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Short Review: Special Edition

Apicomplexan autophagy and modulation of autophagy in parasite-infected host cells

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ABSTRACT

Apicomplexan parasites are responsible for a number of important human pathologies. Obviously, as Eukaryotes they share a number of cellular features and pathways with their respective host cells. One of them is autophagy, a process involved in the degradation of the cell's own components. These intracellular parasites nonetheless seem to present a number of original features compared to their very evolutionarily distant host cells. In mammals and other metazoans, autophagy has been identified as an important contributor to the defence against microbial pathogens. Thus, host autophagy also likely plays a key role in the control of apicomplexan parasites, although its potential manipulation and subversion by intracellular parasites creates a complex interplay in the regulation of host and parasite autophagy. In this mini-review, we summarise current knowledge on autophagy in both parasites and their host cells, in the context of infection by three *Apicomplexa*: *Plasmodium*, *Toxoplasma*, and *Theileria*.

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Autophagy in *Apicomplexa*

The core machinery for autophagy is evolutionarily conserved in most of the eukaryotic phyla, however *Plasmodium*, *Toxoplasma* and *Theileria* possess a reduced repertoire of recognizable autophagy-related proteins. Except in *Toxoplasma*, they noticeably lack clear orthologues of the initiating kinase ATG1/ULK1/2, and all lack proteins involved the nucleation of autophagosomes [Table 1]. Apicomplexan parasites also lack the equivalent of mammalian lysosomes, so they rather resemble fungi and plants by degrading autophagosome cargo in vacuoles with a proteolytic function. For example, in *Plasmodium*-infected red blood cells, autophagosomes fuse with the digestive food vacuole that is better known for degrading haemoglobin that the parasite imports from the erythrocyte cytosol [1]. *Plasmodium* sporozoites and merozoites are the developmental stages invasive for hepatocytes and erythrocytes, respectively, but they do not possess a food vacuole. However, it has been proposed that post-invasion of hepatocytes, *Plasmodium berghei* ATG8-decorated micronemes (an invasion-related organelle) are expelled from the parasite and degraded by enzymes present in the parasitophorous vacuole (PV) lumen [2]. Proposing the PV as a degradative compartment is an interesting concept, as invasive *Toxoplasma* tachyzoites leave behind a residual body of unused material after their division by endodyogeny, which vanishes quite rapidly as parasites develop in the vacuole. Therefore, the PV might be an important interface between the parasite and its host cell for nutrient acquisition, where import of autophagosome-recycled parasite material from the lumen back into the parasite might be facilitated. One should point out that post-invasion of leukocytes or erythrocytes, *Theileria* parasites reside only very transiently within a PV that is rapidly degraded, leaving the parasites exposed to the host cell cytosol [3]. If secretory autophagy occurs, then lysosomes in the host cell cytosol could be the digestive compartment for *Theileria*-derived autophagosome cargo.

Autophagy in *Plasmodium* parasites

A better understanding of autophagy regulation in malaria-causing *Plasmodium* species has taken on renewed urgency due to the recent description of artemisinin-resistance mutations occurring in *Plasmodium falciparum Atg18 (PfAtg18)* [4]. In addition, previously, resistance to another anti-malaria drug (chloroquine) was associated with alterations in PfATG8 distribution [5]. Although PfATG18 has not yet been characterised, studies on PfATG8 are well documented (14 papers in PubMed). Particularly, a surprisingly common observation was the localisation of PfATG8 on a non-photosynthetic plastid, present in most apicomplexan parasites, called the apicoplast [6,7]. This led to the proposition that PfATG8 has a non-canonical function in apicoplast biogenesis [1] or, since apicoplasts also bind Phosphatidylinositol 3-phosphate (PI3P) produced by Vps34, its membrane might be the site of phagophore formation [2]. Once formed, the maturation of autophagosomes is associated with them becoming decorated with PfRab7, and then fusing with the food vacuole for degradation of their cargo [1]. PI3P binds to FYVE-domains [8] and the single parasite FYVE domain-containing protein also locates to the food vacuole [9], where it might participate in fusion of the autophagosome with the food vacuole membrane. The function of autophagy in *Plasmodium* blood stages is largely unexplored, but one proteomic study suggested that PfATG8 could be involved in parasite ribophagy and piecemeal microautophagy of the nucleus [10].

In the absence of a recognizable ATG1 orthologue (see Table 1, [11]) it's intriguing as to how malaria parasites regulate the initiation of autophagy and one can only hypothesize that another unidentified parasite kinase activity might play an ATG1-like role. Clearly, little is known and one possibility is that post-translational modifications of ATG proteins play a dominant role in regulating autophagy. In Table 1 we have indicated the phosphorylation status collated from PlasmoDB (http://plasmodb.org) of the different PfATG proteins and most are phosphorylated at more than one site. One can see that only PfATG5, PfATG7 and PfATG12 are not phosphorylated in infected red blood cells. cAMP-dependent protein kinase A (PKA) likely plays an important regulatory role, as PfATG4 (T625), PfATG8 (T83), PfATG11 (S243, S465), PfVps34 (T47, S90, S1036, S1362), PfVps15 (S250) and PfRab7 (S72) are all phosphorylated *in vivo* at typical PKA sites. Other phospho-sites and the two in PfATG18 (S42, S375) are not typical of PKA suggesting that additional parasite kinases must be responsible. Clearly then, kinases and phosphatases are likely key players in the regulation of parasite autophagy.

Autophagy in *Plasmodium*-infected host cells

Although autophagy is well studied as reticulocytes develop into normocytes, a process during which organelles including the nucleus are eliminated during erythropoiesis [12], little is known about host cell autophagy in *Plasmodium*-infected mature erythrocytes. However, in *P. berghei*-infected hepatocytes the PV membrane (PVM) is decorated with LC3 ("microtubule-associated protein 1A/1B-light chain 3", the mammalian orthologue of ATG8), ubiquitin, SQSTM1/p62 and lysosomes in a process resembling selective autophagy [13]. As *P. berghei* development is dampened in host hepatocytes deficient in autophagy, it gave rise to the proposition that host cell autophagy was occurring at the PVM to supply the parasite with nutrients necessary for optimal growth [13,14]. Moreover, in human hepatocytes infected with *Plasmodium vivax,* interferon-gamma (IFN-γ) stimulation also enhances LC3 and lysosome recruitment to the PVM [15]. However, this IFN-γ mediated induction of autophagy seemed detrimental to liver-stage *P. vivax* infection, in contrast to the role described promoting *P. berghei* development [13,14]. Moreover, IFN-γ mediated induction of autophagy appeared non-canonical, as it did not involve activation of the mammalian ATG1 orthologue ULK1. Thus, during liver stage infection the parasite provokes hepatocyte autophagy to help it grow, while the host appears to respond to infection by IFN-γ stimulated autophagy to eliminate the parasite.

Table 1 Putative homologues of *Saccharomyces cerevisiae* Atg proteins in *Theileria*, *Plasmodium* and *Toxoplasma*. Yeast sequences were used as a BLAST query in PiroplasmaDB.org, PalsmoDB.org, and ToxoDB.org, respectively. Evidence of *in vivo* phosphorylation was also searched for in the PlasmoDB.org and ToxoDB.org databases. *nf*: no homologue found,*: distant homologue (e-value >10e^{-5}).

Functional group	Yeast protein	*T. annulata* orthologue	*P. falciparum* orthologue	Phosphorylation	*T. gondii* orthologue	Phosphorylation	Features and possible function
Atg1 complex	Atg1	multiple possible hits	multiple possible hits		TGME49_316150*	No	Ser/Thr protein kinase; regulated by the TOR complex; recruitment of Atg proteins to the phagophore assembly site
	Atg13	*nf*	*nf*		*nf*		Regulatory subunit through phosphorylation by TORC1 or PKA
	Atg17	*nf*	*nf*		*nf*		Scaffold protein
	Atg29	*nf*	*nf*		*nf*		Ternary complex with Atg17 and Atg31; not found in mammals
	Atg31	*nf*	*nf*		*nf*		Ternary complex with Atg17 and Atg29; not found in mammals
	Atg11	*nf*	PF3D7_0216700*	Yes	*nf*		Scaffold protein for phagophore assembly in selective autophagy; not found in mammals
Atg9 and its cycling system	Atg2	*nf*	*nf*		TGME49_304630	Yes	Interacts with Atg18
	Atg9	*nf*	*nf*		TGME49_260640	Yes	Transmembrane protein, possible membrane carrier for phagophore formation
	Atg18/A tg21	TA03100	PF3D7_1012900	Yes	TGME49_288600 TGME49_220160	Yes	PtdIns3P-binding protein; potentially involved in driving membrane elongation
PtdIns3K complex	Vps34	TA20360	PF3D7_0515300	Yes	TGME49_215700	Yes	PtdIns 3-kinase
	Vps15	TA04815	PF3D7_0823000	Yes	TGME49_310190	Yes	Ser/Thr protein kinase
	Vps30/Atg6	*nf*	*nf*		TGME49_221360	Yes	Component of the PtdIns3K complex
	Atg14	*nf*	*nf*		*nf*		Component of the PtdIns3K complex
Atg8 and Atg12 Ubiquitin-like conjugation systems	Atg8	TA03605	PF3D7_1019900	Yes	TGME49_254120	No	Ubiquitin-like; conjugated to PE at the autophagosome membrane; involved in autophagosome cargo recognition and possibly in membrane elongation
	Atg7	TA06610	PF3D7_1126100	No	TGME49_229690	Yes	E1-like enzyme
	Atg3	TA03605	PF3D7_0905700	Yes	TGME49_236110	Yes	E2-like enzyme
	Atg4	TA13550	PF3D7_1417300	Yes	TGME49_206450*	Yes	Cysteine protease; deconjugating enzyme for Atg8
	Atg12	TA11895*	PF3D7_1470000	No	TGME49_321300	Yes	Ubiquitin-like
	Atg10	*nf*	*nf*		*nf*		E2-like enzyme
	Atg16	*nf*	*nf*		TGME49_200280*	Yes	Interacts with Atg5 and Atg12
	Atg5	TA04165	PF3D7_1430400*	No	TGME49_230860*	Yes	Conjugated by Atg12
Other	Rab7	TA17640	PF3D7_0903200	Yes	TGME49_248880	Yes	Late endodomes/lysosomes marker; involved in the final maturation of late autophagic vacuoles in mammals

Autophagy in *Toxoplasma* parasites

Toxoplasma tachyzoites (rapidly dividing forms of the parasite) can generate autophagosome-like structures upon experiencing stress, for instance in the case of nutrient deprivation, both for extracellular [16] and intracellular [17] parasites. Electron microscopy imaging revealed the presence of cytoplasm-containing double-membrane autophagosomes and potential autophagolysosomes in starved parasites [16,17]. In *Toxoplasma*, GFP-fused TgATG8 was used to detect and quantify autophagic vesicles [16]: upon starvation, the protein re-localizes from the cytosol to punctate structures that by immuno-electron microscopy resemble autophagosomes. Noticeably, prolonged starvation triggers significant parasite mortality and leads to the disruption of the mitochondrial network in *Toxoplasma* tachyzoites. The fact that this can be prevented by the use of a chemical inhibitor of autophagy suggests that autophagic cell death could be involved [17]. Functional investigation of a *Toxoplasma* ATG9 homologue (a protein potentially important for the early steps of autophagosome formation), revealed a possible role for canonical autophagy in the parasites for surviving stress conditions, either as extracellular parasites or within host immune cells [18]. Altogether, these data suggest that canonical autophagy could be part of an integrated stress response pathway in *Toxoplasma*, although there is no clear demonstration of a fully functional parasite catabolic autophagy.

Surprisingly, under normal intracellular growth conditions, TgATG8 localizes to the membrane of the apicoplast [19,20], as described above for *Plasmodium*. This peculiar organelle harbours essential metabolic pathways, and cell lines deficient for TgATG8 [21] and related proteins TgATG3 [16] and TgATG4 [19] (that regulates TgATG8 membrane association), have converging phenotypes showing loss of both the apicoplast and parasite viability. This illustrates that part of the autophagy machinery is used for associating TgATG8 to the apicoplast, where it plays a vital role in organelle inheritance during cell division [21]. This important function appears clearly distinct from canonical autophagy and highlights that apicomplexan parasites may have subverted at least part of the machinery for performing a specialized non-canonical function [22].

Host cell autophagy for the control of *Toxoplasma*

Toxoplasma gondii is an obligate intracellular parasite that invades a wide range of vertebrate host cells. In these, autophagy has been identified as an important contributor to the defense against microbial pathogens (including viruses, bacteria and parasitic protists) [23]. Not only does autophagy allow the selective delivery of intracellular pathogens to the lysosomes for their degradation (a process called xenophagy), but microbial antigens generated through this process can also be used for the activation of innate and adaptive immunity. The recruitment of LC3 to single-membrane phagosomes surrounding intracellular bacteria has also been described recently and termed LC3-associated phagocytosis (LAP) [15,24]. This suggests that observing LC3 around pathogens can no longer by itself be taken as an evidence for the presence of autophagosomes, and that some other unconventional compartments involving autophagic markers might be involved in their elimination.

In mammalian cells, efficient control of *T. gondii* infections is achieved by IFN-γ, a cytokine that triggers the activation of a diverse array of effector pathways, including NO production, nutrient starvation, and the induction of immunity-related GTPase (IRG proteins - rodent-specific GTPases), or GBP (guanylate-binding proteins) proteins that damage the PV membrane [25]. In recent years, a number of reports have suggested a role for the host cell autophagy machinery in the control of *Toxoplasma* tachyzoites. In the mouse model, IRGs promote the elimination of *Toxoplasma* by associating with the PV in an IFN-γ-dependent way, leading to the disruption of its membrane, and exposure of the parasite to the host cytoplasm and its eventual elimination [26]. Early reports were already describing the recruitment of both IRGs and autophagy protein LC3 to the vicinity of the PV [27] [26]. Since then, numerous studies have convincingly shown that several members of the host autophagy machinery are important for IRG recruitment at the PV membrane and subsequent parasite clearance. Noticeably, ATG3, ATG5, ATG7, ATG12 and ATG16L1, which are all involved in the mechanism of LC3 conjugation to membranes, are also important for IFN-γ-inducible IRG localization to the PV membrane [28]. LC3 is thus likely the key player in this, and it was indeed confirmed that this protein, and also its homologues GABARAPL1 and GABARAPL2, are needed for targeting of the IRGs to the PV [29]. Once associated with the PV membrane, LC3 could 'tag' it for targeting by IRGs, and maybe even act as a scaffold to recruit the GTPases for subsequent parasite elimination [30]. However, to date there is no proof of a direct interaction between LC3 and IRGs. Nevertheless, it seems clear that this mechanism is independent of canonical autophagy, as interfering with the function of ULK1, ULK2, ATG9 and ATG14 did not impact on LC3 and IRG recruitment to the PVM, or subsequent elimination of parasites [30,31].

Noticeably, human cells do not express IRGs, but GBPs, which can also be recruited to the PVM through the LC3 conjugation system [31]. However, GBPs are not essential for IFN-γ-dependent parasite elimination and a recent study in human cells has shown core autophagy proteins involved in LC3 conjugation are important in the control of parasite growth in a different way: in this case, they seem to be required for a process that results in wrapping the PV in multiple host membranes to limit parasite growth [32].

CD40 [a member of the tumor necrosis factor receptor superfamily] signaling has also been shown to trigger autophagic elimination of *T. gondii* independently of IFN-γ and IRGs [33–36]. This anti-parasitic activity also depends on proteins of the autophagy machinery, and the sequestration and degradation of intracellular tachyzoites occurs possibly through classical autophagy, although this remains to be more firmly established.

Finally, in parallel with the implication of host autophagy in the control of intracellular microbes, there is also a growing list of pathogens that seem to be able to antagonize the host autophagy machinery, or even exploit it to enhance their replication. A couple of reports suggest *Toxoplasma* might be able to do so in

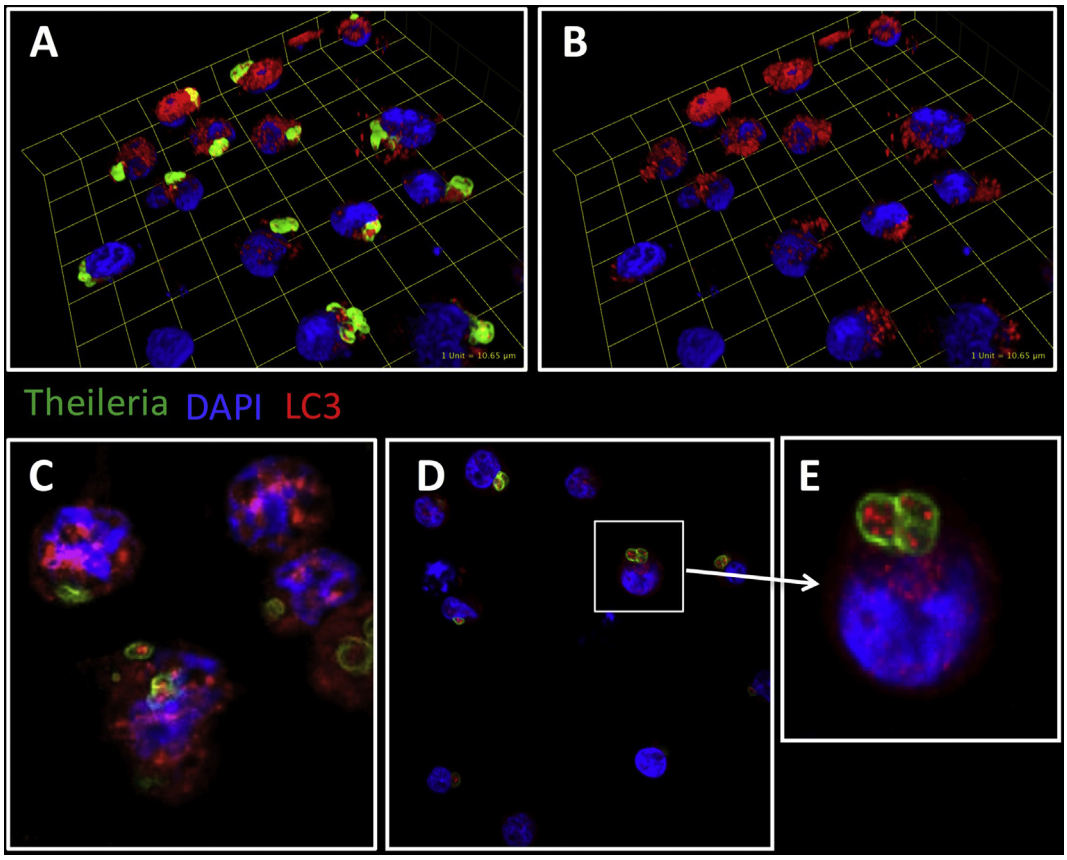

Fig. 1 **LC3 can be detected around and inside *Theileria annulata*-infected macrophages.** Visualisation of intracellular parasites (green; 1C12 monoclonal antibody) [56] and LC3 (red; rabbit polyclonal anti-LC3α/β antibody that recognises endogenous host LC3 [Santa Cruz Biotechnology, cat. No sc-292354] and Alexa Fluor 594-conjugated anti-rabbit Ig antibody) in *T. annulata*-infected macrophages. DAPI was used to counter-stain macrophage nuclei. The 3D-representations of spatial co-localizations of parasite and LC3 (A), as revealed by the removal of parasite staining (B). Confocal microscopy (C, D) also shows LC3 staining within the parasite, magnified in (E). Images were acquired by Spinning Disk Confocal Microscopy and analysed by ImageJ and Volocity software.

the case of permissive and non-immune host cells. For example, *T. gondii* can activate the epidermal growth factor receptor (EGFR)/Akt pathway to prevent the host autophagy machinery from targeting the parasite for lysosomal degradation [37]. Moreover, *T. gondii* is able to induce a significant recruitment of the autophagy machinery in HeLa cells or primary fibroblasts, where genetic inactivation of the autophagic function limits parasite growth, suggesting a beneficial role for host cell autophagy in the development of the parasites [38].

In conclusion, a clearance function of the autophagy machinery enhances pathogen killing in host cells that have been activated for anti-parasitic function, while in permissive host cells tachyzoites may co-opt the autophagy machinery for their own benefit.

Modulation of autophagy by *Theileria*: at the crossroads of infection, immunity and tumorigenesis

Theileria belongs to the same *Apicomplexa* phylum as *Plasmodium* and *Toxoplasma*, but it has developed distinct mechanisms in its parasitic life cycle that make these parasites unique among the known *Apicomplexa* [39]. Unlike *Toxoplasma* and *Plasmodium*, *Theileria* parasites do not reside within a PV within host leukocytes, so are exposed to the host cell's autophagy machinery [3,40]. Nevertheless, the host autophagy machinery does not appear to react to the presence of the parasite that surprisingly persists in the cell in an almost 1:1 leukocyte:parasite ratio. Thus, even when *Theileria* is somewhat exposed due to the absence of the PV, infected host cell autophagy is not induced [41]. How these parasites are able to avoid cell autophagy to survive in infected leukocytes is still unclear. Recent work suggests that *Theileria* might block some of the autophagy pathways in the infected host cell, because when *Theileria* is killed with an anti-parasite drug, dead parasites are immediately engulfed by LC3-positive structures (Latré de Laté and Pineda, unpublished).

Another extraordinary difference to *Plasmodium* and *Toxoplasma* is that *Theileria* transforms its host cells into an immortalized, highly proliferative and disseminating phenotype, properties shared by many cancer types [39]. This is presumably achieved through mechanisms triggered by the parasite to manipulate leukocyte signal transduction pathways that are also relevant for tumorigenesis. Indeed, in infected

macrophages, *Theileria annulata* maintains a Hypoxia Inducible Factor (HIF)-1α-driven transcriptional programme typical of Warburg glycolysis [42], a hallmark of cancer cells. Thus, *Theileria* infection represents a valuable model for studying cancer and lymphoproliferation. In this regard, defects in autophagy have been associated with increased tumorigenesis in some cancers [43], so perhaps *Theileria*'s regulation of host autophagy is the mechanism underlying parasite survival and leukocyte transformation, events that would not be independent, but closely intertwined, although the exact mechanisms are still unknown. *Theileria* induces the oncomiR miR-155, via the c-Jun transcription factor and AP-1 activity [44]. In turn, miR-155 modulates cell autophagy, promoting it to clear intracellular mycobacteria [45], or suppressing it during osteoarthritis and cancer [46,47]. Furthermore, cAMP-PKA signalling is upregulated by *Theileria* infection [48] and it is well established in different cell types that cAMP-PKA signalling negatively regulates autophagy (see accompanying review and [49–52]). cAMP-PKA-mediated phosphorylation of LC3 blocks autophagy. This is mimicked when cAMP production is stimulated by forskolin (an adenylate cyclase activator) and prevented by PKA inhibitor H89. Under conditions of costimulation, LC3 phosphorylation is diminished, leading to autophagy [49]. As augmented cAMP-PKA signaling is characteristic of *Theileria*-infected leukocytes, increased LC3 phosphorylation can be observed and this likely contributes to infection, perhaps inducing blockade in the host's autophagy response. It is also well known that *Theileria* infection induces increased JNK kinase activities [53,54]. Recently it has been shown that loss of JNK2 leads to accumulation of smARF and lysosomal degradation of the adaptor p62 (sequestosome-1, SQSTM1) [55] implying that constitutive induction of JNK2 following *Theileria*-infection could also contribute to the observed stable levels of p62 that reflect a blockade of the host leukocyte's autophagic response. Consistent with this notion, one can observe a decrease in p62 expression only when infected leukocytes are treated with a pro-oxidant anti-parasite drug (Latré de Laté and Pineda, unpublished). Interestingly, when expression of LC3 and phosphorylated LC3 (p-LC3) is evaluated in infected macrophages, a positive staining is also observed within the parasites [Fig. 1]. Whether this is a result of parasite uptake of host LC3 is still unclear and requires further investigation, but it strongly suggests that host and/or parasite autophagy play a relevant role in parasite survival within the infected cell, and perhaps by sequestering host LC3 within itself, *Theileria* inhibits autophagy.

In conclusion, to survive, it appears that while exposed within the cytoplasm of host leukcoytes *Theileria* parasites might modulate the induction of autophagy by targeting at least three different steps in the autophagy induction process: cAMP-PKA-mediated phosphorylation of LC3, JNK2-mediated blockade in p62 degradation, and LC3 sequestration. Understanding how *Theileria* regulates host autophagy will provide novel pathways to improve our current knowledge of autophagy both in infection and in cancer.

Conflicts of interest

The authors have no conflict of interest to declare regarding this manuscript.

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