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DOI
10.1093/mtomcs/mfac063

Publication date
2022

Document Version
Final published version

Published in
Metallomics : integrated biometal science

Metallomics, 0, 2022, mfac063
https://doi.org/10.1093/mtomcs/mfac063
Advance access publication date: 24 August 2022
Perspective

Maximum iron loading of ferritin: half a century of sustained citation distortion

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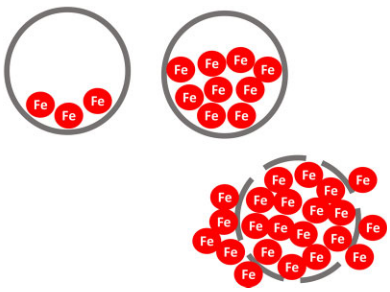
Abstract

Analysis of citation networks in biomedical research has indicated that belief in a specific scientific claim can gain unfounded authority through citation bias (systematic ignoring of papers that contain content conflicting with a claim), amplification (citation to papers that don't contain primary data), and invention (citing content but claiming it has a different meaning). There is no a priori reason to expect that citation distortion is limited to particular fields of science. This Pespective presents a case study of the literature on maximum iron loading of the ferritin protein to illustrate that the field of metallomics is no exception to the rule that citation distortion is a widespread phenomenon.

Keywords: ferritin, iron loading, iron homeostasis, iron core, citation distortion

Graphical abstract



Reversibility of loading ferritin with 4500 Fe is a misconception.

The ferritin-loading problem

Ferritin is the protein that we, and many other species, use to store iron until it is required in metabolism. Research on its structure and function started with its first purification from horse spleen and its initial characterization by Laufberger in 1937[1] and has continued very much into our present times. The first crystal structure of the apo-protein (that is, devoid of iron) was determined in 1978 by Harrison and co-workers[2] revealing a picture of a hollow sphere-like structure made up of 24 subunits of mass ca 20 kDa (shortly thereafter shown to be of two types, heavy, H-, and light, L- chains[3]), adding up to a total mass of ca 480 kDa, and ready to accommodate a large amount of iron as a ferrihydrite-like 'core' in its interior. This milestone was followed by decades of intense studies which have brought us partial insight into the molecular details of Fe(II) ion uptake and controlled conversion into Fe(III), but much less so into the questions how, and in what form, the Fe(III) is eventually stored as a core inside the ferritin, and how it is subsequently reliberated as soluble Fe(II) ion (recent reviews[4–7]). Figure. 1 is an overall picture of a ferritin molecule outlining its approximate spherical structure that surrounds the large internal space available for iron storage.[8]

How many Fe ions can actually be taken up to saturation by a ferritin molecule is a relevant question from the perspective of, e.g. biochemistry (mechanism and form of storage), of nanotechnology (ferritin as a device to make defined nanoparticles of various metals), of medical technology (ferritin as a nanocarrier of aggressive drugs). Overall, the biochemical literature over the last half a century is quite adamant in its many times repeated answer: the maximum loading is 4500 Fe. Here, I argue that (i) the number is very likely to be incorrect (that is, a serious overestimation), and (ii) the claimed number's authority is based on multiple improper scholarly citation.

The citation problem

By analysis of a citation network in biomedical research on the β amyloid protein Greenberg has shown how citation distortions

Received: April 22, 2022. **Accepted:** August 19, 2022

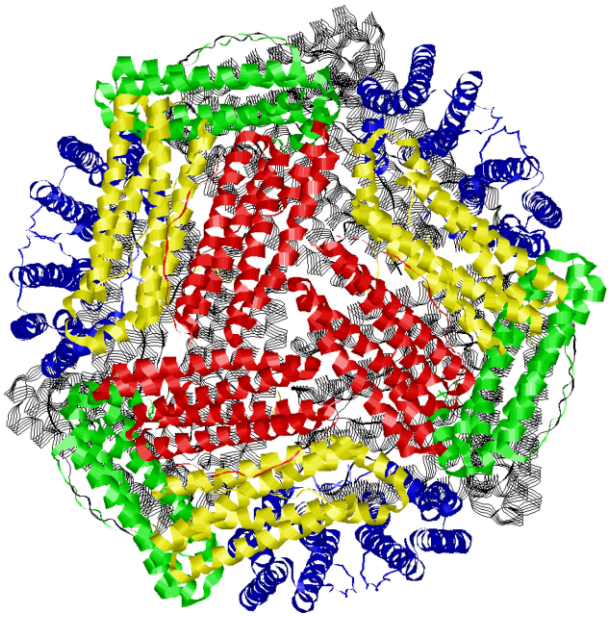


Fig. 1 Overall crystal structure of a ferritin molecule. The figure is based on 2JD7.pdb.[8] The view is approximately along one of the 3-fold channels of the 24-meric ribbons structure with 9 subunits on the far side as strands in black. Irons in the 24 catalytic ferroxidase centers have been omitted and the iron core is absent as an X-ray core structure has never been determined for any ferritin.

create unfounded authority.[9] Here, an equivalent problem is identified in inorganic biochemical literature that appeared over a very long period of time with continuation into the present. The 1978 ferritin crystallization paper[2] states that 'The large cavity inside the molecule can store up to 4500 Fe atoms', and this claim is backed up by citation of 3 previous publications: the first one is a paper in which no Fe number is given (improper citation), the second one gives Fe as a percentage of apo-protein mass, from which I calculate 1807 instead of 4500 Fe (erroneous citation, or invention), and the third one does give a number of 4000–5000, however, without experimental data and without any references (amplification, or dead-end citation). In other words, the number of 4500 has been given apparent authority by citation of previous studies, but in actuality these do not contain any experimental data to support the value. This is but one example of what turns out to be widespread practice in the ferritin literature over the last half a century.

Some of these citation 'trees' are rather more complex than the one just given, as they involve repeated branching, often via not particularly easily available chapters in books or book series. An example is given in Fig. 2 where one of the sub-branches eventually leads to one single paper, in which indeed a number of 4300 is calculated on the basis of experimental results. This paper from 1965 by Fischbach and Anderegg[10] (whose 4300 Fe conclusion I will criticize, below) turns out to be one out of a grand total of two papers in the complete ferritin literature reporting a number close to 4500 based on actual data. For the purpose of the citing authors[11] at the top root of the inverted tree a link to this single paper would have sufficed since they chose to ignore all reported numbers along the tree that were significantly different from 4500 (citation bias).

In recent times the citation trees in the ferritin loading story tend to simplify, e.g. in a 2022 crystallography paper by Wu et al.[12] where the 4500 number is claimed to be documented in three preceding papers none of which actually turns out to provide data and/or references in support of the number (three dead-end citations; see Fig. S1). A 2021 review by Song et al.[13] even takes a shorter route: only a previous paper by the same group is cited, which does not provide data and/or references for 4500 Fe (redundant self-citation and dead-end citation; see Fig. S2). Supplementary Table S2 lists 39 primary papers from the period 1965–2022 that claim the ca 4500 Fe number and thereto cite (usually a combination of): (a) papers that do not provide data/references in support of 4500 (dead-end citation); (b) papers that give numbers <3000 Fe (incorrect citation or invention); (c) papers that do not give a number at all (amplification); (d) the 1965 Fischbach-Anderegg paper (a questionable source, see below).

And the simplest citation of course is no citation at all. The ferritin literature is full of papers (with recent increasing incidence) in which the belief that 'ferritin can take up to 4500 Fe ions' is simply stated as an obvious fact that does not require literature support. Supplementary Table S3 lists 53 primary papers from the period of 1969–2022 in which the 4500 statement is made without any literature reference.

The 4500 Urtext and its decendants

We now turn our attention to what is presumably the Urtext of the 4500 number. The 1965 paper by Fischbach and Anderegg[10] describes a study of the behaviour of horse spleen ferritin in the analytical ultracentrifuge. The Schlieren pattern shows a sharp optical absorption peak from apo-protein and a broad, flat pattern from ferritin molecules with different Fe loadings. Multiple fractions are then separated at different radial positions from a CsCl density gradient and, after dialysis against buffer, each fraction is subjected to protein and Fe content determination. Thus, the sample with the highest sedimentation coefficient is found to have a mg protein per mg iron ratio of ca 2.95. Using an apoprotein molecular mass of 465 kDa[14] and a presumed core composition $(FeOOH)_8(FeO{\cdot}OP_3H_2)$[15] this gives a maximum iron loading of 2822 Fe per ferritin molecule. Fractions from lower density contain less iron.

However, the CsCl run also results in some protein precipitation, which is collected and subjected to 'further fractionation by zonal centrifugation' (no details given). The fastest sedimenting fractions are named 'full ferritin' and are claimed to have a protein/iron mass ratio of ca 1.90, which translates into 4277 Fe per ferritin molecule. This number for precipitated protein is the single basis for the 'widely accepted' view that ferritins can be loaded up to a maximum of 4500 Fe.

That native horse spleen ferritin purified according to the then prevailing method of ammonium-sulfate fractionation due to Granick (1942), contained a certain amount of aberrant protein was already noted by Granick who reports a fraction that he names 'non-crystallizable ferritin', which 'perhaps represents denatured protein'.[16] In a similar vein, when Niitsu and Listowsky in 1973 repeated the density gradient fractionation experiments, they measured a typical loading of 2000 Fe, but also found a sedimented fraction that contained about 3500 Fe and exhibited a 'lower solubility' than other ferritin fractions.[17] Dognin and Crichton (1975) also tried to reproduce the Urtext experiment: they do not report any precipitation, but the determined loading was in the range 49–3390 Fe.[18] A more recent attempt at reproduction was reported by May et al. (2010)[19] who found values between 29 and 3465 Fe with the additional observation that the heaviest material (>3600 Fe) sedimented to the bottom of the tube. Use of a steeper gradient resulted in significantly poorer resolution, however, the 'fastest sedimenting Gaussian component' (not

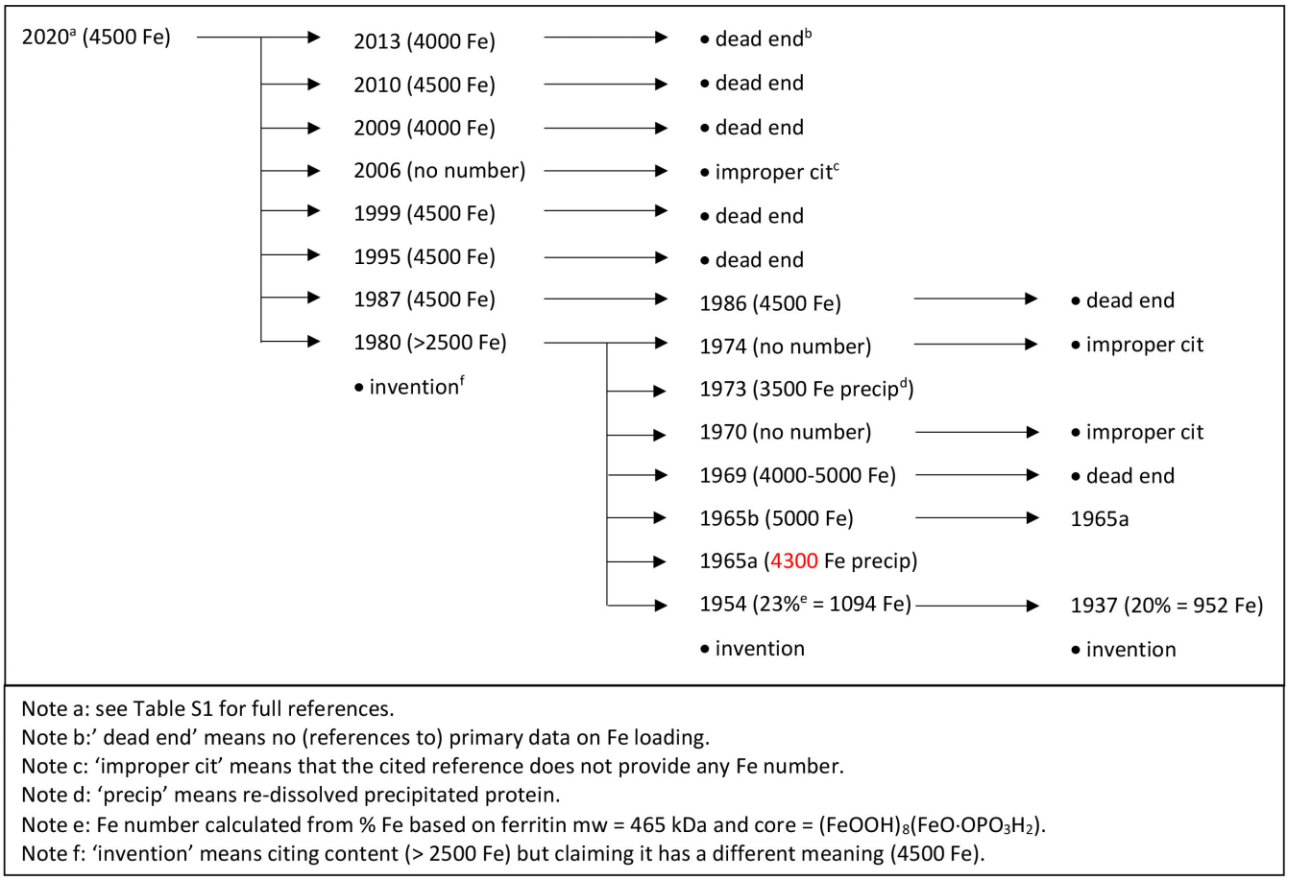


Fig. 2 Citation tree for a claim that ferritin can incorporate up to 4500 Fe. The example is for a 2020 paper by Alenkina et al.[11] and involves 18 references in the period 1937–2013, only one of which is evidence based (indicated in red).[10]

Table 1. Maximum iron loading of apo-ferritins. The maximum iron (Fe-max) numbers are for in vitro loading of ferritin from different species and comprise a complete overview of the literature.

Year	Species	Fe-max	Remark
1978[a]	horse spleen ferritin	3060	Turbidity above 2500 Fe Some precipitation at 3450 Fe
2010	horse spleen ferritin	2000	Some precipitation with 2500 Fe
2012	horse spleen ferritin	4300	
1993[a]	*E. coli* bacterioferritin	1800	
2003	*E. coli* bacterioferritin	2700	Precipitation at higher loading
1993[b]	*E. coli* ferritin	2000	Precipitation at higher loading
2006	*P. furiosus* ferritin (expressed in *E. coli*)	2700	Precipitation at higher loading

Note [a]see Table S4 for full references.

further defined) of the heaviest fraction now gave an apparent sedimentation coefficient that would extrapolate to 4320 Fe. The fraction was not analytically characterized.

Taken together, density gradient studies over a period of 45 years suggest a maximum iron loading of native horse spleen ferritin somewhere in the range of 2000–3500, where overloading to some 4300 Fe affords 'unusual' samples of compromised solubility and crystallizability.

In vitro loading of apo-ferritin

This preliminary conclusion is substantiated by a series of studies in which apo-ferritins were subjected in vitro to loading with increasing amounts of iron. Results for ferritins from a variety of sources are given in Table 1. The data generally indicate a maximum loading in the range of 2000–3000, with repeatedly reported precipitation when higher values are employed.

The table contains one outlier: Lopez et al. (2012)[20] report that they can load a sample of horse spleen apo-ferritin with 4300 Fe. This is perhaps not surprizing in the presence of potentially metal ion liganding 100 mM imidazole buffer, however, after exhaustive dialysis versus water they recover exactly 4300 Fe (± 14) from ferritin as measured by ICP-AES. Since this is only the second report in 47 years (and until today also the last one) of a measured loading of 4300 Fe, its subsequent citation history (2013–2022) is highly surprising: of 35 generated citations (Table

S5) only 2 care to mention the 4300 number and, furthermore, do so in a perfunctory manner. No allusion is made to its unique position as the second 4300 paper only in now 57 years nor to it being in contradiction with several other reports (Table 1).

Is the maximum loading of ferritin dependent on the protein (species, type, subunit composition) or on the conditions of core formation? The literature provides insufficient data to answer this question. Table 1 is all we have at this time. All studies were done at a single pH (6.0–7.5) except for the 2003 paper which reports an optimum maximal loading at pH 6.5. Some studies use anaerobic Fe(II) solutions, others do not, or do not specify. In none of the studies of Table 1 phosphate was added, whose presence is known to result in amorphous, rather than (poly)crystalline, cores.[21,22]

The present situation sums up as follows: although it may be widely accepted that ferritin can store up to 4500 Fe, over the last 57 years only two publications appeared that could support this view. The first report is on a fraction of questionable integrity; the second report (only sporadically and perfunctorily cited for its claimed Fe loading number) does not indicate any integrity problems, however, the maximum-loading number of 4300 Fe given in both reports conflicts with a value range of 2000–3000 Fe that transpires from a series of papers on the loading of apo-ferritins (Table 1).

More recently, some authors have claimed that up to 4500 Fe can be stored 'theoretically' in a ferritin molecule.[23–25] All these papers fail to cite any reference where the relevant theory might be found (citation amplification). The complete ferritin literature does not contain any such theory.

Reduction of the 4500 number to ca 3000 can be expected to have direct consequences, e.g. for the chemical modelling of the core composition (a long-standing and unsettled discourse) and for the quantitative capacity of ferritin in technological applications. A direct implication of significance occurs in the emerging, and heavily debated, field of magnetogenetics: Barbic has recently argued that in order for a ferritin molecule, covalently attached to an ion channel, to be operable as a switch by means of an externally applied magnetic field, it is necessary that the ferritin is loaded to its maximal capacity of 4500 Fe.[26] By implication, if the 4500 number is transferred to the realm of fantasy, then so is Barbic's ferritin-modulated magnetogenetics model.

Conclusions

(1) The ferritin literature of over half a century provides little support for the widely accepted view (cf Tables S2 and S3) that ferritin can store up to 4500 Fe. A rather more reasonable working hypothesis would be that ferritins can be loaded with up to perhaps some 3000 Fe, and that it is difficult to determine a hard limit presumably because overloading leads to increased binding of iron on the outside of the protein resulting in decreased protein solubility.

(2) The ferritin literature of over half a century exemplifies an accumulation of citation distortion in the form of (trees of) dead-end citations, incorrect citations, redundant self-citations, and, above all, claims based on no citations at all.

(3) The complete data set on maximal iron loading of ferritin is scanty. New systematic studies under well described, varying conditions are called for.

Supplementary material

Supplementary data are available at *Metallomics* online.

Conflicts of interest

The authors have no conflicts of interest to declare.

Data availability

All data are incorporated into the article and its online supplementary material.

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