Most Autophagic Cell Death Studies Lack Evidence of Causality

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KEYWORDS

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ABSTRACT

Autophagy plays a critical role in maintaining cellular homeostasis and is implicated in various physiological and pathological processes, including cancer, neurodegeneration, and metabolic disorders. Although typically associated with cell survival, autophagy has also been proposed to contribute to cell death, referred to as autophagic cell death (ACD). Despite its importance, the identification of ACD remains contentious due to inconsistencies in experimental methodologies and terminological misuse. In this study, we evaluated 104 research articles published in 2022 that claimed to demonstrate ACD. Articles were assessed based on established criteria, including evidence for autophagy, evidence for cell death, exclusion of apoptosis, and experimental designs demonstrating causality. Our findings reveal that only 12.5% of the articles fulfilled all the criteria for ACD, while 37.5% provided only correlation-level evidence. Additionally, 32.7% of the articles relied on viability loss rather than direct evidence of cell death, and 45.0% of studies utilizing autophagy inhibition to prove causality failed to demonstrate actual inhibition of autophagy. Inconsistent terminology was also prevalent, with cases of ‘autophagy-mediated cell death’ being incorrectly classified as ACD and instances where the term ACD was misused as an umbrella term to describe the coexistence of autophagy and cell death, which should have been labeled ‘autophagy-associated cell death.’ These issues highlight a lack of rigor in current practices, with reliance on correlation-level evidence and inappropriate experimental designs undermining the robustness of many studies. This analysis underscores the need for greater rigor in experimental approaches and article revision processes.

INTRODUCTION

Autophagy is a physiological process in which cellular components such as proteins and organelles are recycled via lysosomal activity. This process is characterized by the engulfment of the target cargo by autophagosomes, which then fuse with the lysosomes to break down complex molecules and structures into simple building blocks. Autophagy serves as both a catabolic pathway triggered as a response to starvation to provide cells with nutrients, and a cytoprotective mechanism that eliminates potentially harmful substances, such as misfolded proteins, dysfunctional mitochondria and pathogens. Due to its critical role in cellular homeostasis, disruption of the autophagy machinery, resulting in either excessive or insufficient autophagy, can lead to undesirable outcomes for the cell, such as cell death or neoplastic transformation. These disruptions can have severe consequences at the organismal level, potentially leading to malignant, neurodegenerative, or metabolic disorders [1].

The role of autophagy in cell death is complex and not as straightforward as one might expect from a cytoprotective pathway. Autophagy has been proposed as an alternative programmed cell death mechanism, where cells lose the ability to maintain themselves due to excessive self-digestion, leading to their demise [2]. However, given its apparent role in cell survival, discussions about its true role in cell death have been controversial at best [3,4]. Excessive autophagy has been observed in many cases of cell death, raising the question of whether cells are dying by autophagy (Autophagic Cell Death, ACD) or are activating autophagy as a survival strategy in an attempt to avoid death (Autophagy-Associated Cell Death) [5]. Shen and Codogno proposed following criteria to identify autophagic cell death: increased autophagic influx, prevention of death by suppression of autophagy, and exclusion of apoptosis [6]. Similarly, the Nomenclature Committee on Cell Death (NCCD) specified that autophagic cell death should only be identified if inhibition of autophagy suppresses the cell death [7].

More recently, the terms ‘autophagy-mediated cell death’ (where excessive autophagy triggers apoptosis or another type of cell death) and ‘autophagy-dependent cell death’ (where autophagy itself is the primary cause of cell death) have been introduced to distinguish between different mechanisms and clarify the role of autophagy in these processes [8,9]. However, since excluding other modes of cell death is one of the criteria for identifying ACD, the concept of ‘autophagy-dependent cell death’ essentially becomes synonymous with ACD [10]. Furthermore, within the spectrum of ACD, there is a specific form known as autosis, which is characterized as an autophagy-dependent, non-apoptotic cell death process with distinct morphological and biochemical features [11].

Even though it has been more than a decade since autophagic cell death was first distinguished from autophagy-associated cell death, and numerous follow-up reviews and guides have been published [10], it remains unclear whether current studies claiming to demonstrate autophagic cell death meet the established criteria and show a causal relationship between autophagy and cell death. In this study, we aimed to investigate the evidence provided in studies claiming to demonstrate ‘autophagic cell death’ and analyse how they interpret their findings.

RESULTS

1. Database Construction and Article Characteristics

We investigated a total of 137 articles from 94 different journals. After excluding 7 review articles, 21 articles that did not contain relevant claims, and 4 articles for which the full texts were not accessible, 104 articles from 76 different journals (75.9%) were included in the final analysis. Among the excluded articles, one with an 'autophagy-associated cell death' claim (Article ID = 10; see Supplementary Document) was omitted as it correctly used the term and provided correlation-level evidence, which does not meet the criteria for an autophagic cell death claim.

We entered all relevant information regarding these articles to an online spreadsheet (Airtable) creating a database for our analysis (see Supplementary Document for the complete database).

98 out of 104 (94.2%) articles contained ‘autophagic cell death’ (ACD) claim, 5 (4.8%) contained ‘autophagy-mediated cell death’, 3 (2.9%) contained ‘autophagy-associated cell death’, 3 (2.9%) contained ‘autosis’ and 2 (1.9%) contained ‘autophagy-dependent cell death’ claims. There were 7 (6.7%) articles with multiple claims in their manuscripts. It is important to note that, except for the omitted article (ID=10), in all 3 instances where authors used ‘autophagy-associated cell death’ they also used ‘autophagic cell death’ as if they were synonyms (see Supplementary Document).

2. Evidence for autophagy

Surprisingly, 8 out of 104 articles (7.7%) failed to provide appropriate evidence for autophagy. Among the remaining 96 articles, which provided evidence, the vast majority (n=88) employed LC3-II measurement in combination with other methods to monitor autophagy. Other commonly used strategies included demonstrating a reduction in p62/SQSTM1 levels (n=49) and visualizing autophagic vacuoles (n=27) through various techniques (see Supplementary Document).

3. Experimental Design to Demonstrate Autophagic Cell Death

Of the 104 articles, 60 (57.7%) lacked specific experimental designs to distinguish autophagic cell death (Figure 1A). Among these, 10 provided no evidence at all, and 11 demonstrated autophagy induction alone as evidence for ACD without any supporting data. The remaining articles without specific experimental designs produced correlation-level evidence. In these studies, while demonstrating autophagy correlating to cell death, 12 articles relied on viability loss to indicate cell death, and 27 actually measured cell death (Figure 1A).

In the remaining 40 articles (38.5%), there were experiments specifically designed to reveal the causal relationship between autophagy and cell death. However, since in 22 of these articles there was a reliance on viability loss instead of actual cell death measurement, only 22 remaining articles (21.2% of all articles) provided actual causality-level evidence for ACD (Figure 1A).

The most common strategy to prove causality was to inhibit autophagy and observe whether cell death or viability loss was prevented, a method used in 39 articles (Figure 1D). While 3 articles employed the opposite approach by activating autophagy and measuring the increase in cell death, in 6 articles (5.8%) both strategies are utilized to provide more robust evidence of causality (Figure 1D).

Of the 40 articles where autophagy was inhibited to prove causality, 33 relied solely on small molecule inhibitors, while 1 opted for genetic inhibition. 6 articles utilized both small-molecule and genetic-based inhibition to achieve more reliable results (Figure 1E). It is important to note that more articles used autophagy inhibitors overall. In total, small molecule inhibitors were used in 48, genetic inhibition was used in 5, both approaches together were used in 8 articles (see Supplementary Document). However, despite utilizing autophagy inhibition, 11 of these 61 articles did not measure viability and/or cell death following the inhibition, and thus failed to provide causality-level evidence.

Among all studies using small molecule inhibitors of autophagy, 3-methyladenine (3-MA) was the most popular choice, used in 27 studies, followed by chloroquine in 26 studies and Bafilomycin A1 in 12 studies (see Supplementary Document). Four studies used non-specific inhibitors that are not listed in the 'Genetic and Pharmacological Regulation of Autophagy' table in the 'Guidelines for the Use and Interpretation of Assays for Monitoring Autophagy' [12]. Additionally, 13 studies employed more than one type of small molecule inhibitor, with 8 of them utilizing 3-MA and chloroquine (see Supplementary Document). In 18 of the studies, inhibition of autophagy was not demonstrated experimentally, thereby diminishing the robustness and reliability of their conclusions. Finally, only 27 out of 40 articles reported less than 50% rescue of cells from death after autophagy inhibition, suggesting that while autophagy may contribute to cell death, it may not the primary mechanism (see Supplementary Document).

4. Non-apoptotic evidence of cell death

Out of 104 articles, 14 (13.5%) failed to provide any evidence for cell death (Figure 2A). 36 articles (34.6%) demonstrated only viability loss and attributed that loss to cell death without further proof. Remaining 54 articles provided evidence for loss of membrane integrity as an indicator of cell death (Figure 2A).

5. Experimental Design to Omit Apoptosis

Apoptosis was experimentally excluded in 26 articles (25.0%) (Figure 1C and 2B), with 16 of these articles simply demonstrating its absence. In contrast, 10 studies showed that apoptosis inhibitors were ineffective in preventing cell death and/or viability loss (Figure 2B). Amongst articles experimentally excluding apoptosis, 13 demonstrated a causal relationship between autophagy and cell death (Figure 1C).

6. Presence and Influence of Apoptosis, and Other Claims.

Apoptotic status was unknown in 22 articles (21.2%) (Figure 2C). As mentioned earlier, 26 articles demonstrated that apoptosis either did not contribute to cell death or viability loss, or was completely absent.

In 8 articles, the inhibition of apoptosis prevented cell death and/or viability loss (Figure 2C), indicating that apoptosis was the primary mechanism of cell death. However, in 7 of these 8 studies, inhibition of autophagy also prevented cell death and/or viability loss (see Supplementary Document), suggesting that the mechanism should have been identified as autophagy-mediated apoptosis. While 2 of these articles accurately labelled the process, the remaining studies incorrectly referred to it as 'autophagic cell death'.

Additionally, 3 other articles claimed ‘autophagy-mediated cell death’ but only demonstrated a correlation between autophagy and cell death, making ‘autophagy-associated cell death’ a more appropriate term in these cases (Supplementary Document). In summary there were 5 articles claiming ‘autophagy-mediated cell death,’ but only 2 of them presented evidence that justified this classification.

Moreover, 11 articles demonstrated that inhibiting autophagy also inhibited apoptosis, a clear indication of 'autophagy-mediated apoptosis'. However, all were incorrectly identified as 'autophagic cell death' by the authors. Similarly, 3 articles demonstrated that activating autophagy further intensified apoptosis which was again incorrectly identified as 'autophagic cell death' (see Supplementary Document).

2 articles claimed ‘autophagy-dependent cell death’, and both lacked the necessary supporting evidence. There were 3 articles claiming ‘autosis’, of which only 1 provided sufficient evidence for ACD (Figure 2D). Since the identification of ‘autophagy-dependent cell death’ and ‘autosis’ also requires satisfying the established criteria for ACD, we analysed these claims accordingly.

7. Reclassification of Autophagy-Related Cell Death Claims Based on Evidence Provided

The graph (Figure 2E) illustrates the distribution of articles according to the appropriate terminology assigned based on the evidence they provided, rather than the claims explicitly made by the authors. Specifically, it highlights the number of articles that should have been classified under the terms ‘autophagic cell death’, ‘autophagy-mediated cell death’, or ‘autophagy-associated cell death’, based on their experimental evidence. Specifically, ACD claims were accepted only when a causal relationship between autophagy and cell death was demonstrated, alongside the omission of apoptosis, as recommended by established guidelines [6,8,9,12]. This data can also be seen in Figure 1C. The graph further distinguishes between studies relying on cell death evidence (blue bars) and viability loss data (pink bars). This differentiation underscores the significant reliance on less stringent criteria by nearly half of the studies, as viability loss does not necessarily equate to direct cell death. It is important to underline that since we only included studies with a type of ‘autophagic cell death’ claim, in ideal conditions the number of articles re-classified as ‘autophagy-associated cell death’ should have been zero. It is also important to note that 21 studies provided no evidence for any type of claim and are therefore not represented in the graph (see Supplementary Document).

DISCUSSION

Autophagy is a crucial mechanism with implications for a wide range of physiological and pathological processes, including cancer, neurodegenerative diseases, and immune responses. Despite its importance, the precise nature of autophagy as a cell death mechanism remains a topic of intense debate, making the accurate identification of autophagic cell death (ACD) essential for advancing research in this field.

In this study, we systematically evaluated 104 research articles that claimed to demonstrate ACD, uncovering significant limitations in the experimental designs utilized. While less than half of the articles presented evidence suggesting a causal relationship between autophagy and cell death, fifteen of these relied on viability loss rather than direct measurements of cell death, leaving only 22 (21.2%) studies that demonstrated autophagy as a cause of cell death. However, according to established guidelines, the exclusion of apoptosis is also a crucial criterion for accurately identifying ACD [6,8,9,12]. When this criterion was applied, only 13 articles (12.5%) met all the necessary requirements, highlighting a substantial gap between claimed and verified evidence.

However, a counter-argument could suggest that the authors may have used the term 'ACD' as a shorthand to indicate the correlation between autophagy and cell death, even if such a practice is not recommended and the term ‘autophagy-associated cell death’ should have been used instead [6], [12]. Nonetheless, even if this were the case, our analysis revealed 10 articles with no evidence whatsoever, 11 articles that demonstrated only autophagy without providing any information on cell death, and an additional 12 articles that relied solely on viability loss rather than direct evidence of cell death.

Notably, the previously proposed nomenclature does not appear to be widely adopted by researchers. The vast majority of claims referred to ACD, while the usage of other terms was quite rare. For instance, only four articles (one of which was omitted as it did not include autophagic cell death claim) used the term ‘autophagy-associated cell death’ but in reality, more studies demonstrated ‘autophagy-associated cell death,’ which requires only correlation-level evidence. There were a handful of other claims including five ‘autophagy-mediated’, three ‘autosis’, and two ‘autophagy-dependent’ cell deaths, with only four articles providing the necessary evidence (two for ‘autophagy-mediated’ and one for ‘autosis’). When examining the actual evidence provided by the studies, 27 demonstrated ‘autophagy-associated cell death’, 13 identified ‘autophagy-mediated cell death’, and 13 provided evidence for ‘autophagic cell death’ (Figure 2E).

In addition to challenges with nomenclature, our findings highlight concerning practices, which further complicate the interpretation of results, including the reliance on viability data instead of direct measurement of cell death, which was observed in 34 studies overall (32.7%) and in 22 studies which provided causality-level evidence (50.0%).

Other concerning practices were observed in studies involving autophagy inhibition. The most commonly used inhibitor was 3-methyladenine (3-MA), a non-specific phosphatidylinositol 3-phosphate inhibitor that blocks the early stages of autophagy [12] but can also promote autophagy in certain systems [14], and it was employed in 27 of 40 studies (67.5%). Practices to enhance robustness, such as using multiple inhibitors (13 of 40 studies, 32.5%) or combining genetic inhibition with small-molecule inhibitors (6 of 40 studies, 15.0%), were relatively uncommon. Additionally, 18 of 40 studies (45.0%) utilized autophagy inhibitors like 3-MA or chloroquine (CQ) without experimentally demonstrating the inhibition of autophagy. In cases where inhibition evidence was provided, it often involved an increase in LC3-II levels. While this is an acceptable indicator of inhibition with inhibitors like bafilomycin A or CQ that block LC3-II turnover [12], its dual use as a marker of autophagy induction in the same studies complicates the interpretation of autophagy inhibition. Lastly, in most of the 27 studies, autophagy inhibitors rescued fewer than half of the dying cells, suggesting that other mechanisms significantly contribute to cell death.

In summary, our analysis reveals significant inconsistencies and limitations in the current literature on autophagic cell death, particularly concerning experimental rigor and terminological precision. Only a small fraction of studies (12.5%) met established criteria for ACD, underscoring the need for more robust experimental designs and adherence to standardized guidelines. Addressing these shortcomings will not only advance our understanding of the role of autophagy in cell death but also reflects the broader responsibility of the scientific community, including authors and reviewers, to uphold rigor and precision in both research and its evaluation.

MATERIALS AND METHODS

1. Article retrieval and database creation

The following search string was used in Web of Science to retrieve articles published in 2022 that claimed to demonstrate autophagic cell death: ALL=("autophagy-associated cell death") OR ALL=("autophagic cell death") OR ALL=("type II cell death") OR ALL=("(type II) cell death") OR ALL=("autosis") OR ALL=("autophagy-mediated cell death") OR ALL=("autophagy-dependent cell death").

Information regarding each article, including its title, DOI number, the journal in which it was published, and details of the experimental designs and methods used, was exported to an online spreadsheet database (Airtable).

2. Investigation of the claims and exclusion criteria

We investigated each article to determine the specific claim made by the authors—whether it was autophagic cell death, type II cell death, autophagy-mediated cell death, or autophagy-associated cell death—and identified where and in what context these claims were made. We included only those articles where the claim of autophagic cell death was relevant to the main purpose and hypothesis of the study. Therefore, all of the included articles contained an intervention strategy (such as chemical treatment or RNA interference) which authors claimed to have induced autophagic cell death. Reviews and articles that merely mentioned autophagic cell death without substantial context were excluded.

3. Analyses of the evidence

The evidence of autophagic cell death was analysed on multiple levels by asking various questions derived from the criteria originally proposed by Shen and Codogno [6]. We sought to answer the following questions:

1. Is there sufficient evidence for autophagy?
2. Is there sufficient evidence for cell death?
3. Is apoptosis excluded?
4. Is there an experimental design to demonstrate autophagic cell death?

We evaluated all evidence presented in each study by investigating what was measured by using which method and whether the result of this measurement is a reliable indicator of the related claim (autophagy, cell death or apoptosis) according to the available guides. When multiple measurements were carried out to support a single claim, we focused on the strongest evidence. Additionally, we assessed whether the experimental design could effectively identify autophagic cell death.

Beyond these questions, we also investigated whether the data produced by the experiments were correctly interpreted.

3.1. Is there sufficient evidence for autophagy?

We determined the answer to this question in accordance with the ‘recommended methods for monitoring autophagy’ table presented in ‘Guidelines for the Use and Interpretation of Assays for Monitoring Autophagy’ [12]. Briefly, if visualization of autophagic vacuoles, measurement of LC3-II, and/or measurement of SQSTM1/p62 was performed, we considered it sufficient evidence for demonstrating a change in autophagy. However, other markers, including transcription-level expressional changes in Beclin1 and other autophagy-related proteins, spectrophotometric measurement of total acridine orange (and other acidotropic dye) signals as well as changes in the activity of signalling pathways such as mTOR and JNK alone, were considered insufficient.

3.2. Is there sufficient evidence for cell death?

Results obtained from methods measuring loss of membrane integrity, such as lactate dehydrogenase assays, dye exclusion tests, and specific markers were considered sufficient evidence to demonstrate cell death. Additionally, indicators of apoptosis, including Annexin-V/PI staining, caspase activity, PARP cleavage, and DNA fragmentation were considered sufficient evidence to demonstrate apoptosis. We distinguished apoptosis from non-specific cell death, as the presence of apoptosis indicates that the death may not be autophagic.

Results obtained from methods measuring viability loss, such as formazan-based or similar metabolic activity assays, measurement of total DNA or protein content, and counting the number of cells or colonies, were considered insufficient evidence for cell death. This is because viability loss may result from decreased proliferation rates rather than increased cell death [13]. However, to distinguish viability loss from no evidence, we included it in our findings as a separate entity.

We accepted Annexin-V/PI, live/dead assays, as well as other similar dual markers as non-apoptotic indicators of cell death as PI or other dead cell indicator component of such assays measures loss of membrane integrity.

3.3. Is apoptosis excluded?

We consider apoptosis to be excluded when its absence is proven and/or when there is an experimental approach to exclude it. Absence of apoptosis should be demonstrated via an acceptable method (listed in 3.2.) in the setting where cell death occurs. An acceptable experimental design should demonstrate that cell death persists despite the suppression of apoptosis through chemical (such as caspase inhibitors) or genetic (such as caspase siRNA) inhibition.

3.4. Is there an experimental design to demonstrate autophagic cell death?

Experiments where suppression of autophagy prevents cell death and/or further activation of autophagy increases cell death were considered as appropriate designs to identify autophagic cell death.

We considered targeting proteins and using small molecules listed on “genetic and pharmacological regulation of autophagy table” in “Guidelines for the Use and Interpretation of Assays for Monitoring Autophagy” [12] as appropriate strategies to inhibit autophagy. Other strategies such as small molecule inhibitors of non-specific signalling pathways and genetic targets that are not essential for autophagy were considered inappropriate.

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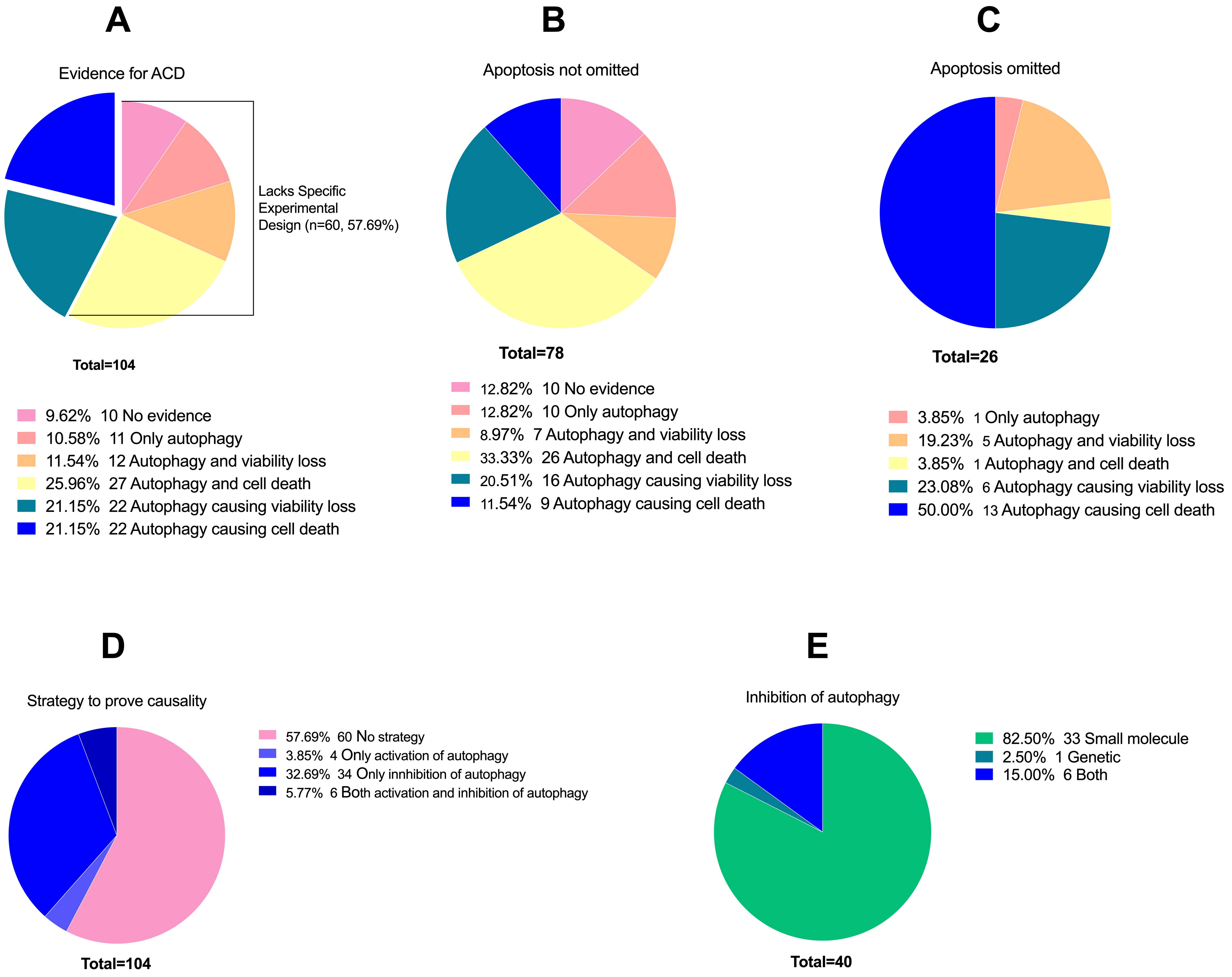
FIGURE LEGENDS

Figure 1. Distribution of articles based on evidence for autophagic cell death (ACD), strategies to prove causality, and types of autophagy inhibition.

(A) Distribution of articles according to the evidence provided for ACD, without considering the status of apoptosis. (B) Distribution of articles that did not omit apoptosis as a potential mechanism of cell death. (C) Distribution of articles that omitted apoptosis to meet the criteria for ACD. (D) Distribution of articles based on the strategies employed to prove causality. (E) Distribution of articles based on the type of autophagy inhibition performed. In all graphs, numbers indicate the number of articles meeting the specified criteria.

Figure 2. Distribution of articles based on evidence for cell death, strategies to omit apoptosis, effects of apoptosis, alternative claims, and evidence-based reclassification of claims.

(A) Distribution of articles according to the evidence provided for cell death. (B) Distribution of articles based on the strategies employed to omit apoptosis. (C) Distribution of articles according to the cellular effects of apoptosis. (D) Distribution of articles based on claims other than ACD. Blue bars represent claims with sufficient evidence, while pink bars represent claims with insufficient evidence. (E) Distribution of articles reclassified based on the appropriate terminology assigned according to the evidence provided. Blue bars represent articles relying on cell death, and pink bars represent articles relying on viability loss. In all graphs, numbers indicate the number of articles meeting the specified criteria.


A
Evidence for ACD
Lacks Specific Experimental Design (n=60, 57.69%)
Total=104
9.62% 10 No evidence
10.58% 11 Only autophagy
11.54% 12 Autophagy and viability loss
25.96% 27 Autophagy and cell death
21.15% 22 Autophagy causing viability loss
21.15% 22 Autophagy causing cell death
B
Apoptosis not omitted
Total=78
12.82% 10 No evidence
12.82% 10 Only autophagy
8.97% 7 Autophagy and viability loss
33.33% 26 Autophagy and cell death
20.51% 16 Autophagy causing viability loss
11.54% 9 Autophagy causing cell death
C
Apoptosis omitted
Total=26
3.85% 1 Only autophagy
19.23% 5 Autophagy and viability loss
3.85% 1 Autophagy and cell death
23.08% 6 Autophagy causing viability loss
50.00% 13 Autophagy causing cell death
D
Strategy to prove causality
57.69% 60 No strategy
3.85% 4 Only activation of autophagy
32.69% 34 Only innhibition of autophagy
5.77% 6 Both activation and inhibition of autophagy
Total=104
E
Inhibition of autophagy
82.50% 33 Small molecule
2.50% 1 Genetic
15.00% 6 Both
Total=40

A
Non-apoptotic Evidence of Cell Death
Total=104
13.46% 14 No evidence
34.62% 36 Viability loss
51.92% 54 Loss of membrane integrity
B
Omision of Apoptosis
Total=104
75.00% 78 No omission
15.38% 16 Apoptosis is absent
2.88% 3 Inhibition of apoptosis does not prevent viability loss
6.73% 7 Inhibition of apoptosis does not prevent cell death
C
Effects of Apoptosis
Total=104
21.15% 22 Status of apoptosis is unknown
25.00% 26 Apoptosis is absent or non-contributory
46.15% 48 Apoptosis is present but its impact is unknown
2.88% 3 Inhibition of apoptosis prevents viability loss
4.81% 5 Inhibition of apoptosis prevents cell death
D
Claims other than ACD
Autosis
Autophagy-dependent cell death
Autophagy-mediated cell death
0
1
2
3
4
Number of Articles
Sufficient Evidence
Insufficient Evidence
E
Evidence-based reclassification of claims
Autophagic cell death
Autophagy-mediated cell death
Autophagy-associated cell death
0
10
20
30
40
50
Number of Articles
Accepting only actual cell death data
Accepting viability loss as cell death