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Human autophagy measurement: an underappreciated barrier to translation

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Preclinical research shows that autophagy is a modifiable process that holds promise for preventing human age-related disease. However, this knowledge has not been clinically translated. Here, we discuss recent developments in the ability to measure human autophagy, and why it is a critical step for translation.

Autophagy: a promising target for age-related disease

Autophagy is a recycling process that degrades unwanted and damaged cellular macromolecules. It slows the hallmarks of ageing [1] and is thought to delay multiple age-related diseases, such as atherosclerosis and Alzheimer's disease [2,3], two of the largest unmet clinical needs of our time. *In vitro* and *in vivo* preclinical research also show that autophagy is modifiable both pharmacologically and nutritionally [4]. For example, autophagy can be increased through calorie restriction or inhibitors of the mechanistic target of rapamycin complex 1 (mTORC1), such as rapamycin. We know this from decades of autophagy research in cell and animal models but, conversely, we know little about how autophagy behaves in humans. This contradiction arises because measuring human autophagy has proven to be difficult. To understand why, we must understand autophagic flux and why its measurement only provides an accurate result under strict physiological conditions.

Measuring autophagic flux in humans

To meaningfully measure autophagy as a functional test, we need to measure how much material is sequestered by autophagosomes and subsequently destroyed in lysosomes (Figure 1A) over a defined period, a process termed 'autophagic flux' [5]. The current gold standard for determining autophagic flux in cell culture involves measuring autophagy proteins (typically LC3-II by immunoblot) in paired replicate samples with a chemical inhibitor to block lysosomal degradation [5] (Figure 1B,C). This is easily achieved in cell culture because samples can be split and cultured in parallel with and without lysosomal inhibitors. However, cell culture models are unlikely to reflect autophagy in a physiologically relevant context notably due to the use of nutrient-rich culture media.

Autophagy is nutrient sensitive, because one of its roles is to access nutrients and energy locked away in macromolecules to maintain homeostasis. High concentrations of nutrients (e.g., amino acids) and insulin decrease autophagy, while low nutrient and insulin concentrations increase autophagy. This nutrient-related autophagy response is regulated through mTORC1 signalling. As a result, isolating primary cells, such as leukocytes, from human samples and culturing them in nutrient-rich culture media changes mTORC1 activity and consequently autophagic flux. Thus, culturing primary cells *ex vivo* in artificial media and measuring autophagic flux does not yield a physiologically accurate measure of autophagy (Figure 2A).

Researchers have instead attempted to measure autophagy in humans and animals by measuring autophagic machinery transcript or protein abundance. However, despite their common use, these snapshot measurements of autophagic transcripts and proteins do not correlate well with autophagic flux [6]. This limits their ultimate usability for studying autophagy.

Nevertheless, a few studies have assessed autophagic flux *ex vivo* in human samples cultured in artificial media. For example, the lysosomal inhibitors bafilomycin and leupeptin have been used in combination on human liver biopsies cultured in artificial medium to measure autophagic flux [7]. Autophagic flux has also been measured in leukocytes [8]. While that study demonstrated autophagic flux in mice using intraperitoneal injection of the lysosomal protease inhibitor, leupeptin, the same inhibitor incubated with human leukocytes cultured *ex vivo* failed to demonstrate autophagic flux [8].

Instead, physiological autophagic flux has been measured in organotypic human blood samples [9]. Rather than culturing human cells in an artificial medium, the blood cells (peripheral blood mononuclear cells; PBMCs) were maintained in their natural medium: blood. Paired blood samples were incubated briefly with and without the lysosomal inhibitor chloroquine (Figure 2B). PBMCs were then isolated from these whole-blood cultures and subjected to western blotting and fluorescence microscopy for measurement of LC3-II and, thus, autophagic flux (Figure 1C). Furthermore, the method is adaptable to ELISA [10] and flow cytometry [11] to more accurately measure the autophagy protein LC3-II. Thus, it is possible to measure autophagic flux in physiologically intact human samples.

Measurement of autophagic flux in blood and its relevance to other tissues

Measurement of autophagic flux by adding lysosomal inhibitors to whole blood, which is physiologically intact, will yield a faithful measure of autophagy in PBMCs (or other blood cell populations). However, several important questions remain. We must determine the correlation between autophagic

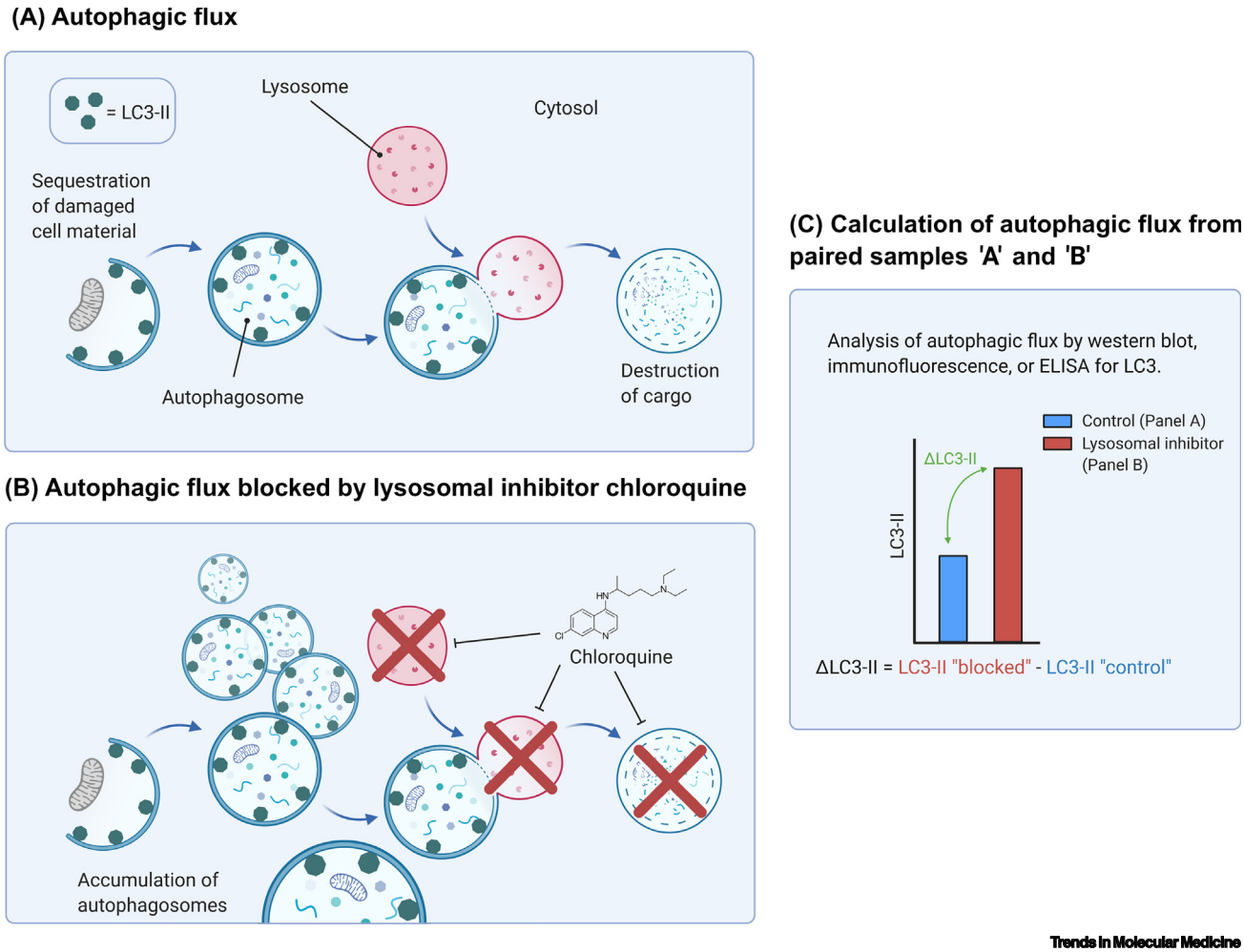

Figure 1. Autophagic flux and its measurement using a lysosomal inhibitor. (A) Damaged material that is sequestered inside an autophagosome fuses with the lysosome for eventual destruction. Autophagosomes contain the autophagy protein LC3-II, which is also degraded upon lysosomal fusion. (B) If a lysosomal inhibitor is added to a paired replicate sample that is run in parallel, the rate of increase of the autophagy protein LC3-II can be used to measure autophagic flux. (C) The autophagic flux is calculated as ΔLC3-II = LC3-II 'blocked'- LC3-II 'control', where control is a paired untreated sample. Figure created with BioRender (BioRender.com).

flux in blood cell populations with other disease-relevant tissues (such as the brain). This could be explored in animal model systems under different conditions using mice that transgenically express ratiometric fluorescent probes, such as tandem fluorescent (mCherry-EGFP)-labelled LC3 [5]. Such findings would provide a better understanding of how autophagic flux measured in accessible cellular populations, such as those in blood, can inform about autophagy-related human conditions, including ageing, atherosclerosis, and Alzheimer's disease.

Additionally, given that we know almost nothing about autophagic flux in humans, research needs to address the most basic human characteristics. It is widely assumed that autophagic flux decreases with ageing. However, this assumption has not been directly tested in humans; neither have other relevant parameters, including sex or obesity. Genetics is also an important and complex variable: humans carry appreciable genetic variation in autophagic and lysosomal systems associated with neurodegenerative disease [12]. Elucidating the interaction between these key variables and autophagy will improve human study design, and inform how we can change autophagic flux using lifestyle and pharmacological interventions.

Finding the autophagometer

Although autophagic flux can be measured in humans using the methodology detailed in [8], the ultimate goal must be to identify a biomarker of autophagic flux. An autophagy biomarker would permit two significant advancements: (i) retrospective characterisation of clinical specimen collections and biobanks;

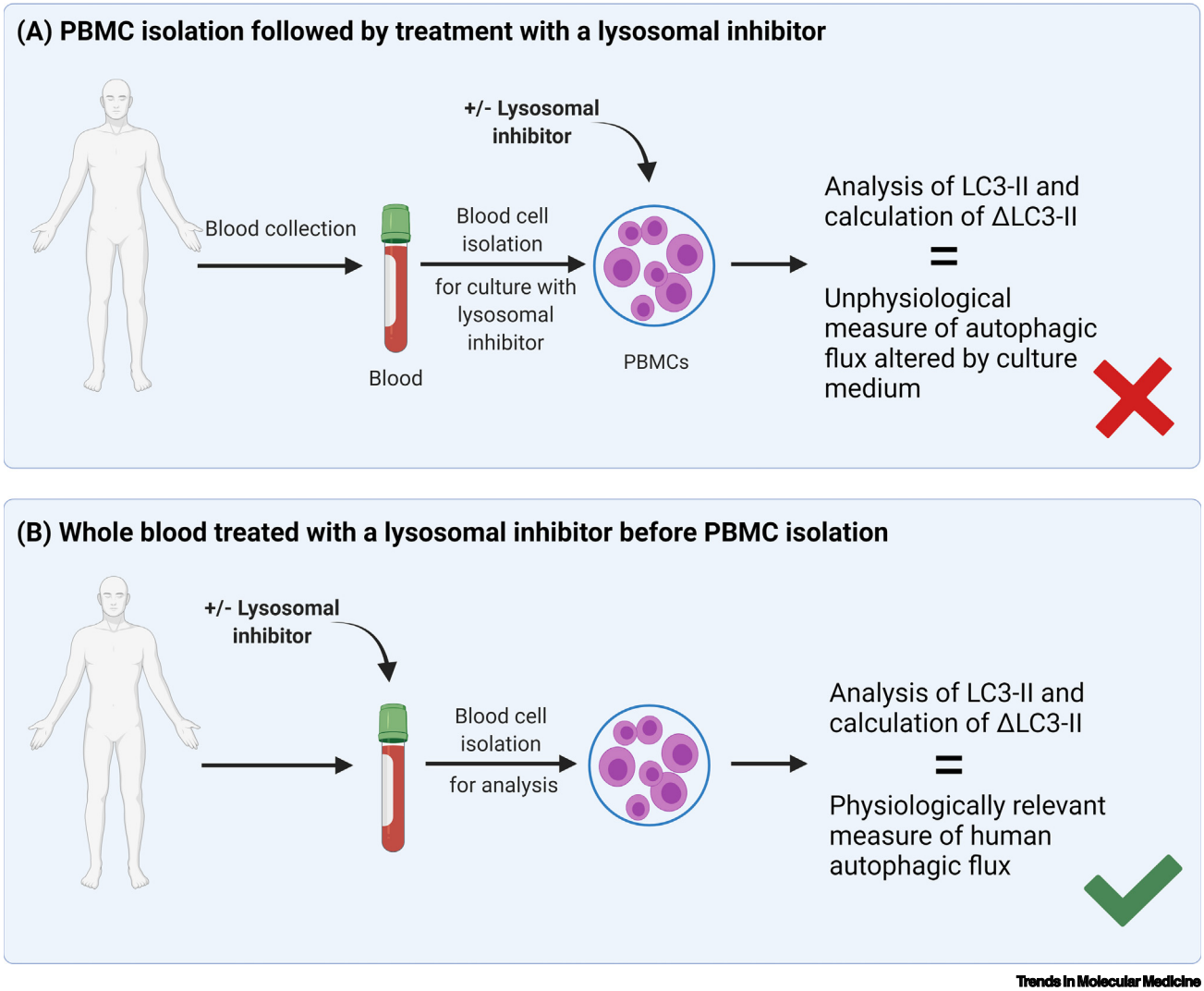

Figure 2. Strategies for measurement of autophagic flux in human blood. (A) Peripheral blood mononuclear cells (PBMCs) are isolated from blood and cultured *ex vivo* in artificial culture medium in both the presence or absence of a lysosomal inhibitor. (B) Whole blood is treated and cultured *ex vivo* both in the presence or absence of a lysosomal inhibitor and PBMCs are then isolated. Figure created with BioRender (BioRender.com).

and (ii) technological developments to measure autophagy easily in a clinical setting. Clinical assessment of a patient's autophagy will allow practitioners to provide interventions or advice to change it to promote healthy ageing.

Identifying biomarkers from cell and animal models in which autophagy is genetically or chemically modified could be accomplished using proteomics and metabolomic analysis of biofluids. A major advantage of an -omics approach is a nonbiased identification of targets; however, initially, exosomal proteins are excellent candidates because secretory exosomes come from within the lysosomal system itself. These hits would subsequently require validation in human cohorts by correlating identified molecules with autophagic flux measured in whole blood (Figure 2B), only now possible with newly developed methodology [9].

Concluding remarks

The tools to measure physiological autophagic flux in humans are now available and need to be applied to understand how autophagy behaves in human ageing and age-related disease. However, these methods do not allow measurement of autophagic flux in tissues other than blood, such as the brain. Given that our current understanding of autophagy in humans is very limited, any research to address these shortcomings will yield important

insights and take us closer to translating decades worth of basic autophagy research.

Declaration of interests

T.J.S. and J.B. are listed as inventors on a related patent, PCT/AU2020/050908.

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