

## Review

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# A Bacterial Component to Alzheimer's-Type Dementia Seen via a Systems Biology Approach that Links Iron Dysregulation and Inflammagen Shedding to Disease

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**Abstract.** The progression of Alzheimer's disease (AD) is accompanied by a great many observable changes, both molecular and physiological. These include oxidative stress, neuroinflammation, and (more proximal to cognitive decline) the death of neuronal and other cells. A systems biology approach seeks to organize these observed variables into pathways that discriminate those that are highly involved (i.e., causative) from those that are more usefully recognized as bystander effects. We review the evidence that iron dysregulation is one of the central causative pathway elements here, as this can cause each of the above effects. In addition, we review the evidence that dormant, non-growing bacteria are a crucial feature of AD, that their growth *in vivo* is normally limited by a lack of free iron, and that it is this iron dysregulation that is an important factor in their resuscitation. Indeed, bacterial cells can be observed by ultrastructural microscopy in the blood of AD patients. A consequence of this is that the growing cells can shed highly inflammatory components such as lipopolysaccharides (LPS). These too are known to be able to induce (apoptotic and pyroptotic) neuronal cell death. There is also evidence that these systems interact with elements of vitamin D metabolism. This integrative systems approach has strong predictive power, indicating (as has indeed been shown) that both natural and pharmaceutical iron chelators might have useful protective roles in arresting cognitive decline, and that a further assessment of the role of microbes in AD development is more than highly warranted.

**Keywords:** Alzheimer's disease, bacteria, dormancy, dysbiosis, eryptosis, iron, LPS, systems biology, ultramicroscopy

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## INTRODUCTION

Alzheimer's-type dementia (AD) is a neurodegenerative disorder and the most common form of dementia, already in 2013 affecting 44.4 million people globally; this number is expected to affect 75.6 million by 2030 [1]. The current cost is reckoned at \$604 billion per year and this figure is expected to triple by 2050 [2]. Due to the increasing prevalence of the condition, the cost to the public health and elderly care systems to support these individuals is increasing exponentially, and posing major financial challenges [3].

Arguably, the major hurdle in understanding AD is the lack of any integrative and comprehensive knowledge about its etiology and pathogenesis (and there may be many pathways that lead to it), as the onset and risk of AD development is still mostly unexplained (and animal models are of questionable relevance) [4]. Since our genomes changed but little in the last 50 years, but the incidence of AD increased considerably [5], this increase can only to a limited extent be explained by genetic factors [6, 7], notwithstanding the signals detectable in twin and gene association studies [8, 9]. Although dementia is properly diagnosed via cognitive impairment, and true diagnoses of AD can only be done postmortem, specific lesions that characterize AD include extracellular senile plaques and intracellular neurofibrillary tangles with synaptic and neuronal loss [10–13]. In particular, the production of senile plaques, a central event in AD [14], is a result of the cleavage of the amyloid- $\beta$  protein precursor (A $\beta$ PP). A $\beta$ PP has important developmental functions in cell differentiation and possibly in the establishment of synapses [15, 16]; however, it is also expressed by neurons in response to cell injury [17]. Neurofibrillary tangles are composed of the tau protein [18]. In healthy neurons,  $\tau$  is an integral component of microtubules, which are the internal support structures that help transport nutrients, vesicles, mitochondria, and chromosomes from the cell body to the ends of the axon and backwards [19]. In AD, however,  $\tau$  becomes hyperphosphorylated [18, 20]. This phosphorylation allows tau proteins to bind together and form tangled threads [21], a process that can be reversed by iron chelation [22].

Recent evidence suggests that neuroinflammation may play a major role in the pathological processes of AD progression [23–31]. Indeed, inflammation and microglial activation are known as common components of the pathogenesis of a number of

neurodegenerative diseases, including AD, Parkinson's disease, Huntington's disease, multiple sclerosis, and amyotrophic lateral sclerosis [32]. Several neuroinflammatory mediators, including complement activators, chemokines, cytokines, and oxygen radical species, are expressed and released by microglia, astrocytes, and neurons in the AD brain. While minor signs of neuroinflammation can be found in the normal aging brain, the AD brain faces a much stronger activation of inflammatory systems, indicating that an increasing amount of (or qualitatively different) immunostimulants are present. In recent papers, we have also reviewed the comprehensive evidence that in AD the neuroinflammation is probably a systemic inflammatory condition [33, 34]. In one sense, however, the above are all manifestations or accompaniments of AD, and what we seek are the most important causative pathways. It turns out that central to all of these diseases is iron dysregulation [35, 36].

Figure 1 provides an overview of the article in the form of a 'mind map', while Table 1 lists some of the symptoms (some causative) accompanying the pathology of AD. This wide strategy necessarily involves a systems biology approach [37–41] as we recognize (e.g., [36, 42–47]) that this is the only reasonable strategy for approaching complex biochemical networks, each of whose components may contribute partially to the phenotype of interest.

A typical systems biology strategy (e.g., [42, 43]) has the following four elements: first we identify the actors that are most involved, and how they interact. 'Actors' for these purposes may be enzymes or other biochemical elements, or higher-order physiological processes (such as those in Table 1). We then adduce the order or pathway of such interactions (as in Fig. 2, below). Latterly (though we are not yet ready for this in the present problem), we seek to make quantitative these interactions, and predict their relative fluxes, contributions, and so on. We next turn to some of the main actors, starting with iron dysregulation.

## IRON AND AD

Strongly and causatively related to this neuroinflammation in AD is the involvement of unliganded iron and its accompanying oxidative damage in AD etiology [48–61]. Specifically, AD is characterized by elevated brain iron levels [62–64] and the accumulation of copper and zinc in cerebral amyloid- $\beta$  (A $\beta$ ) deposits (e.g., in senile plaques) [65–73].

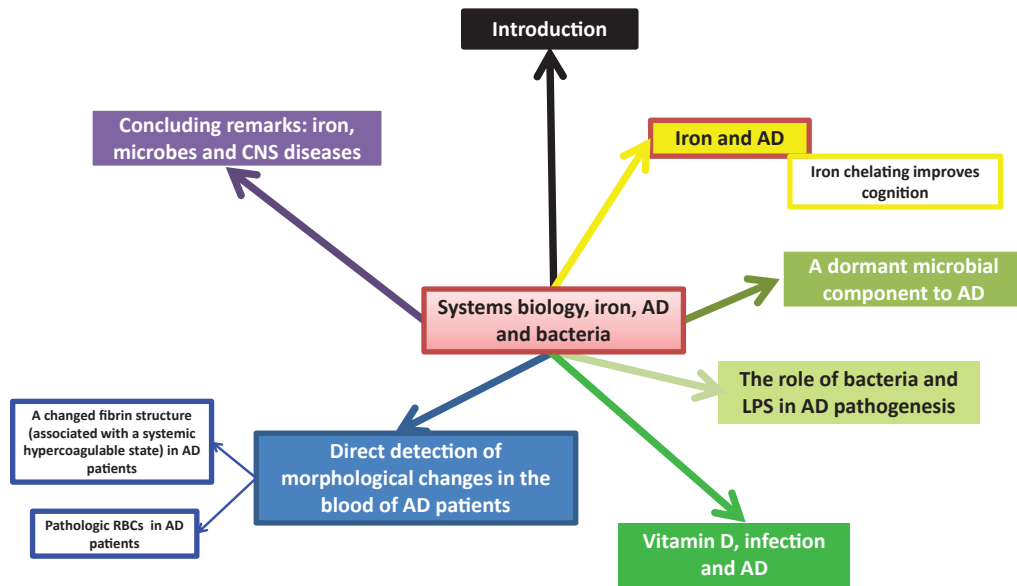


Fig. 1. A mind map summarizing the content of this paper.

Table 1

Some of the most well-known Alzheimer's-type dementia symptoms. Some may be causative

Most well-known (some causative) Alzheimer's-type dementia symptoms

- Pathological loss of microglia, astrocytes and neurons
- Neurofibrillary tangles composed of hyperphosphorylated tau
- Cerebral amyloid- $\beta$  ( $A\beta$ ) or senile plaques
- Upregulation of complement activators, chemokines, cytokines
- Reactive oxygen species generation
- Iron dysregulation
- Accumulation of metals in cerebral  $A\beta$  deposits (e.g., in senile plaques)
- Neuroinflammation

There is evidence in the literature that the iron status of AD patients, particularly the serum ferritin (SF) levels, as measured systemically, might have clinical relevance, as this is an indication of iron dysregulation [33, 58, 72, 74, 75]. Increased iron levels are also closely linked to hematological pathology in AD, and this is indicative of systemic inflammation, which also plays an important role in the pathogenesis of the condition [54, 76, 77]. Recently, we showed that, in a randomly chosen AD population, 60% of the patients had increased SF levels, causing adverse effect on red blood cell (RBC) structure [33] as well as causing significantly thinner fibrin fiber diameters, resulting in abnormal clotting [78].

Pathology, in the presence of increased SF levels to both RBCs and fibrin formation, is indicative of a systemic inflammatory involvement of iron in AD. In the recent Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort study, increased SF levels were also measured in cerebrospinal fluid and found to be neg-

atively associated with cognitive performance [79]. Systemically elevated SF levels therefore may have great clinical relevance in AD, as they may be useful as markers of cognitive performance.

Currently, the main therapeutic approaches in AD either attempt to prevent  $A\beta$  production (e.g., by the use of secretase inhibitors) or to clear  $A\beta$ . However, there is convincing evidence that  $A\beta$  does not spontaneously aggregate on its own, but that there is an age-dependent reaction with excess brain metal (copper, iron, and zinc), which induces the protein to precipitate into metal-enriched plaques [65]. In AD there is also a dramatic increase in brain iron content and in fact there are higher iron concentrations inside the  $A\beta$  plaques [80], suggesting that disturbances in brain iron homeostasis may contribute to AD pathogenesis [81, 82].

It is well known that excessive poorly liganded iron may cause oxidative damage [35, 83, 84], and there is ample evidence that suggests that oxidative stress and

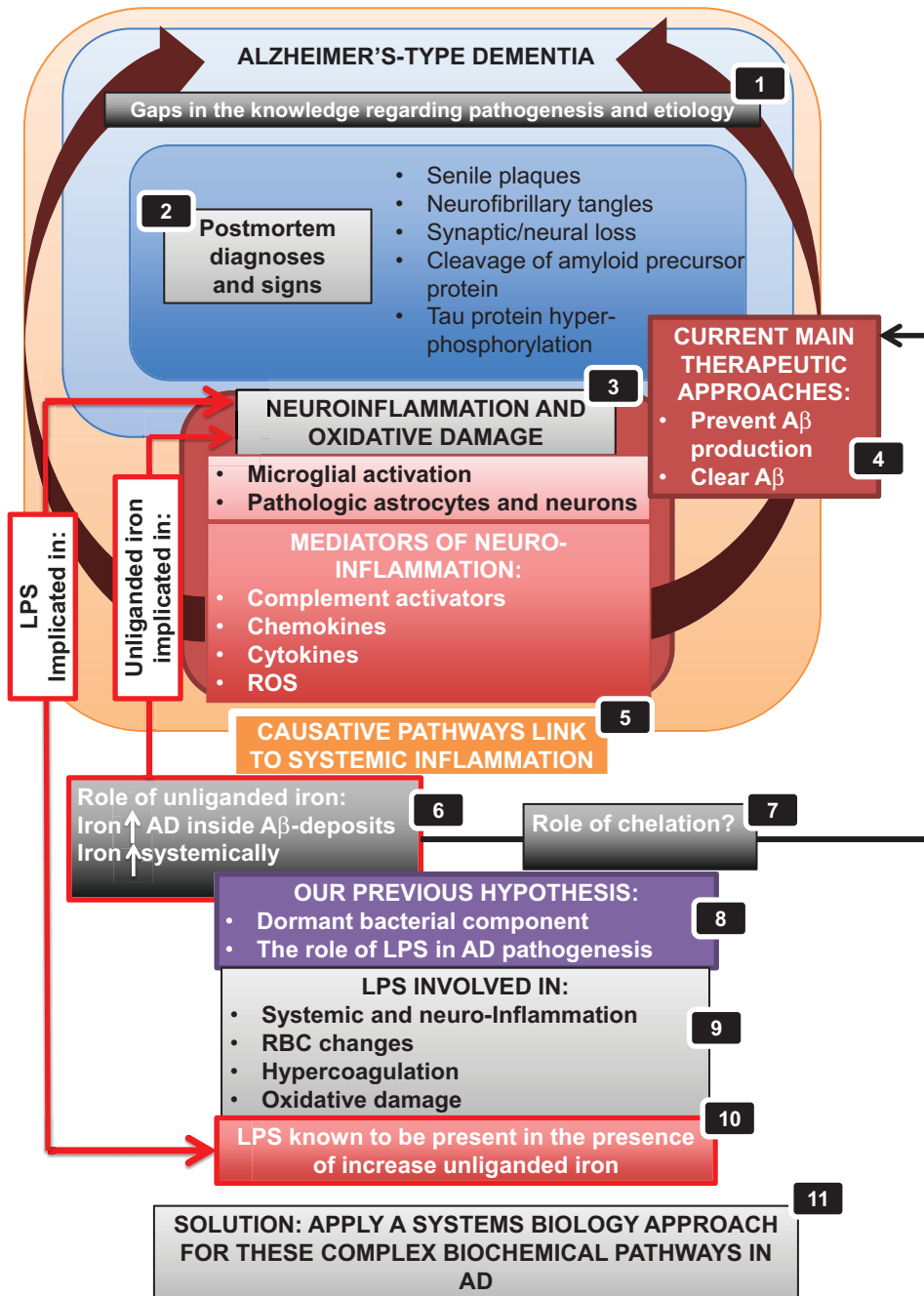


Fig. 2. The order or pathway of major and potentially causative interactions in Alzheimer's-type dementia between enzymes or biochemical elements, following a systems biology strategy.

therefore aberrant redox activity is one of the earliest pathological changes in AD, and that there is a link between systemic and brain oxidative stress [50, 85].

Oxidative stress plays a significant role in the pathogenesis of AD [86–89]. Oxidative stress in AD results in increased levels of lipid peroxidation,

DNA, and protein oxidation products (HNE, 8-HO-guanidine, and protein carbonyls, respectively) inside AD brains [90]. Oxidative stress participates in the development of AD by promoting A $\beta$  deposition [91], tau protein hyperphosphorylation, and the subsequent loss of synapses and neurons. In AD, much as

with the prion protein in prion diseases [35, 36, 92], A $\beta$  can become a pro-oxidant and when complexed to iron, this can result in hydrogen peroxide formation; this process can underlie the increased oxidative stress burden [93]. The relationship between oxidative stress and AD suggests that it is an essential part of the pathological process; poorly liganded iron can participate in the Fenton reaction ( $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-$ ), and the highly reactive hydroxyl radical  $\text{OH}\cdot$  may be the main culprit [35]. In addition, the Haber-Weiss reaction  $\text{Fe}^{3+} + \text{O}_2^{\bullet-} \rightarrow \text{Fe}^{2+} + \text{O}_2$  reverts the  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  such that the 'iron' then becomes catalytic rather than stoichiometric [35, 94]; this is why the unliganded iron is so particularly toxic.

In a series of articles, including a number of reviews, we have shown that poorly liganded iron is key to a great variety of diseases [33, 95–97]; it also affects erythrocyte morphology and coagulation properties (touched on briefly later in this paper) [96, 98].

Ultimately, oxidative stress may be due to the combined action of mitochondrial dysfunction, increased metal levels, inflammation, and the presence of A $\beta$  peptides [99]. However, there is a link between all the above-mentioned and the pathological presence of iron. Increased oxidative stress results in inflammation, which can be both neuroinflammation or systemic inflammation [100], and the pathologic levels of iron have been associated with both inflammation and oxidative stress in AD [23, 91]. We tend to like ideas with predictive power (such as unitary explanations for diseases with comorbidities, for which see also [101]). Thus, if iron is so important to the pathogenesis of AD, one might then suppose that its chelation (that stops the Fenton and Haber-Weiss reactions) would be expected to improve it [102, 103]. The next section looks at this.

#### *Iron chelating improves cognition*

Starting with a Lancet paper that is now a quarter of a century old [104], it has been shown that the removal of pathologic levels of free iron improves cognitive function in AD. Metal chelators such as clioquinol and desferrioxamine, and natural antioxidants such as curcumin and ginkgo extract, have had some success in altering the progression of AD symptoms [90, 105–107]. More recent and important papers, to the same effect, come from the group of Perry and colleagues [51, 54] and that of Youdim [108], while similar beneficial effects of iron chela-

tion can be observed with Parkinson's disease and models thereof [109–112]. We do find it slightly surprising that these indications have not been more widely picked up.

A fine control of iron regulation might play an important role in systemic iron overload [113] including AD [114], as there is a known association between diet and risk of dementia [115]. Except for pharmaceutical intervention, it is well known that a healthy diet rich in polyunsaturated fatty acids and polyphenols may have a positive effect on general health brain function [116]. In particular, the Mediterranean-type diet has a positive effect on the healthiness of AD patients [117–120], due to the presence of naturally occurring iron chelating agents found in fruit and vegetables as these agents are known scavengers as a result of their ability to chelate iron [118, 121–124]. Another route might also be calibrated phlebotomy in AD, to reduce iron stores [125].

#### **A DORMANT MICROBIAL COMPONENT TO AD**

While metals can certainly contribute significantly to the explanation of the development of AD via these Fenton-type pathways, we have recently suggested that they may do so by another and parallel means, explicitly involving the awakening of a dormant bacterial component [34, 126]. This follows from the recognition that the growth of microbes *in vivo* is normally limited by the availability of free iron [127–132]. Others too have noted the presence of an authentic blood microbiome even in 'normal' controls, based on macromolecular sequencing and other molecular approaches (e.g., [126, 133–138]), although sequencing methods cannot of themselves reflect replicative potential, of course.

In this sense, a 'classical', related, and well-known example is that of *Helicobacter pylori* and gastric ulcers. These latter had long been assumed to be due to the over-activity of the gastric  $\text{H}^+$ -ATPase (which can certainly contribute). However, the pioneering (and initially 'controversial') work of Barry Marshall and Robin Warren showed unequivocally that they were inevitably accompanied, and the disease was essentially caused, by a hard-to-culture and little-known microaerophilic organism, subsequently codified as *H. pylori* [139–142]. Our major thesis here (and elsewhere) is that it will turn out that a very large number of chronic, inflammatory diseases, that share many observable symptoms, will also turn out

to be due to hard-to-culture organisms, many or most of which will turn out to be well known to science. The issue is that they typically lie dormant, and thus (by definition) resist culture by means that normally admit their culture.

The point of 'dormancy' is particularly important, as most clinical microbiologists typically consider or define microbial propagules (cells potentially capable of replication) as being 'alive' (i.e., so capable) or not under any conditions tested ('dead'). However, a considerable literature (reviewed by ourselves, e.g., [126, 143–146]) and others (e.g., [147–152]) indicates that most microbes in nature are non-growing and can appear operationally 'dead', yet can recover culturability, by a process referred to (virtually by definition) as 'resuscitation'. They are thus not operationally 'dead' and are typically and more properly referred to as 'dormant' (or, commonly in clinical microbiology, 'persistent' [150, 152–156]). One needs then to recognize that dormancy is an **operational** property that depends both on the cell (singular [157]) being assessed and on the means used to detect it [158]. **This cannot be emphasized too strongly:** the designation of a microbe as dormant implies that it is not just a property of the microbe alone but of the means by which we assess it, a phenomenon reminiscent of the "Schrödinger cat paradox" in the philosophy of quantum mechanics. One important consequence (see e.g., [126, 159–164]) of this ability of microbes to enter non-replicating physiological states is that they do not fulfill the Henle-Koch postulates regarding the microbial causality of diseases, at least in their ordinary form [165].

Particularly, the neurotoxic lipopolysaccharides (LPS) from their cell walls may be of importance (see below), since LPS molecules are highly inflammatory agents, that can even induce cell death [126]. **It is of course the cell death that is the proximate cause of the loss of cognitive function.** We summarize all of these pathways in Fig. 3. The especial attractions of this scheme are that (i) it provides for the necessary systems-level understanding, (ii) the elements hang together and are 'coherent' within the meaning of that term as used in the Philosophy of Science [126, 166], and (iii) it is rich in both predictive and explanatory power.

While recognizing the importance of various kinds of infectious agents in the pathogenesis of AD (see [34, 162, 167–198]), and that also depend for their growth on the availability of free iron, we next turn to the question of the role of prokaryotes and their inflammatory components in the pathogenesis of AD.

## THE ROLE OF BACTERIA AND LPS IN AD PATHOGENESIS

Recently, immunoblotting demonstrated bands corresponding to LPS in four AD brain specimens, which were positive when screened by immunofluorescence [199]. Bacterial endotoxins may be involved in the inflammatory and pathological processes associated with AD [200]. Indeed a number of studies indicate that the LPS-induced neuroinflammation can drive A $\beta$  formation (e.g., [201–206]).

Interestingly, it has been observed that chronic infusion of the bacterial LPS into the fourth ventricle of rats reproduces many of the inflammatory and pathological features seen in the brain of AD patients [200, 201].

Previously we have reviewed the extensive published accounts suggesting a possible link between LPS presence and the pathological process of AD [34, 126, 207–211]. It is also well known that LPS presence is at least one of the causes of inflammation [212–214], and one of the hallmarks of inflammation is a hypercoagulable state [215–221]. Previously, we have seen changes in erythrocytes (RBCs), as well as hypercoagulation in the presence of LPS, where we added LPS to whole blood of healthy individuals or to platelet poor plasma [34]. We also reported on the presence of bacteria, which will indeed point to the presence of LPS, in whole blood of AD and Parkinson's disease patients, and also in fact inside RBCs [34]. We also discussed in detail the reasons why we might find bacteria in typically "sterile" blood, and suggested that these bacteria may be dormant (as operationally defined).

## VITAMIN D, INFECTION, AND AD

While, in a sense, 'everything is connected to everything else', the role of the systems biologist is to highlight those metabolic networks and other processes whose variation (whether as a dependent or an independent variable – see [222]) are most pertinent to the outcomes of interest. Leaving aside the well-established roles of vitamin D in calcium and bone metabolism, it does seem to have a considerable impact on the immune system. To this end, there are some interesting clues (e.g., [223]) that link inflammation, infection, and vitamin D metabolism (and indeed elements of iron and vitamin D metabolism [224]), as well as AD [225–231]. Although the degree, and any mechanisms, of causality remain to

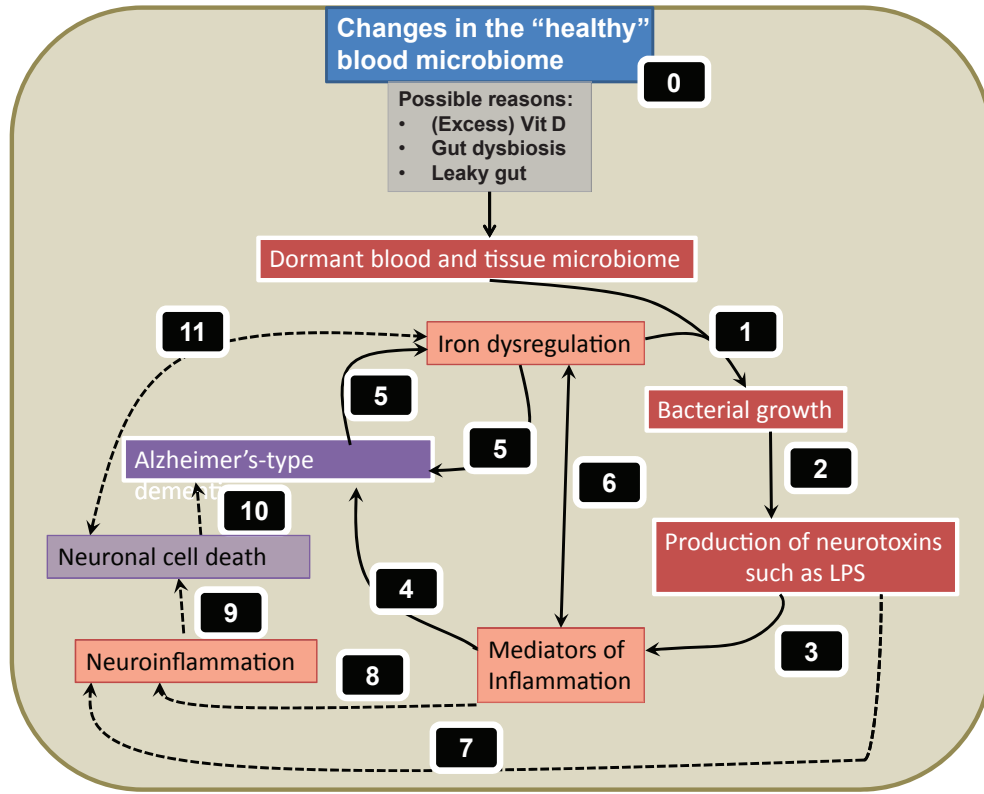


Fig. 3. A generalized systems scheme for microbial/iron-driven inflammatory disease in Alzheimer's-type dementia.

be seen, and the inter-relations are complex and non-linear [232, 233], there is an emerging consensus among a significant group of workers that chronic infection is intimately linked to detailed vitamin D status, and that this may provide a way in to useful therapies for a variety of chronic, inflammatory diseases (e.g., [101, 234–237]). The first issue concerns what in fact we mean by 'vitamin D'. Specifically, vitamin D may typically refer to two distinct forms: ergocalciferol (vitamin D<sub>2</sub>) and cholecalciferol (vitamin D<sub>3</sub>), with some question as to whether D<sub>2</sub> is indeed useful as a vitamin supplement [238, 239]. The structures and metabolic products of vitamin D<sub>2/3</sub> (of which only the hydroxy derivatives are in fact active, and the 1 $\alpha$ ,25-dihydroxyderivative especially) are given in Fig. 4.

In particular, Mangin and colleagues [235] have suggested that that low 25(OH)D is a consequence of chronic inflammation rather than the cause, and that tissue bacteria were responsible for an inflammatory disease process which results in high 1,25(OH)<sub>2</sub>D and low 25(OH)D (see also [237]). 1,25(OH)<sub>2</sub>D activates the vitamin D receptor (VDR) [240–244], a transcription factor that serves to induce the expres-

sion of over 900 genes, including for antimicrobial peptides [101, 223, 245–251] such as cathelicidin and beta defensins which attack (presumably non-dormant) pathogens [252]. In general, the innate immune system is enhanced and the adaptive immune system is inhibited by 1,25(OH)<sub>2</sub>D [235]. The general scheme, essentially as redrawn from [235], is given in Fig. 5. Other papers have also highlighted a relationship between low 25(OH)D and AD [226, 229, 230, 253, 254] and tend to imply that vitamin D supplementation should therefore be a solution. Obviously from a systems biology point of view, this does not follow directly, and there is evidence that the opposite can in fact be true [235, 236, 255]; clearly we need to know precisely the different roles of 25(OH)D and 1,25(OH)<sub>2</sub>D, and any effects on the CYP enzymes that produce them. More particularly, however, the complex, variable quality [256], and sometimes apparently contradictory, literature [257] is arguably better explained on the basis that there are separate populations who simply respond differently to vitamin D<sub>3</sub> supplementation [258–260]. Biomarkers (such as taurinuria; [261]) for genuine vitamin D deficiency may help disentangle this. Indeed, the

contradictory nature of any kinds of phenomena in which the 'same' additions are made to the 'same' system with very different results are typically explainable on the basis of uncontrolled variation. Thus the antioxidant ascorbate is actually pro-oxidant if unliganded iron is present [35]. Another explanation of such contradictions here involves the simultaneous presence of agonist and antagonist conformers of the VDR [262–264]. Finally, and in a different vein, the apoptotic versus proliferative effects of NF- $\kappa$ B are determined by the frequency rather than the amplitude of the NF- $\kappa$ B signaling molecule [43, 265, 266]. Vitamin D has significant effects on NF- $\kappa$ B [267–269]. Since there are also significant oscillations in ERK [270], VDR levels are partly dependent on ERK [271], and vitamin D3 also regulates circadian genes [272], these kinds of explanations based on the timing and frequency of oscillations (rather than simple metabolite concentrations) seem well worth exploring.

At all events, the nature(s) of the intracellular pathogens (and the cells in which they reside) is probably very wide, and at least one strategy for their persistence (in terms of their ability to evade the immune systems) is the adoption of cell-wall-deficient morphologies [148] or L-forms [273]. These, as well as more conventional structures, can of course be detected microscopically.

### **DIRECT DETECTION OF MORPHOLOGICAL CHANGES IN THE BLOOD OF AD PATIENTS**

#### *Pathologic RBCs and hypercoagulable fibrin(ogen) in AD patients*

In previous work, we showed that the erythrocytes of AD patients were of highly anomalous shape, especially when serum ferritin levels were simultaneously raised [33] and that there was likely a hypercoagulable state (ascribed to the elevated LPS [34]). Here we now also show that AD RBCs are indeed abnormal, by using RBC and antibody-based fluorescent markers for spectrin (Ab11751) (red fluorescence) and Band-3 (Ab11012) (green fluorescence). Band3 is found in three distinct protein complexes associated with the erythrocyte membrane: an ankyrin-dependent tetrameric band3 complex, a dimeric band3 complex bound to the protein 4.1-glycophorin C junctional complex, and freely diffusing dimeric band3 complexes [274, 275]. Band 3 can also bind to spectrins, the internal scaffold for

erythrocyte shape, via ankyrin, suggesting that band 3 contributes to the membrane-cytoskeleton interactions that help to define erythrocyte shape and stability [276, 277]. Structural alterations to the phospholipids, as well as band 3 and spectrin, cause RBC physical shape changes, which can be detrimental to their normal functioning [97, 278]. Under normal conditions, the neutral phospholipids, phosphatidylcholine, and sphingomyelin are mostly found on the outside, and the charged phosphatidylserine (PS), phosphatidylinositol, and phosphatidylethanolamine, are found mostly on the inner membrane leaflet. However, during inflammation, the erythrocyte membrane leaflet phospholipids becomes more symmetric as PS is externalized, resulting in RBC membrane vesicle formation and ultimately microparticle shedding, with subsequent pathological shape changes of RBCs [279]. PS is normally found only on the intracellular leaflet of the plasma membrane in healthy cells, but during early eryptosis (RBC programmed cell death) [280–284], membrane asymmetry is lost and PS translocates to the external leaflet [285]. For a detailed review on the role of the RBC membrane and changes therein due to inflammation, see [286].

Figure 6A shows a typical example of confocal microscopy of a healthy RBC and Fig. 6B shows a typical scanning electron microscopy (SEM) image of a representative RBC from an age-controlled healthy individual, while Fig. 6C and D show confocal and SEM images of a representative sample from an AD individual. Figure 6A shows intense green fluorescence on the rim of the RBCs and less intense toward the inside of the RBC. There is little to no red fluorescence specifically on the rim of the RBCs indicating the presence of the spectrin. Where there is some red staining, it is more toward the inside of the RBCs and much less intense than the green band3. In the RBCs of the AD individuals (Fig. 6C), the red fluorescence is much more visible, and the red fluorescence is found not only on the inside of the RBCs but also on the rim and outside of these cells unlike the control group. This suggests a structural membrane disorder, typically associated with eryptosis, which is often enhanced by cytoplasmic calcium activity and also characterized by cell membrane scrambling and cell shrinkage [287, 288]. Particularly the disarrangement of spectrin and band 3 positional changes are two important markers to determine structural damage to the membrane that will result in changes to elasticity and pliability of RBCs [286]. SEM images comparing healthy and AD RBC ultrastructure, clearly show that the RBCs from



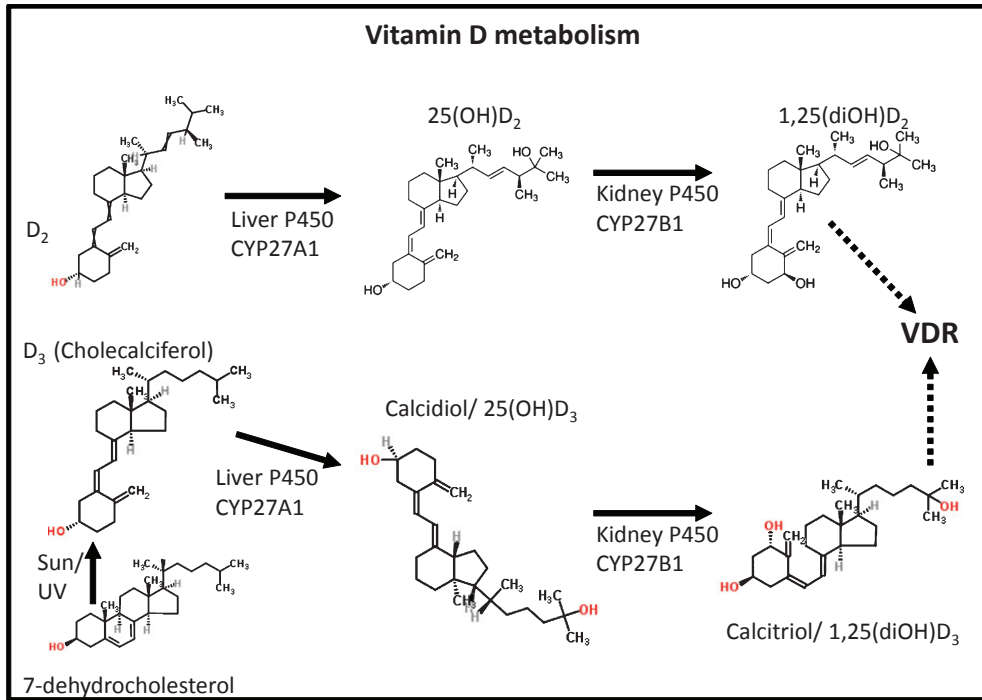


Fig. 4. The structures and major metabolic products of vitamin D<sub>2/3</sub>. The dihydroxylated derivatives are by far the most active in terms of binding to the vitamin D receptor.

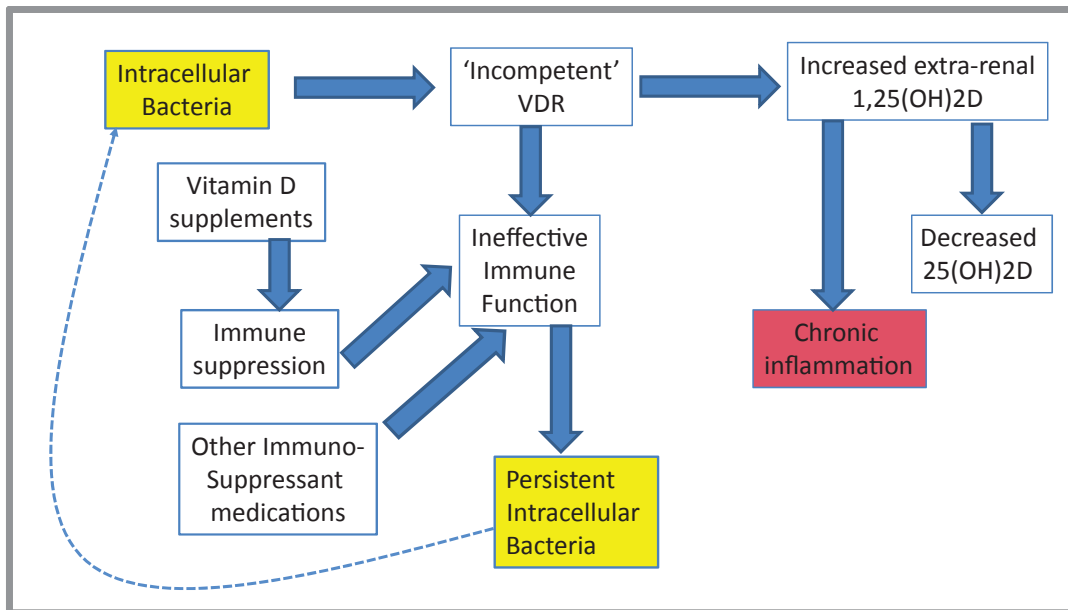


Fig. 5. A general scheme of some of the roles of vitamin D and its metabolites in chronic infection: (essentially as redrawn from [235]).

AD individuals have an eryptotic structure. Eryptosis is visible in most of the RBCs from AD patients, and also in those with Parkinson's disease [95]. Additionally, to the eryptotic structure of the RBCs, bacteria

were also visible with SEM in the same AD sample (Fig. 6E, F).

As well as changes in AD RBCs, we previously found that pathologic fibrin fiber formation

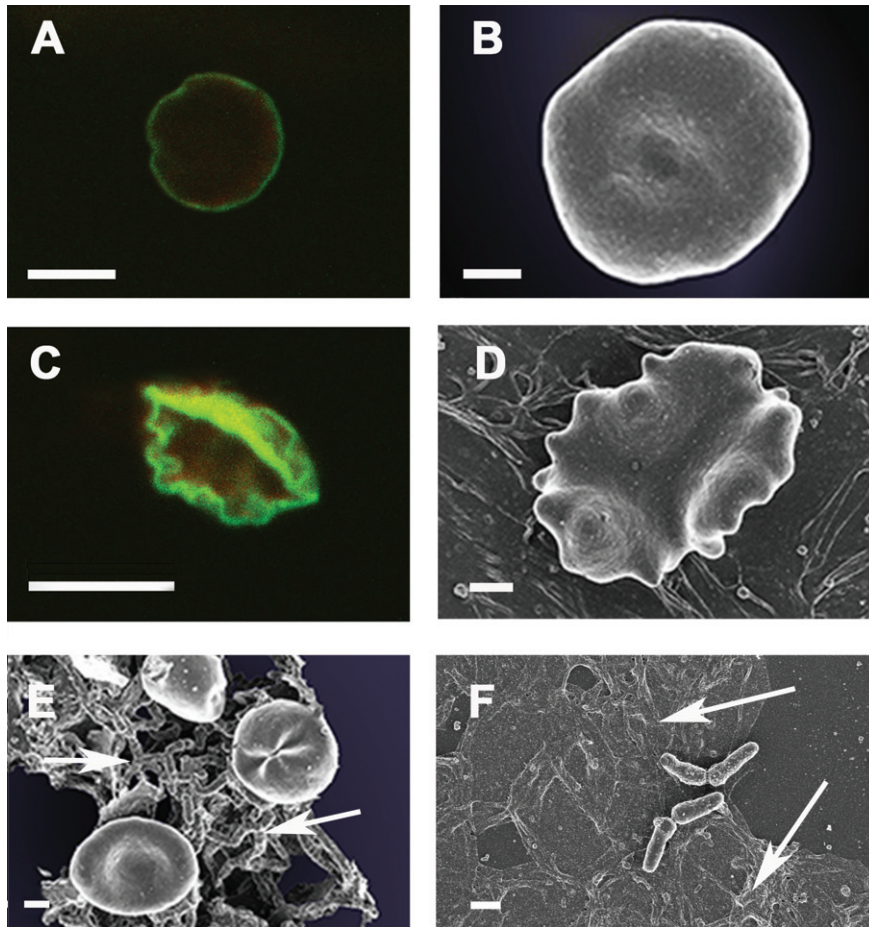


Fig. 6. Confocal and scanning electron microscopy (SEM) of health and Alzheimer's-type dementia RBCs. The fluorescent markers spectrin (Ab11751) (red fluorescence) and Band-3 (Ab11012) (green fluorescence) were used in confocal microscopy. A) Confocal micrograph of a healthy RBC; B) SEM micrograph of a healthy RBC; C) Confocal micrograph of an Alzheimer's-type dementia RBC; D) SEM micrograph of an Alzheimer's-type dementia RBC; E) SEM micrograph showing bacteria between RBCs; and F) of bacteria and matted fibrin. Scale bar of SEM micrographs: 1  $\mu\text{m}$ ; and for confocal: 5  $\mu\text{m}$ .

(associated with hypercoagulation) is also present in AD, and may therefore be used as a further and useful inflammatory indicator [34]. As seen in pathological changes in RBCs, oxidative damage, increased iron levels, and inflammation are also all reasons for the development of hypercoagulability [95–97, 289–293]. Hypercoagulability is closely associated with increased fibrin(ogen) in AD patients, while hypercoagulation has been observed in blood vessels positive for amyloid in mouse and human AD samples [294]. A changed fibrinogen structure has been implicated in the development of neuroinflammation [295, 296], and memory deficits and increased fibrinogen levels in AD are noted to be a strong indicator of cerebrovascular risk, as fibrinogen specifically binds to  $A\beta$ , thereby altering fibrin clot structure and delaying clot degradation [297]. In a previous paper, we

looked at the viscoelastic and ultrastructural properties of AD plasma and whole blood by using scanning electron microscopy, thromboelastography (TEG<sup>®</sup>) and the Global Thrombosis Test (GTT<sup>®</sup>) [34]. TEG<sup>®</sup> analysis showed a hypercoagulable state in AD, while TEG<sup>®</sup> results, where LPS was added to uncitrated blood, showed the same trends as were found with the AD patients. The GTT<sup>®</sup> results (where only platelet activity is measured) were not affected by the added LPS, suggesting that LPS does not directly impact platelet function [34]. See Fig. 7 for an ultrastructural comparison of platelet poor plasma smears (treated with thrombin) from a healthy (age-controlled) individual and from an AD individual.

Although pathophysiological changes in RBCs and fibrin fiber structure are not unique to AD, they are hallmarks of systemic inflammation [96], and as

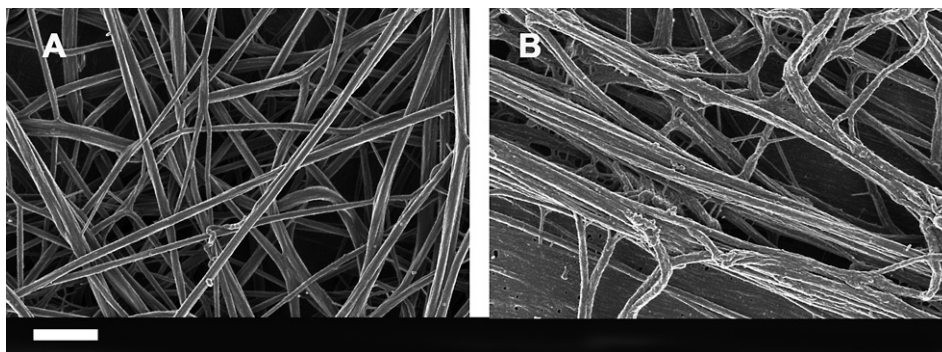


Fig. 7. Platelet poor plasma of A) healthy (age-controlled) individual; and B) an Alzheimer's-type dementia individual. Thrombin (20 U.mL<sup>-1</sup>) was added at a final concentration of 57.7 nM. Scale bar: 1  $\mu$ m.

noted here LPS may play a role in the biochemical pathways that may destabilize RBC and fibrin structure. As RBCs are extremely vulnerable in the presence of pro-inflammatory molecules, hydroxyl radicals, oxidative stress, and LPS, they may possibly be used as a 'healthiness' indicator of AD patients. Currently we have few actual markers of AD status, and we note that the latest NIH guidelines suggest that clinical medicine should focus on precision medicine [298] and that individualized medicine should in the future, form an essential part in the diagnosis and treatment of patients. We therefore suggest that RBC and fibrin morphology could be used as "health indicators". Here we do not of course suggest that they should be used as diagnostic tools for AD per se, but rather as a healthiness indicator of the overall systemic inflammatory status of patients after diagnoses.

## CONCLUSION

Modern molecular biology had become a little obsessed with a presumed need for hypotheses, and it has taken the post-genomic era to remind scientists of the virtues of scientific induction and data-driven biology [299, 300], often intertwined with a systems biology approach. A typically nice example is a hypothesis-free discovery biology paper [301] in which the authors sought to identify those pathways that were most intimately involved in the development of prion disease. Genes involved in iron metabolism were among the most highly involved [301].

In a similar vein, we have brought together the evidence underpinning a coherent and self-consistent view of the linked contributions to AD progression of iron dysregulation, the resuscitation of dormant bacteria, and the shedding of the highly inflammatory

LPS that can induce both cytokines and apoptosis (see Figs. 2 and 3). As with any systems approach, it implies the need for pharmacological interventions at multiple points (e.g., [302–305]). The role of the systems pharmacologist, based on knowledge of the most important pathways proposed herein, is to develop them.

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