directly in abnormal fibrin fiber formation during clotting, and MPs associated with platelets, as well as membrane changes of RBCs, can be visualized by scanning electron microscopy in blood smears from RA individuals. See Figure 3(a) for an example of healthy fibrin fiber formation versus pathological fibrin fiber formation in RA (Figure 3(b)), and a healthy platelet showing a clear cell body and pseudopodia formation with a smooth membrane (Figure 4(a)) versus platelets from RA where activation, spreading, and MP formation are visible (Figure 4(b) and (c)). Red arrows in Figure 4(c) possibly indicate round ultramicrobacteria, which differ from the much more irregularly shaped MPs.

Figure 5(a) and (b) shows a typical healthy RBC with no membrane changes, compared to normal RBCs, of a typical RA individual (Figure 5(c) and (d)), where RBC folding due

\begin{table}
\centering
\caption{An analysis of the co-existing conditions of rheumatoid arthritis patients from a rheumatoid arthritis clinic in South Africa, showing baseline demographics of subjects (n = 38) with Rheumatoid Arthritis}
\begin{tabular}{|c|c|c|}
\hline
Variable & Mean ± (SD) for age, others N (%) & \\
\hline
\hline
Age, years & & \\
Males & 53 ± (13.01) & \\
Females & 55 ± (11.54) & \\
\hline
Chronic medication: & Females & Males & \\
NSAID & 21 (64%) & 4 (80%) & \\
Prednisone & 20 (61%) & 5 (100%) & \\
Chloroquine & 7 (21%) & 2 (40%) & \\
Salazopyrin & 8 (24%) & 1 (20%) & \\
Opioids & 13 (39%) & 1 (20%) & \\
Anti-depressants & 6 (18%) & 1 (20%) & \\
Acid-reducers & 17 (51%) & 3 (60%) & \\
Anti-hypertensives & 14 (42%) & 5 (100%) & \\
Statins & 10 (30%) & 2 (40%) & \\
\hline
Co-morbid conditions: & & & \\
Hypertension & 21 (64%) & 5 (100%) & \\
Dyslipidaemia & 12 (36%) & 1 (20%) & \\
Hypothyroidism & 4 (12%) & & \\
Asthma & 3 (9%) & & \\
Anaemia & 3 (9%) & & \\
Diabetes mellitus & 1 (3%) & 1 (20%) & \\
Gastric reflux & 11 (33%) & 3 (60%) & \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Markers taken as indicators of oxidative stress in RBCs}
\begin{tabular}{|c|c|}
\hline
Marker & Observed effect On RBCs & \\
\hline
Peroxides & Increased in RBCs\textsuperscript{249} & \\
Glutathione & Oxidized in RBCs\textsuperscript{249–251} & \\
Catalase & Increased in RBCs\textsuperscript{250} & \\
Superoxide dismutase & Increased in RBCs\textsuperscript{250} & \\
Malondialdehyde & Increased in RBCs\textsuperscript{250} & \\
Membrane redox system & Upregulated in RBCs\textsuperscript{250} & \\
Caspase-3/Calpain & Increased in RBCs\textsuperscript{251} & \\
Enzyme activity, e.g. ATPases & Altered in RBCs\textsuperscript{249,251–254} & \\
2,3 Diphosphoglycerate & Decreased in RBCs\textsuperscript{251,253,254} & \\
\hline
\end{tabular}
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image}
\caption{Fibrin fiber formation in the presence of thrombin (a) healthy fibrin and (b) rheumatoid arthritis fibrin with matted fibrin. Scale: 1 \textmu m}
\end{figure}
to loss of structural integrity is seen in the presence of fibrin fiber formation (when thrombin is added (Figure 5(c))) and membrane changes are observed with MP formation (Figure 5(d)). The changed membrane surface is also confirmed via roughness analysis of RBC membranes using atomic force microscopy (AFM), where a significantly increased roughness was noted in RA RBCs compared to the case of healthy RBCs (Figure 6).

Figure 4 (a) A healthy platelet with prominent cell body and pseudopodia formation and smooth membrane; (b) two spreaded and activated platelets with microparticle formation (irregularly shaped structures closely associated with membranes (white arrows)) in rheumatoid arthritis; red arrows showing much rounder structures – possibly ultrabacteria; (c) a higher magnification of an RA platelet with microparticles budding off spreaded platelet. The scale bars are (a): 200 nm; (b): 1 μm and (c): 200 nm. (A color version of this figure is available in the online journal.)

Figure 5 (a) and (b) A representative healthy RBC (b is higher magnification showing the membrane); (c) and (d) A representative rheumatoid arthritis RBC with folding (c) and visible membrane microparticle formation. Scale bars for (a) and (c): 1 μm; scale bars for (b) and (d): 100 nm
A great many approaches to assessing disease severity exist, and the tendency to prescribe more drugs to those with more severe disease is necessarily a confounding factor. Databases for medical claims around disease severity have value, but they tend to lack information on important clinical variables, such as the number of tender and swollen joints, which would traditionally be used to assess disease severity in RA. Where comparissons exist, the information regarding, e.g. the presence of swollen joints and disease severity, are not well correlated. Objective measures of variables such as inflammatory cytokines are attractive, but for the patient, the severity, especially of pain, is subjective, and patient-assessed severity indices are consequently common. This said, the patient global assessment (PGA) is noteworthy as while using seven objective criteria based on stiffness and swelling, pain (being presumably too subjective) is not among them. Other studies did find some correlations between swelling, stiffness, and pain of joints.

**Small molecules (sDMARDs) and their role(s) as antibacterials**

The chief recommendations are to start early and monitor frequently. These are seen as the strategy of first resort, with methotrexate, sulfasalazine, and leflunomide being seen as front line drugs (possibly along with low-dose glucocorticoids). It is highly noteworthy that sulfasalazine is in fact an antibiotic (one of the first), as it is split in the intestine into aspirin and the antibiotic sulphaspyridine, while methotrexate shows antibiotic activity against organisms as diverse as *S. aureus* and *Plasmodium vivax*. As mentioned above, “first treatments” are the least likely to be confounded by bias occasioned by the fact that later treatments will be favored by patients with more severe or refractory disease, a phenomenon that probably confounded a study of minocycline and doxycycline. However, and while – like other drugs – they probably have multiple effects, a good many studies indicate the utility of the antibiotics minocycline and doxycycline in treating RA (e.g. literature). After sDMARDs have been tried, the recommendation is to move to a biological.

**Biologicals (bDMARDs)**

The chief biologicals are inhibitors of TNF-α, including monoclonals, and inhibitors of the IL-6 receptor; they all decrease inflammatory symptoms, and it is unclear whether they might have any direct or indirect antibacterial effects. Their chief issue is that, as proteins, they can themselves cause (auto) antigens to be raised, while, as mentioned above, any dampening of immune system response may increase the likelihood of novel or emergent infection.

**Iron chelation as a therapeutic?** If the “dormant microbial” hypothesis is correct, it is to be predicted that...
A role for lipopolysaccharides (LPS) in RA

Recently, we summarized the evidence for a significant involvement of lipopolysaccharide shed by dormant and resuscitating bacteria as underpinning the chronic inflammation characteristic of a variety of diseases, and suggested that LPS may play a role in the pathogenesis of RA.\textsuperscript{21} The presence and role of LPS may be supported by a recent review that provided evidence for the involvement of a microbiome in inflammatory arthritis and rheumatic diseases.\textsuperscript{316} Recently, Scher et al.\textsuperscript{317} also found strong correlates between the presence of \textit{Prevotella copri} and new-onset untreated RA patients.

Certainly, LPS is also known to upregulate all of the cytokines upregulated in RA and mentioned in Table 1. In our recent review,\textsuperscript{21} we also focused on the fact that antibodies could be generated to LPS that – like the anti-\textit{Proteus} antibodies mentioned in detail above – might also serve as autoantibodies of significance in RA and in particular during the flares (that may be ascribed to periods of particular resuscitation activity).

The generalized LPS also exerts its effects via activation of cytokines such as IL-6, and TNF-\textit{\alpha} in response to LPS,\textsuperscript{115} IL-8,\textsuperscript{318} IL-12,\textsuperscript{319} IL-15,\textsuperscript{320} thereby exciting the innate...
immune response. The scheme is typically as follows (extensively discussed in Kell and Pretorius21):

- LPS binds to the toll-like receptor 4 (TLR4).321–325
- Production of a variety of pro-inflammatory cytokines,326–328 where NF-κB plays a prominent role329,330 via a set of canonical pathways illustrated in Figure 7.
- NF-κB translocates to the nucleus to turn on a great many genes in a frequency-dependent fashion, including in particular TNF-α and IL-6.331–333
- At high concentrations of LPS,334,335 it also activates a “non-canonical” inflammasome pathway, which is independent of TLR4336,337 (see Figure 8).

Finally (see above), LPS may catalyze the formation of inflammatory and cytotoxic β-amyloids. Consequently, we might again suggest that appropriate antibacterials and iron chelators (that can hitchhike on the necessary transporters282,339–343) would serve to lower this stimulus, and in contrast to the biologics actually strike at the root causes of the disease.

**Further tests of our hypothesis**

While we have adduced much evidence in favor of the view that recurring infection by (resuscitating dormant) bacteria is in fact a, if not the, major and ultimate cause of RA, albeit there is not a unitary “cause,” our views do come with multiple predictions that may be tested (of course some have been already, see above, in many cases extensively).

- Bacteria should be detectable in relevant tissues of RA patients, whether by culture or by molecular methods (e.g. macromolecular sequencing or antibodies).
- Relevant products such as LPS and other antigens should be detectable in patients vs. controls.
- Their numbers (bacteria and/or inflammatory products) should increase with disease severity and during “flares.”
- Their numbers and activity (hence disease prevalence/severity) should correlate with free iron levels.
- Treatments that lower the activity of bacteria and/or their products should be of significant therapeutic benefit.
- These may include iron withholding, antibacterial, anti-LPS, and anti-amyloid treatments.

Although probably not yet seen as mainstream, a number of therapeutic strategies based on these and other ideas (including the roles of vitamin D metabolites, that for reasons of space we do not discuss here) do indeed seem to have enjoyed success (e.g. literature344–348).
Concluding remarks
As mentioned previously, while it can be difficult (but cf.49) to ascribe causality in complex biochemical networks, an accepted strategy within the philosophy of science, that rather accurately describes the systems biology approach, is to the effect that if a series of ostensibly unrelated findings are brought together into a self-consistent narrative, that narrative is thereby strengthened. This is known as “coherence.”350–353 We have sought, we believe successfully, to bring together the evidence for a coherent narrative that links infection, microbial dormancy, iron dysregulation, and inflammation as part of the etiology of RA. Importantly, the proposals can easily be tested further, both diagnostically and therapeutically.

Authors’ contributions: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript. EP wrote the paper and prepared images; DBK wrote and edited the paper; OA prepared design, interpretation of the studies, and analysis of the data. All authors participated in the comparisons using the 2010 ACR/EULAR classification criteria and the 1987 ACR classification criteria. Results from the Norfolk Arthritis Register. Ann Rheum Dis 2013;72:1315–20
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Acknowledgments
We thank the National Research Foundation (NRF) and Medical Research Council (MRC) of South Africa for supporting this collaboration. This article is paper 10 in the series “The role of the dormant blood microbiome in chronic, inflammatory disease.”

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval
Ethical approval was granted at the University of Pretoria (Human Ethics Committee: Faculty of Health Sciences): E Pretorius.

Funding
This work was also a contribution from the Manchester Centre for Synthetic Biology of Fine and Specialty Chemicals (SYNBIOCHEM) (BBSRC grant BB/M017702/1).

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