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Review

Lipofuscin as the Main Driving Force of Current Age-Related Disease: Justification and Strategies for Removal

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Abstract:
Lipofuscin is indigestible garbage that accumulates in the autophagic vesicles and cytosol of post-mitotic cells with age. Drs. Brunk and Terman postulated that lipofuscin accumulation is the main or at least a major driving factor in aging. They even posited that the evolution of memory is the reason why we get lipofuscin at all, as stable synaptic connections must be maintained over time, meaning that the somas of neurons must also remain in the same locale. In other words, they cannot dilute out their garbage over time through cell division. Mechanistically, their position certainly makes sense given that rendering a large percentage of a post-mitotic cell's lysosomes useless must almost certainly negatively affect that cell and the surrounding microenvironment. Here, I explore the possibility that the accumulation of lipofuscin to some extent underlies all other categories of age-related damage as defined by Dr. Aubrey de Grey. I do not think that lipofuscin removal will reverse/prevent all forms of aging, just the major component facing us currently. In this piece, I will review what is known about lipofuscin accumulation from evolutionary and mechanistic standpoints and discuss ways of removing it from non-dividing (or slowly-dividing) cells.

Keywords: Anti-aging therapy; lipofuscin; SENS; TFEB; intracellular microbe; and synthetic chemotaxis

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Introduction:
Biological aging is a complex molecular process that takes place over time in all organisms. However, organisms have evolved mechanisms to repair various forms of age-related damage. For example, DNA repair enzymes exist that can fix damage in nuclear DNA. Mitophagy enables the degradation of damaged mitochondria. The immune system, when one is young at least, eliminates at least some proportion of one's senescent cells.

Years ago, Dr. Aubrey de Grey posited that there are seven types of damage that accumulate with age in the human body. These include extracellular aggregates, senescent cells, extracellular matrix stiffening, intracellular aggregates, mitochondrial mutations, cancerous cells, and cell loss/tissue atrophy. He devised a strategy to eliminate each form of damage from the body, called Strategies for Engineered Negligible Senescence (SENS) [i]. He is of the belief that all seven categories must be addressed before substantial life extension can be attained.

However, I believe that one of his categories, i.e., intracellular aggregate accumulation, actually underlies many of the other categories of damage - and that the removal of indigestible lysosomal garbage (lipofuscin) could, by itself, substantially extend the human lifespan. It could serve to substantially reverse aging and also prevent it (if the therapy were applied perhaps every decade or so to individuals starting at an age of ~30).

There are two arguments for lipofuscin removal being the most important goal of anti-aging science currently. One is evolutionary and one is mechanistic.

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Evolutionary argument:

In nature, there are only a handful of organisms that can be said to be essentially biological immortal. *Hydra vulgaris* (i.e., *magnipapillata*) is one of such organisms, and the reason for this might be that its indigestible garbage is essentially released from its body over time. It has three cell lineages - ectodermal epithelial, interstitial, and endodermal epithelial. All the epithelial cells in its body column are stem cells that continuously divide - displacing cells toward its extremities. The cells at the extremities slough off eventually [ii, [iii]]. In terms of the interstitial lineage, the differentiated cells that its stem cells produce are closely associated with epithelial cells and so are continuously displaced as well. This is a convenient way to dispose of lipofuscin - i.e., through dilution and cell shedding. However, continuous replacement of neurons may not allow for the stable inter-neuronal interactions required for long-term memory [[iv]]. Lobsters continually grow throughout life; their fully differentiated cells express telomerase, allowing them to keep dividing as needed [[v]]. This includes the cells of their central nervous system, which allows for adult neurogenesis [[vi]]. They also shed their shells periodically. Thus, the same logic appears to apply to them. However, their growth process does not appear to be fast enough to prevent lipofuscin from accumulating over time [[vii]]. Notably, lipofuscin accumulation in eyestalk ganglia [[viii]] is used as a gauge of biological age in lobsters (and myocardial lipofuscin accumulation can be used a marker of chronological age in humans [[ix]]). Lobsters can retain memories, but only for a short time span [[x]]. Naked mole rats are also very long lived, and it has been shown that they have unusually active autophagic systems [[xi], [xii]] (in addition to better anti-cancer defenses [[xiii]]). Even with better autophagy, naked mole rats still do accumulate lipofuscin in their post-mitotic tissues [[xiv]].

It appears as though all animals that age, e.g., flies [[xv]], worms [[xvi], [xvii]], lobsters, naked mole rats, mice [[xviii]], non-human primates [[xix]], and humans [[xx]] accumulate lipofuscin in their post-mitotic tissues. None of the aforementioned organisms seem to possess any evolutionarily "built-in" ways of exporting the lipofuscin that accumulates in their post-mitotic cells from their bodies, presumably because that would be an unnecessary expenditure of energy in light of procreation. Export from the post-mitotic cells themselves is possible through exocytosis [[xxi]], exosome/microvesicle secretion, or secretory autophagy. That part is not too energetically costly. However, from *in vitro* studies, it does not seem as though lipofuscin is exported from post-mitotic cells very often [[xxii]]. It also has never been observed *in vivo* [[xxiii]]. More importantly, if exported *en masse*, there would be nowhere for the garbage to go except to be picked up by tissue-resident phagocytes, which themselves become bloated with lipofuscin - probably mostly from damaged molecules deriving from their own metabolic processes [[xxiv], [xxv]]. (Transfer of lipofuscin to tissue-resident phagocytes through tunneling nanotubes or partial cell fusion is also theoretically possible.) Finally, I have seen no evidence in the literature that tissue-resident phagocytes - full of lipofuscin or not - are able to efficiently leave the body through migration to the gastrointestinal tract, skin, or lungs.

Mechanistic argument:

Lipofuscin can eventually occupy a large portion of the cytoplasm in certain cell types. If it reaches a critical level that negatively affects cellular metabolism in a large enough number of cells in a tissue, it could not only cause pathology, but also cause a downward spiral of functionality due to an exponential increase in the whole-body accumulation of the other six categories of age-related damage as defined by Dr. Aubrey de Grey. For example, damaged mitochondria will not be mitophagized as rapidly and may then start to accumulate. Beta-amyloid that is normally degraded may sit around for longer in the extracellular space and begin to form plaques. Senescent cells may start to accumulate if the tissue-resident immune cells are rendered inert either by phagocytosis of ejected lipofuscin or more likely, the general non-functionality of the parenchymal cells around them leading to dilapidation of the extracellular matrix. Cancer will be more likely to form if many microenvironments throughout the body are corrupted. Similarly, stem cell niche corruption may prevent them

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from replicating efficiently to replenish tissues. And, it is clear that with the extracellular matrix not being tended to properly by its resident cells, stiffening due to cross-links may occur more frequently than they can be cleaved.

It has been argued that lipofuscin is inert. Recently, more researchers are starting to look into whether it may in fact play a pathological role inside a given cell [xx, [xxvi], [xxvii]]. Even if the damaged molecules are mostly sequestered within lysosomes and are not actively harmful to the cell, the fact that many lysosomes become full of garbage and therefore are almost surely unable to perform their normal functions nearly as well just logically seems as though it must be a major problem for the cell. Along these lines, lipofuscin-loaded human fibroblasts display reduced autophagy and decreased survival when exposed to amino acid starvation [[xxviii]]. If a critical threshold is reached in enough cells in a tissue, e.g., the brain, it clearly would be problematic.

With regard to short-lived species, like mice and rats, lipofuscin may not have enough time to accumulate to pathological levels before they die of cancer. It is estimated that 50-90% of aged mice die of cancer [xxix]. Even still, it was shown that in the cerebral cortex neurons of lamina Vb in 630-700-day old rats, lipofuscin occupied 23% of the soma volume [xxx, xxxi]. This could still certainly have a negative physiological effect. Unsurprisingly, we do see a cognitive decline in mice with age [xxxii]. However, even the oldest mice do not develop age-related neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, or amyotrophic lateral sclerosis [xxxiii]. Ostensibly, they simply do not live long enough for sufficient build-up of lipofuscin in their neurons. Other conditions like age-related macular degeneration and sarcopenia do occur in mice, but whether their most severe cases are as bad as the most severe human cases is unclear to me. It is also possible that some mouse tissues accumulate lipofuscin more rapidly than others due to cell type differences in metabolic rates, etc. Some types of human neurons, for example, have not accumulated much lipofuscin by the time others are nearly full [[xxxiv]].

With regard to humans, it has been demonstrated that multiple neuronal cell subtypes become densely packed with lipofuscin granules with age [xxxv, xxxvi, xxxvii]. In large motor neurons of centenarians, lipofuscin constitutes up to 75% of total cytoplasmic volume [xxxviii]. Other post-mitotic cell types also accumulate substantial amounts of lipofuscin [xxxix, xl, ix]. Lipofuscin-laden lysosomes are often much larger than typical lysosomes. The typical size of a lysosome in a fed, unaged cell is ~100 nm-500 nm in diameter [xli]. In contrast, lipofuscin granules are generally 1-5 microns in diameter [xxxviii].

Also, it should be noted that many studies of lipofuscin have relied upon autofluorescence measurements [xlii], but lipofuscin is also very heterogeneous with regard to its structure and composition between different species, tissues, perhaps even cells of the same type, and when found at different concentrations [xx]. Thus, it seems as though relying on a specific wavelength range to visualize a cell's lipofuscin deposits may not be prudent.

If one could remove the lysosomes from laden macrophages stuck at plaques in artery walls, the plaques may eventually regress, given a healthy diet and sufficient exercise as well [[xliii]]. Similarly, with regard to extracellular plaques like beta-amyloid plaque, Le Chatelier's principle may apply. If there are fewer of the soluble protein monomers in solution in that area, the solid mass may dissolve back into solution. Parenchymal cells devoid of lipofuscin could endocytose monomers; as they are degraded, the extracellular plaques could start to dissolve and be taken up as monomers as well - for subsequent degradation. Tau tangles would be autophagocytosed and degraded as well. For the elderly, multiple rounds of lysosomal clearance may be necessary to achieve full results - because, for example, defective mitochondria could have accumulated

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that will be mitophagized after the initial lipofuscin clearance, which may then become lipofuscin inside the lysosomes due to extensive damage of the mitochondrial membranes by free radicals.

Removal strategies *in vivo* (on a cellular basis):

The simplest way to prevent lipofuscin accumulation is to prevent the excessive uptake of biomolecules from external sources via caloric restriction [xliv]. Caloric restriction triggers upregulated autophagy [xlv], meaning that reversal and prevention are possible to some degree through this method. Exercise also increases the degradative capacity of our cells [xlvi]. But, of course, these two methods alone are not enough to reverse/prevent aging in a truly substantial manner.

Dr. Aubrey de Grey, an anti-aging pioneer, first introduced me to the concept of lipofuscin through his 2005 TED talk and book that he co-authored with Michael Rae, "Ending Aging: The Rejuvenation Breakthroughs That Could Reverse Human Aging in Our Lifetime [xlvii]." He proposed a way of eliminating it from the body involving "xenocatabolism". It is based on his realization that lipofuscin, which is typically fluorescent, must be eliminated from human corpses by microbial enzymes, or else graveyards would glow in the dark. He postulated that we can co-opt enzymes from soil bacteria and fungi and install them in our cells to degrade our lipofuscin [xlviii]. However, I do not believe this will be feasible in our lifetimes at least, as lipofuscin is quite heterogeneous - it varies widely in composition between cell types and even cells of the same type, potentially [xlix]. It also varies in composition between different individuals, probably in part due to differences in diet.

Thus, it would likely require an inordinate number of microbial enzymes to degrade the majority of our lipofuscin, almost all of which would still have to be discovered, evolved, or rationally-designed - and many of them may be toxic to our cells. There are some molecular species that seem to be major contributors to lipofuscin or lipofuscin's toxic effects at least in some cell types, like the fluorophore A2E (retina) [l] and the oxysterol 7-ketocholesterol (throughout the body - especially atherosclerotic plaques and the retina) [li], but we cannot neglect other organs or tissues, or they will fail and we will die anyway. It is worth looking into to see if there are major lipofuscin constituents present across a wide variety of cell types that would be very good targets. But it is unlikely, at least in my mind, that a small number of enzymes will be sufficient to degrade the majority of our lipofuscin deposits.

TFEB is the master regulator of autophagy, and its overexpression can increase lysosomal acidity and boost lysosomal hydrolase levels in lysosomes, which could help to shrink existing lipofuscin deposits in lysosomes. Here, it is important to draw a distinction between "false" and "true" lipofuscin. False lipofuscin would be any intracellular garbage that can be digested by cellular machinery if said machinery is maximally expressed. True lipofuscin is that which is indigestible by cells no matter how they are transcriptionally manipulated. But TFEB (i.e., HLH-30) overexpression only grants a 15-20% increase in lifespan for *C. elegans* [lii]. Presumably this is because lipofuscin still accumulates in the intestinal cells over time, even with HLH-30 overexpression. For this truly indigestible garbage, TFEB could still be of use. It also induces the exocytosis of lysosomal contents through fusion of the lysosomal and plasma membranes.

One problem with boosting endogenous exocytosis processes is that they may lose potency with age; oxidatively warped lipids from lipofuscin may insert themselves into the membranes of the lysosomes that contain them and prevent efficient fusion with the plasma membrane. The main problem, however, is that the two most well-known inducers of lysosomal exocytosis, Ca^{2+} and TFEB, do not induce a substantial amount of exocytosis - even in youthful cells [liii, liv]. Perhaps over time, TFEB could work well enough to exocytose all a post-mitotic cell's lipofuscin, but other cellular mechanisms may need

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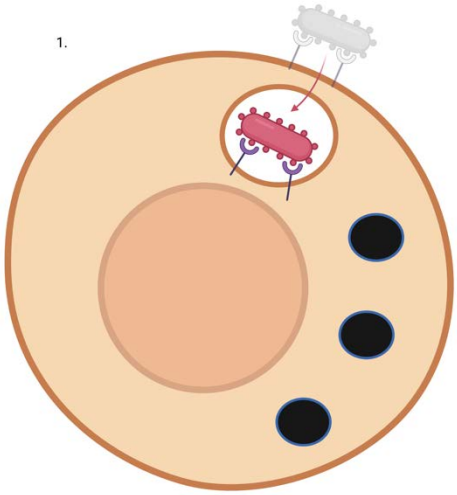
to be combined with TFEB for truly effective exocytosis - and there may be cell type differences in what machinery is required. A small molecule was shown to induce some lipofuscin exocytosis in monkey retinal pigment epithelial cells, which are important in macular degeneration [xxiii]. A final issue is that with lysosomal exocytosis, lipofuscin granules may break apart and/or get caught in various parts of the extracellular matrix - causing a problem by being there. The lipofuscin could also at least eventually be taken up by the same cells that ejected it, neighboring cells that need to be cleared of garbage themselves, or tissue-resident macrophages. It's probably better for the lipofuscin to be inside an intact vesicle that can prevent re-uptake - only to be picked up by bioengineered macrophages.

Lipofuscin could also theoretically be exported from post-mitotic cells through microvesicular secretion of lysosomes or by transferring lysosomes through tunneling nanotubes (TNTs) to bioengineered macrophages delivered to their locale. (However, the diameter of some lipofuscin-laden lysosomes may be too great for them to be transferred through TNTs.) Secretory autophagy may also be relevant here [lv, lvi]. Secretory autophagy utilizes autophagosomes, which are double-membrane vesicles. Perhaps a pathway involving the exocytosis of lysosomes engulfed in single-membrane vesicles could also be possible [lvii].

A.



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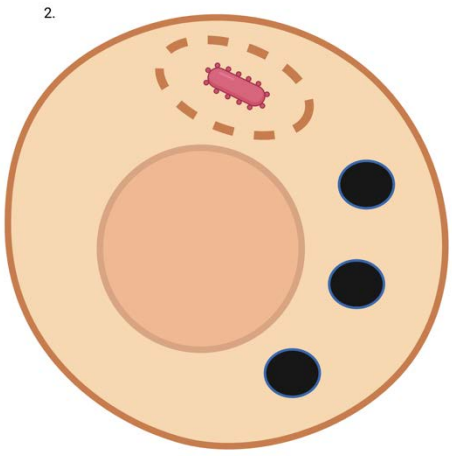

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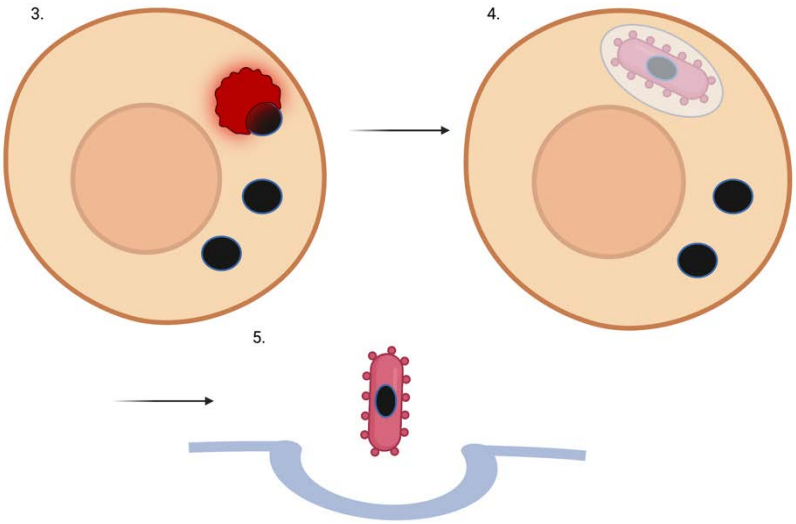

Figure 1. **Lipofuscin-laden lysosome removal from a non-dividing or slowly-dividing cell using a "trash-collecting" microbe.** A) The intracellular microbe enters the target post-mitotic cell. B) The intracellular microbe escapes the vacuole. C3. The intracellular microbe phagocytoses a lipofuscin-laden lysosome. Of course, not all of a target cell's lysosomes may be full of lipofuscin. But over time, through random chance, even with new lysosomes being generated naturally or via TFEB induction - many if not most of the garbage-filled lysosomes will be removed. C4. Amoebae that have picked up indigestible lysosomal cargo re-enter a vesicle. C5. Full amoebae are ejected from the cell via exocytosis.

Instead of trying to determine which combination of proteins would promote each post-mitotic cell type to efficiently rid itself of its lipofuscin, an intracellular microbe could be harnessed to enter post-mitotic cells and engulf lipofuscin-laden lysosomes directly, then escape the cell. The smallest white blood cell with phagocytic properties has a diameter of 12-15 microns (neutrophils). Some post-mitotic cells have a diameter of around 10 microns. Thus, instead of trying to bioengineer a leukocyte to be intracellular, it might be best to use a micro-organism with a diameter of ~3 microns so that they can enter the cell and engulf lysosomes that have grown to a relatively large size due to lipofuscin accumulation. If the micro-organism could expand (i.e., if it had a ruffled membrane) to accommodate even larger cargo, that would be ideal. Perhaps phagocytic bacteria (~4-5 microns in diameter) [lviii] would be easier to work with than amoebae in terms of having them survive inside host cells and not damage said cells. However, one micro-organism of the appropriate size and with phagocytotic properties is an amoeba known as *Massisteria voersi* [lix].

It might be necessary to prevent the attempted digestion of the phagocytosed material - in order to prevent metabolic failure of/cytotoxicity to the microbe. Also, a mechanism may need to be in place that could prevent phagocytosis of more than one target. Finally, perhaps a mechanism should be put in place to induce growth [lx], detachment, or lysis (to avoid microbial stress-mediated detrimental effects like chromosomal instability or, potentially, toxin secretion) upon phagocytosis frustration - possibly via a timer-based mechanism involving a temporal promoter cascade as in early, intermediate, and late viral gene expression.

An even smaller microbe may be required to treat certain cells; the somas of cerebellar granules cells, for example, are only around 5 microns in diameter. Perhaps a single microbe could treat all target cells if it can identify smaller human cell types based on surface proteins - inducing shrinking of the microbe if it recognizes a smaller human cell type [lx]. (Being

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smaller initially might help with cell entry; the microbe could potentially grow a bit after entering a target cell and escaping the endosome. Encountering a smaller cell, plausibly with smaller lysosomes in general - even with lipofuscin accumulation - could trigger a counting mechanism wherein it picks up more than one lysosome in the target cell [lxi]. This may increase the "cleaning" efficiency of this approach.)

Non-replicating intracellular bacteria can be rapidly generated by introducing into them a plasmid encoding the *dnaA* gene that has a temperature sensitive origin of replication and then deleting the *dnaA* gene from the bacterial genome [lxii]. When the temperature is increased, the bacteria will lose the plasmid and become non-replicating; ampicillin can then be added to the culture to lyse the dividing cells while sparing the non-dividing cells. However, those bacteria elongate substantially and may have other metabolic abnormalities. (Moreover, it is plausible that bacteria that are so elongated could potentially have trouble entering certain target cells.) Alternatively, RelA overexpression induces the large scale production of pppGpp and ppGpp. These two molecules are known together as (p)ppGpp, and they are known to drastically slow bacterial growth [lxiii, lxiv]. Thus, (perhaps truncated) RelA overexpression could be the baseline state in the trash-collecting intracellular bacteria [lxv]. However, some amount of RelA may be necessary for the vector to survive in host cells [lxvi].

Only two genes are required to change a normally extracellular bacterium to an intracellular bacterium, an invasin gene and the listeriolysin O gene from *Listeria monocytogenes* [lxvii]. Perhaps those two could be utilized here as well for an amoeba. Moreover, ActA could potentially be utilized to promote intracellular movement of the amoebae [lxviii]. The phagocytic receptors on the amoebae would be redirected to LAMP-1, a lysosomal transmembrane protein. The amoebae could also secrete Bcl-2 to prevent stress-induced apoptosis of host cells - in case its presence inside the cell is excessively detrimental to the metabolism of that cell [lxix]. However, further bioengineering may certainly be necessary to prevent host cell cytotoxicity.

The amoebae could be bioengineered to phagocytose lysosomes only, perhaps via selective recognition of targets heavily decorated with LAMP-1 [lxx]. (If LAMP-1 is too non-specific in terms of its localization on other autophagic/endocytic vesicles, an AND-gated receptor system could certainly be developed - similar to SynNotch [lxxi].) After an amoeba binds its target, there could then be a pause using a synthetic gene circuit like a temporal promoter cascade, allowing for the engulfment process to proceed and be completed. Then, the amoebae would escape via extrusion, budding, actin-mediated protrusion [lxxii], ejection, expulsion, or exocytosis [lxxiii]. Notably, *F. tularensis*, an intracellular bacterium, is known to re-enter the endocytotic compartment after cytoplasmic replication - perhaps at least in part due to its ability to then be exocytosed, spreading to neighboring cells [lxxiv]. A microbe with a T3SS might be able to translocate proteins across the vesicle into the cytoplasm to force its exocytosis. There is a *Mycobacterium tuberculosis* surface protein that promotes engulfment of the bacteria into LC3-associated autophagosomes [lxxv].

(Of course, prior to an amoeba engulfing a lysosome, autophagosomal engulfment must be prevented. ActA would help - through motility-dependent and motility-independent mechanisms [lxxvi, lxxvii]. Also, expression of USP30 [lxxviii, lxxix] on the surface of the amoebae could help. Production of at least the former protein should be halted when the amoeba has engulfed its target and needs to be autophagocytosed.)

TFEB expression [lxxx] by the microbes prior to engulfment - using a time delay synthetic gene circuit, could help to consolidate garbage by promoting autophagosome-lysosome fusion and get most of the lipofuscin in vesicles rather than

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some of it being free in the cytoplasm. It would also be important to induce TFEB expression after engulfment, but prior to leaving - in order to force the generation of new lysosomes, so the cell is not left at a serious deficit. A small molecule that increases TFEB activity could perhaps alternatively be used instead prior to and periodically throughout treatment - such as the one currently being developed by Generian. Incorporating TFEB activity would likely increase the overall treatment time, as it would generate new lysosomes that may be targeted by the intracellular microbes, but over time, eventually all the lipofuscin-laden lysosomes would be removed as well - and it seems much safer/like it would lead to a much more thorough "deep clean".

Testing the importance of lipofuscin accumulation in aging *in vitro* now:

In order to test the theory that lipofuscin is the main issue with regard to age-related disease perhaps for the next few hundred years at least, it appears that we must either figure out how to induce a particular post-mitotic cell type (e.g., a neuron derived from an iPSC) to export its garbage using a gene vector or remove its lipofuscin-laden lysosomes with host cell-autonomous means. Then we could see how long it lives in culture as opposed to normal cells of the same type. However, while primary cell cultures often die after a few days-weeks [lxxxi], the cause of death may not be related to normal aging. It may instead be related to infection or hyperosmolality due to evaporation of the media [lxxxii]. It was shown, however, that at ~day 25, neurons *in vitro* have an aged phenotype, including dysfunctional mitochondria, higher reactive oxygen species production, and at least one feature of senescence [lxxxiii]. It was also shown that defective autophagy was linked to this senescence-like phenotype - and that aged neurons in culture accumulate lipofuscin [lxxxiv]. Thus, if the lipofuscin were removed from the cells and new, functional lysosomes were introduced exogenously or generated through TFEB, their aged phenotype may reverse.

Another approach would be to study aged skeletal muscle cells that contain large quantities of lipofuscin taken from biopsies of elderly humans [lxxxv]. Their lipofuscin-laden skeletal muscle cells may also eventually die from other causes than normal aging due to culture conditions. But by removing the lipofuscin from those cells, one could at least see if markers of health are restored in the cells; i.e., defective mitochondria could be mitophagized, any cytoplasmic lipofuscin granules could eventually be autophagocytosed and delivered to lysosomes, and a senescence-like phenotype could eventually be reversed.

To get a cell to export its garbage, perhaps one could force lysosomes out to the periphery via JIP4 inhibition [lxxxvi] while overexpressing the VAMP7, Stx4, and SNAP23 [lxxxvii] - and then administer Ca^{2+} and the calcium ionophore A23187 to the cell to induce fusion of peripheral lysosomes with the plasma membrane [liii]. TFEB would subsequently be overexpressed to induce lysosomal biogenesis [lxxxviii]. This approach could be applicable to many cells in culture. (Other inhibitors like TMEM55B could also be inhibited via siRNA; the concept of inhibiting inhibitors is also clearly applicable to secretory autophagy.)

Another approach that may work and would be applicable to many cells in culture would be to target galectin-8 (or TRIM16 directly) to LAMP-1 while overexpressing Sec22b, Stx3/4, and SNAP23/29 to force the selective encapsulation of lysosomes into an autophagosome destined for secretory autophagy [lxxxix, xc, xci]. Also, autophagosome-lysosome fusion could be cyclically inhibited via small molecule or the SARS-CoV-2 viral protein ORF3a to bias at least nascent autophagosomes toward secretory autophagy [xcii, xciii]. Targeting a plus-end directed motor protein to the cells' autophagosomes would also be of use to move them to the periphery. Furthermore, TRIM16 could potentially be engineered so as not to bind to mIL-1β or any other native cargo - if necessary. However, TRIM16-mediated secretory autophagy has only been observed

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in the context of protein cargo, to my knowledge. Thus, I am not sure if this approach will work exactly as described. However, whole organelle secretion is possible via cellular mechanisms [xciv, xcv].

In reality, any protein targeted to LAMP-1 with an LC3-interaction region (LIR) [xcvi] and the ability to bind to Sec22b, in the context of Stx3/4 and SNAP23/29 overexpression, might be able to mediate secretory autophagy of lysosomes.

The efficiency of secretory autophagy for these vesicles specifically could potentially be enhanced by engineering/evolving all the proteins in the pathway to have more affinity for their respective binding partner(s). VEGAS [xcvii] may be of use here to enhance the binding affinity of these proteins - or perhaps coiled coils [xcviii] could simply be added, at least in some cases. One could also overexpress all of the altered proteins. (TFEB overexpression would again be helpful to replenish the cells' lysosomes after each round of export.) This approach of increasing the interaction affinity of all the proteins in the pathway and overexpressing the altered proteins also applies to lysosomal exocytosis.

Two host cell-autonomous means occur to me. Both involve a FluidFM-based approach [xcix]. Every few days or so, a single cell could be injected with large iron particles conjugated with antibodies against LAMP-1. Then, a magnetic field could be applied to cluster the lysosomes to one small area at the plasma membrane. They would then be removed by the needle. TFEB would subsequently be induced from an AAV vector genome in the nucleus via small molecule to replenish the intracellular supply of functional lysosomes. The second means would involve the bioengineered amoebae described before. Amoebae containing large quantities of magnetic nanoparticles could infect or be injected into host cells. They could then engulf the target lysosomes. After giving them some time to do so, a magnetic field could be applied near the cell to cluster them in one small area at the plasma membrane. Then, they could be withdrawn via needle. TFEB again would be utilized here.

Eventual *in vivo* strategy for delivery and pick-up of the "trash-collecting" microbes:
The trouble with trying to intravenously administer large liposomes or viral vectors, which can contain much more complex payloads, is that they cannot reach many intraparenchymal target cells throughout the body because the vascular endothelium serves as a stringent barrier. Some intracellular microbes can transcytose across the intestinal epithelium - but this is not necessarily helpful for the vascular endothelium [c]. There are some pathogens that can cross the blood-brain barrier, but this is typically under conditions of systemic inflammation or bacterial-mediated cytolysis of vascular endothelial cells to cause gaps in the endothelium [ci]. Basically, we do not know nearly as much about effecting safe microbial transcytosis/paracellular passage across the vascular endothelium as we do about white blood cell extravasation [cii] - although we do not know all the mechanisms of leukocyte diapedesis either. Thus, in the more immediate future, to get the trash-collecting microbes across the vascular endothelium, we may have to employ a somewhat complex, two-step delivery system that involves carrier white blood cells.

The two-step system would involving first delivering a gene vector to vascular endothelial cells - this would then inducibly allow for the transmigration of bioengineered white blood cells containing the trash-collecting microbes. Basically, clinicians would seek to infect all or the majority of a patient's vascular endothelial cells [ciii] with a herpes simplex virus type 1 (HSV-1) vector. (Different pseudotypes of HSV-1 or bi-specific antibodies may be necessary to ensure that all vascular endothelial cells throughout the body are transduced; one marker may not be enough - as the gene expression pattern of vascular endothelial cells in different anatomical locales are different [civ].) They would then inducibly express from the viral genomes (upon small molecule administration) synthetic, luminal adherence proteins and chemokines [cv].

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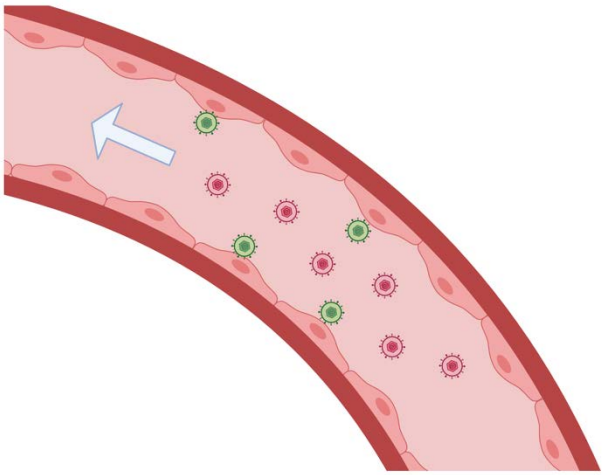
These would allow for the attachment and transmigration of bioengineered macrophages. After transmigration, perhaps chemorepellents could be secreted abluminally to direct them away from the vascular endothelial cells, across the rest of the vascular wall, and into the parenchymal tissue (except if one is targeting smooth muscle cells or pericytes in the vascular wall - which may also need to be treated). The macrophages could then randomly migrate around until binding to a cell type of interest, at which point they could (directionally) donate trash-collecting microbes to the target cell.

In fact, the trash-collecting microbes could replicate within the carrier white blood cells up to a moderate copy number [cvi] - restrained by AI-2-based quorum sensing, perhaps [cvii]. The carrier white blood cells could then continuously donate the trash-collecting microbes to target cells via microvesicular secretion, secretory autophagy, transient TNTs, or even partial cell-cell fusion; they could enter target cells, engulf their lysosomes, and escape.

A.



B.

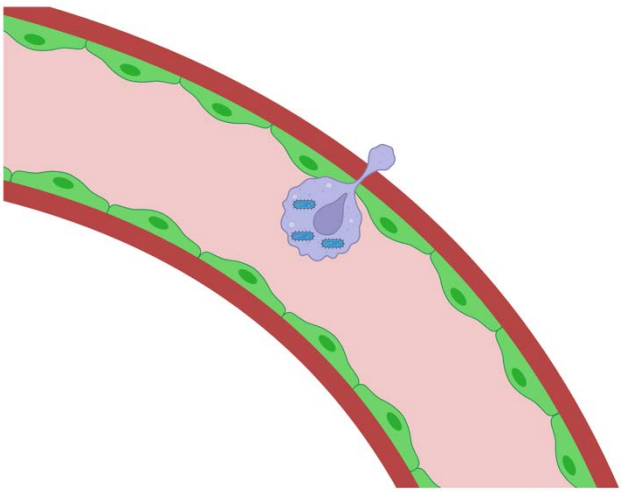


Figure 1: A) Gene vectors are administrated intravenously - and they bind to vascular endothelial cells, are internalized, and transduce them to allow control over synthetic adherence proteins and chemokines that facilitate the extravasation of

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bioengineered white blood carrier cells. B) The bioengineered white blood cells carrying intracellular microbes are "smuggled" across the vascular endothelium in many regions throughout the body.

The vascular endothelial glycocalyx may make the delivery of large gene vectors difficult. AAV vectors are able to reach the vascular endothelial cells beneath the glycocalyx, at least in many regions [cviii, cix]. I suggested HSV, however, because I thought multiple proteins may be required to ensure extravasation of the bioengineered white blood cells carrying intracellular, trash-collecting microbes - perhaps multiple proteins that vary based on anatomical locale. But perhaps a single, synthetic chemokine will be enough. Multiple AAVs can be employed if necessary. (An adenoviral vector is also a possibility, perhaps [cx].) If a larger gene vector is required and can only transduce only a small number of vascular endothelial cells throughout the body - that could still be enough, or at least very helpful. Imaging via HSV1-TK could let us know when the bioengineered white blood cells have reached the tissue parenchyma in various regions - and a small molecule could be intravenously administered to promote the replication of the bioengineered white blood cells in those regions. The intracellular microbes in the bioengineered white blood cells would keep pace with that division - restrained by quorum sensing. The two-step delivery process could, in this sense, still potentially save a given patient a lot of intraparenchymal infusions in different anatomical locales.

The only other option that I think makes any sense is to manually inject parenchymal regions throughout the body with bioengineered white blood cells carrying the trash-collecting microbes. The bioengineered white blood cells could be induced to replicate up to a sufficient number once there via small molecule. That solution isn't pretty, but if lipofuscin is truly the main culprit in age-related disease, we simply have to do whatever is required. More likely, we could combine the aforementioned two-step delivery system and intraparenchymal injections for full coverage. Please keep in mind that the small molecule-induced replication option works for the two-step system as well of course, and that in both scenarios there would be continuous donation of trash-collecting microbes from each carrier white blood cell while they randomly migrate around a given tissue.

There are two important considerations for the two-step delivery system. One is that clinicians should perhaps slowly infuse the patient with bioengineered macrophages after inducing the synthetic luminal adherence proteins and chemokines, so as not to create too much stress on the vasculature. The other is that one may need to SIE in terms of the HSV-1 vector to make sure that all or the majority of vascular endothelial cells are transduced - but without ending up with too high of a vector copy number per given cell. SIE would ideally be on the level of intracellular capsid trafficking. For example, the degradation system cited here [cxi] could ensure the degradation of incoming capsids before they transfer their DNA to the nucleus [cxii]. A synthetic gene circuit imbuing network-dosage compensation could also be of use in this situation potentially [cxiii]. Given that the average EC lifespan is >1 year [cxiv], there is a clear therapeutic window here if the vasculature can be thoroughly transduced first. The extent of vascular endothelial transduction could be visualized via the HSV-1 thymidine kinase. And a kill switch could be included to destroy the vector genomes (based on CRISPR, for example) after lipofuscin removal treatment.

This covers the delivery aspect perhaps, but there is still the issue of pick-up. If a large quantity of bare trash-collecting microbes were to migrate through various tissues to an extraction point, it might severely damage those tissues - as microbes have not adapted to migrate safely through human tissue. It is unclear to me what percentage of the damage we see is due to an inflammatory immune response, however. Stealth microbes [cxv] that we one day perfect may be able to migrate on their own without incurring damage.

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Possibly the most facile approach currently is to utilize phagocytic bacteria delivered via bioengineered macrophages - wherein they do not replicate up to quorum sensing number in the host macrophages. After the two-step transmigration process, the macrophages will migrate around randomly via a synthetic gene circuit - perhaps involving Cdc42 [cxvi]. They will then bind a target cell using the SynNotch system, which will initiate the overexpression of M-Sec and LST1. The resulting TNTs between the macrophage and possibly multiple target cells will allow the leukocyte to deliver phagocytic bacteria to the target cells. Miro1 [cxvii] from the host cells and ActA [cxviii] from the microbes would probably facilitate the delivery process. (The macrophages lysosomes must be marked with a phagocytosis inhibitor so the microbes engulf them.) Then, the bacteria will engulf target cell lysosomes and escape non-lytically - possibly in the same way that whole organelles are expelled from cells. Once they have escaped, and the PrfA promoter is no longer functional due to the absence of host cell glutathione [cxix], they can lyse the vesicle around them if there is one through LLO secretion [cxx] (on a timer-mechanism that stops after a period of time sufficient for vesicle lysis). Alternatively, they can simply swim inside the vesicle [cxxi]. In any of the above scenarios, the bacteria could also greatly increase the thickness of their capsules [cxv]. (They could have the capsule from the beginning - but it may interfere with intracellular movement and phagocytosis.)

The thick capsule could shield them from re-entering the same cell or entering neighboring cells, and potentially allow them to migrate safely on their own to the peritoneal cavity via magnetosome-based [cxxii] magnetotaxis [cxxiii]. There, they could be withdrawn via suction-based laparoscopic removal. I am uncertain if a thick capsule is compatible with migration - it seems likely that an encapsulated bacteria could also be motile. If not, the capsule would still potentially protect them from (re-)uptake by parenchymal cells until the bioengineered macrophages get there and pinpoint their locations via a bacteria-produced small molecule (as mentioned before).

Alternatively, they could be stationary once exported from the target cell and then be picked up by another wave of bioengineered macrophages; macrophages often migrate through our tissues harmlessly. The trash-collecting microbes could secrete a chemoattractant molecule when they are full and extracellular. To amplify the chemoattraction signal from individual microbes, one could bioengineer them to express a pro-drug activating enzyme, so that an exogenous small molecule could be converted into a chemoattractant signal. A new wave of bioengineered macrophages that are bereft of microbes initially would then be administered - the vascular gene vector would be induced via small molecule - and they would extravasate. Then - once chemorepelled across the vessel wall, they would hone in on their microbial targets. The bioengineered white blood cells could recognize the membrane surrounding the full amoebae or the amoebae themselves (depending on whether a double-membraned or single-membraned vesicle is used for export) via a T-cell receptor [cxxiv]-type strategy, and that could trigger migration to an extraction point. Perhaps engulfment of the first microbe would trigger a counting mechanism [lxi], and when perhaps ~10 amoebae have been engulfed - the bioengineered white blood cells could be induced to migrate to an extraction point.

Withdrawal could be effected by implanting small biodegradable beads that slowly release a chemoattractant molecule [cxxv] inside small devices that serve as a trap for the bioengineered macrophages, perhaps via conjugation of antibodies to the interior of the trap that target a certain surface protein of the bioengineered macrophages. The extraction point could be laparoscopically-inserted “traps” for the bioengineered white blood cells as I just mentioned, the bloodstream (where they can be filtered out through dialysis), or the intestinal lumen. Magnetism could also be used to draw bioengineered macrophages loaded with magnetic nanoparticles to a singular, intraperitoneal extraction point, where they can be removed laparoscopically [,].

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This strategy for *in vivo* treatment will likely require immunosuppression throughout the duration of the treatment. As mentioned before, the gene vector in the vascular endothelial cells can self-target with CRISPR/Cas9 after being induced via small molecule following treatment. Perhaps a gene vector can incorporate elements such as ICP47, a MHC class I inhibitor [cxxviii], etc. to prevent an immunological response. It would still be necessary to immunosuppress the patient during the vector envelope biomolecular/capsid degradation stage. Empty HSV-1 vectors or immunopheresis could be used to soak up or remove antibodies [cxxix]. HSV-1's gD protein has also been engineered so that it evades multiple neutralizing antibodies, although this protein variant was incorporated into new virions at lower levels than the wild-type version [cxxx]. With regard to the intracellular microbe, perhaps a fully immunologically "stealthed" vector [cxxxi, cxv] can eventually be developed through bioengineering. However, it is also important to note that the microbe could potentially be shielded from the extracellular environment at every step throughout the treatment, leaving only cell-autonomous innate immunity as an issue.

Regardless of whether this two-step delivery system is feasible in terms of the vascular endothelial glycocalyx, we could at least test the importance of lipofuscin removal *in vivo* by using this system in combination with genetically engineered mice to have one or more genes already present in their genomes - driven by vascular endothelial promoters. (Here, we may not even need synthetic chemokines - if the vascular endothelium of the mice secretes human chemokines and the mice have humanized immune systems. The human chemokines would have to be engineered to at least bind to mouse glycosaminoglycans - if they don't already - to allow for the Chemokine Cloud model to function [cxxxii].)

The two-step transmigration delivery system with vascular endothelial transduction and bioengineered leukocytes also applies to treating other human health conditions like genetic disease. The bioengineered leukocytes could also continuously export copies of a viral vector while wandering around within a target tissue. (One of the proteins the virus normally encodes that is essential for the replication of its DNA would be deleted and expressed by the bioengineered leukocyte. Thus, the viral DNA would be able to replicate exclusively in the nucleus of the leukocyte. It would do so up to a particular copy number - restrained by a plasmid copy number control mechanism [cxxxiii] - thus providing new genomes for packaging in subsequently secreted virion particles while preventing the leukocyte from being overwhelmed.) The viral vector could deliver CRISPR components to target cells. Additionally, the leukocytes could protect our cells against viral infections by secreting a gene vector encoding double-stranded RNA-activated caspase oligomerizer (DRACO) [cxxxiv] - or they could be used for treatment and simply secrete the DRACOs themselves. SIE and maybe a synthetic gene circuit for network-dosage compensation should be employed when continuous gene vector export is desired.

Discussion:

Finally, the reason why I believe an phagocytic intracellular microbe may be the best approach to clear lipofuscin is that they already carry many gene networks capable of allowing entry into a wide variety mammalian cell types, phagocytosis of micron-sized objects, and non-lytic escape from a wide variety of mammalian cell types. Screens can be performed to identify mutants that are even more adept at these three processes. Identifying or rationally-engineering/evolving proteins that can initiate lysosomal exocytosis, secretory autophagy with regard to lysosomes, or microvesicular secretion of lysosomes in all the necessary target cell types - may not be particularly facile. Also, all of the protein-based approaches would initiate mass export of lysosomes, which may be detrimental to the patient's health even when done slowly - and especially if done throughout the whole body simultaneously.

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Target cell-based mechanisms of lysosomal export would also potentially be taxing for them in terms of membrane lipid dynamics. In contrast, the microbes can enter one at a time and pick up cargo, keeping it encapsulated and theoretically preventing re-uptake by the same cell, neighboring cells, or tissue-resident phagocytes. If the microbe buds from the carrier leukocyte or is secreted through secretory autophagy, it would be delivering lipid contents to a given target cell. It would also engulf the target lysosome using its own membrane lipids. Finally, they can give off a small molecule signal that specifically allows bioengineered phagocytes to hone in on their location and phagocytose them.

However, a phagocytic microbe-independent strategy could also certainly be possible. One could employ an DNA or RNA vector delivery system as mentioned earlier with regard to a plasmid copy number control-type mechanism. There is also the option of simply employing intracellular microbe secretion. An extracellular microbe with a T3SS could be delivered to target cells to inject them with effectors (although we may have to bioengineer some of the relevant secretion substrates if they can't be secreted efficiently even after addition of a relevant secretion tag) [cxxxv]. The granzyme-perforin pathway was also recently proposed as a protein delivery system [-based delivery [, - loaded liposomes [

Other mechanisms like adding cell-penetrating peptides and/or toxin translocation domains to effector proteins secreted by the bioengineered leukocytes might work, but they might not be very effective - especially if many different proteins must be delivered in moderate-large quantities [cxl, cxli].

Together, I call both phagocytic microbe-dependent and phagocytic microbe-independent *in vivo* lipofuscin removal approaches, "Clearance of Undigested Rubbish by Encapsulation" (CURE). That is because regardless of how the lipofuscin is ejected from post-mitotic cells, it ends up in a bioengineered leukocyte that will at some point migrate to an extraction point.

Conclusion:
Through synthetic biology, I believe we can clear lipofuscin from our tissues. There is a research article showing that TFEB overexpression can shrink lipofuscin deposits - but this work was done in the context of a transgenic mouse model with overproduction of an aggregating protein, which I would define as "false" lipofuscin [cxlii]. Namely, if the protein ceased to be produced and the cells weren't already completely overwhelmed, it could be degraded over a reasonable amount of time by endogenous lysosomal hydrolases.

As we have never really had a way to clear true heterogeneous, age-related lipofuscin from our cells - it would be extremely interesting to put the "garbage catastrophe theory of aging" to the test with new techniques. If clearance greatly extends the lifespan of non-dividing cells in culture, a new delivery system may have to be designed to get these trash-collecting microbes to the target cells *in vivo*; a system of withdrawal would also be necessary. However, it is important to remember that cells in culture don't necessarily always die from the same things that they do *in vivo*.

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