Autophagy as a novel therapeutic target in vascular calcification

Pharmacology & Therapeutics-PT-23593

Authors' response to reviewers' comments

Dear Dr. Curtis,

We are extremely grateful for your prompt dealing with our review article entitled 'Autophagy as a novel therapeutic target in vascular calcification'. We are pleased to submit the revised manuscript now for your consideration.

We thank the reviewers for their highly constructive comments and suggestions, and we have revised our manuscript accordingly. This has entailed editing and adding new text, which are highlighted in red. We have also listed each of reviewers' comments and provided our detailed response as below.

We believe our manuscript is much improved and we hope that it is now considered suitable for publication in Pharmacology & Therapeutics.

Yours sincerely,

On behalf of the authors,

Dr. Dongxing Zhu

Authors' response to reviewers' comments

Reviewer 1

1. Is the sense of it logical or is it clunky (example)?
   The Review is well written in a Logical way. In my view the Review would benefit from just focusing on media calcification (calcification on VSMC) rather than mixing it up with atherosclerosis and valvular calcification. It is often mixed in the text and thereby not logic to follow. Additionally, the Authors only mention VSMC calcification in their Abstract!

Authors' response: We thank the reviewer for their consideration of this point. We have removed the text in section 3.1 on endothelial cells, section 4.1 describing the role of autophagy in plaque necrosis in atherosclerosis, and the entire section 4.4 autophagy in calcific aortic valve disease (CAVD). Our review manuscript is now focused on vascular media calcification.

2. Is it all necessary or can some of it be deleted as 'off topic' (examples)?
   As mentioned above it would be important to follow VSMC calcification rather than a mixture of very different disease entities. I still believe that media sclerosis and arteriosclerosis are very different diseases.

Authors' response: We thank the reviewer for highlighting this point. We agree with the reviewer

that media sclerosis and arteriosclerosis are very different diseases, although they may share some similar common mechanisms. As described above, we have removed the text in section 3.1, section 4.1 and the entire section 4.4 to focus our manuscript on vascular medial calcification.

3. Is it comprehensive or have they missed citing anything important (examples)?
   It should be clearly stated that there are mTOR dependent and independent ways of inducing autophagy. The Review would benefit to show differences and overlaps of apoptosis, necrosis, necroptosis and autophagy.

Authors' response: We thank the reviewer for consideration of this point. We have stated both the TOR dependent and independent autophagy pathways under section 2-The key regulators of autophagy. Under the same section we have also added text on cell death and autophagic cell death and its crosstalk with apoptosis. As commented by reviewer 2, it is not mTOR in yeast, but TOR. We have revised this term accordingly. The additional text has been added as below (Page 7, line 14-23).

Furthermore, autophagy pathway could also be regulated by TOR independent pathways like inositol signalling pathways (Sarkar, et al., 2005), Ca^{2+}/calpain pathway (Williams, et al., 2008), cAMP pathway (Williams, et al., 2008) etc. It is also interesting to note that autophagy pathway, which is mainly a cellular degradation pathway is intimately linked to cell death and is different from other programmed cell death mechanisms like apoptosis, necrosis and necroptosis. Autophagy cell death is often seen associated with increased number of autophagosomes inside the cell (Yonekawa & Thorburn, 2013). However, there is a complex crosstalk between autophagy and apoptosis where the calpain-mediated cleavage of Atg5 can trigger cell death by apoptosis (Yousefi, et al., 2006). In this review we have only focused on autophagy as a cytoprotective pathway.

Reviewer 2

1. Introduction

Page 5 – Fix none to non in the following sentence: "In this review we focus on macroautophagy (referred hereafter as autophagy) which could be both selective or none selective on the basis of the cargo engulfed."

Authors' response: This has been corrected, Page 5, line 9-15.

Please be clearer about the difference between selective and non-selective autophagy (i.e degrading cytosolic components vs. bulk degradation). "On the basis of the cargo engulfed" makes it sound like only certain organelles (such as mitochondria and peroxisomes) can be degraded by selective autophagy but not others (like the ER). Do you mean to say selective autophagy can be regulated by different autophagy receptors depending on the organelle?

Authors' response: We thank the reviewer for consideration of this point. Additional text has been added under introduction as below to describe both the selective and non-selective autophagy, Page 5, line 10-16.

In this review we focus on macroautophagy (referred hereafter as autophagy) which could be both selective and non-selective. Non-selective autophagy degrades cytoplasmic contents,

however selective autophagy can degrade specific substrates like mitochondria (Lazarou, et al., 2015), ribosomes (Kraft, Deplazes, Sohrmann, & Peter, 2008), bits of ER (Wilkinson, 2019), peroxisomes (Kim, Hailey, Mullen, & Lippincott-Schwartz, 2008), bacteria (Bauckman, Owusu-Boaitey, & Mysorekar, 2015) etc. using specific autophagy receptors.

2. The key regulators of autophagy

• Page 6 – Though the authors are correct in saying most of the initial molecular work on autophagy was carried out in yeast, for this review the authors should focus on mammalian genes and proteins. I found it confusing when the authors talk about mammalian Target of Rapamycin, but then mention the yeast proteins (it is not mTOR in yeast, but TOR).

Authors' response: This has been corrected from mTOR to TOR (Page 6, line14).

• Page 7 – Add the following references found in text (Fimia, Kroemer, & Piacentini, 2013) and (Dikic and Elazar, 2018, Kaur and Debnath, 2015, Mizushima, 2007) in the reference list.

Authors' response: These references are now added on Page 20-23.

3. Autophagy in maintenance of vascular cell function

• Page 7 – Define ECs and VSMCs in text

Authors' response: We have defined VSMCs in text (Page 8, line1). The endothelial cell section has been removed as suggested by Reviewer 1.

• Figure 1 – Phagophore assembly – Vps15 and Atg6 are listed in text but missing in figure (as previously mentioned though, please stick to mammalian proteins, i.e Beclin1 not Atg6)

Authors' response: These corrections are now made in our revised Figure 1.

- Autophagosome expansion and completion – Atg16 and Atg10 are listed in text but missing in figure.

Authors' response: These corrections are now made in our revised Figure 1.

- Autophagosome lysosome fusion - Include LC3-PE complex on autophagosome surface. LAMP-I and LAMP-II are not discussed in text but are in the figure. Do these need to be in the figure?

Authors' response: We have included LC3-PE complex on autophagosome surface, and we have removed LAMP-I and LAMP-II from revised Figure 1.

- Refer to each part of the figure (a,b,c and d) as discussed in order in the text.

Authors' response: We have now referred to each part of the Figure 1 under section 2-The key regulators of autophagy (Page 6-7).

- Figure 2 – Label cell on figure (is it VSMC?)

Authors' response: The cells are now labelled as VSMC in revised Figure 2.

• Perhaps rearrange figure so that 'PDGR and high Pi' are placed above b. Cytoprotective Autophagy heading to make the cause of this autophagy clear

Authors' response: We have rearranged the Figure 2 accordingly.

• Label cell as 'healthy' not health
Authors' response: The cell is now labeled as Healthy VSMC in revised Fig 2.

• The induction of autophagy by oestrogen, rapamycin and metformin is not mentioned in text and is not referenced.
Authors' response: We have described and referenced the induction of autophagy by oestrogen, rapamycin and metformin in our revised manuscript.

Page 12, line 5-7: Treatment with rapamycin significantly reduced aortic calcification and release of pro-inflammatory cytokines including tumour necrosis factor alpha (TNF-α) and Interleukin 6 (IL-6), with enhanced survival noted in these mice (Frauscher et al., 2018).

Page 16, line 5-8: Interestingly metformin, (used to treat type 2 diabetes), has been shown to induce autophagy to restore β-GP-induced impairment of mitochondrial biogenesis and apoptosis in VSMCs, along with blocking the phenotypic transition of VSMCs (Ma, et al., 2019) (Fig 2).

Page 17, line 2-8: Furthermore, treatment with estrogen attenuates arterial calcification by blocking the osteoblastic differentiation of VSMCs and this is via induction of the autophagy pathway (Peng, et al., 2017) (Fig 2). This protective effect of estrogen treatment is enhanced by the addition of rapamycin (Peng, et al., 2017) (Fig 2).

Additionally, rapamycin has been reported to inhibit vascular calcification in the DBA/2 diabetic mouse model (Frauscher, et al., 2018).

• The accumulation of ROS in the cell suggests there is more in the cells as a result of cytoprotective autophagy.
Authors' response: We have changed the colour for ROS in revised Figure 2, so that it looks distinctive from cytoprotective autophagy.

• New information is presented in the figure legend and not discussed in text and also not referenced. Please discuss in text and refer to the figure accordingly.
Authors' response: Both the Figure 2 legend and text has been modified accordingly as suggested by the reviewer (Page 8-10, and Page 15-17).

• Is High Pi intracellular or extracellular which leads to cytoprotective autophagy?
Authors' response: Intracellular Pi leads to cytoprotective autophagy. Dai *et al* has reported that knockdown of the sodium-dependent phosphate cotransporter Pit1 attenuates high Pi-induced autophagy in VSMCs. Pit1 plays an important role in transporting extracellular phosphate into intracellular space. It has been mentioned in our manuscript and revised Figure 2 legend (Page 10, line 24-25).
High Pi increases PiT-1 expression, which leads to elevated levels of intracellular Pi. This further enhances Runx2 expression and the osteogenic transition of VSMCs (Fig 2).

Reference in our manuscript: Dai, X. Y., Zhao, M. M., Cai, Y., Guan, Q. C., Zhao, Y., Guan, Y., Kong, W., Zhu, W. G., Xu, M. J., & Wang, X. (2013). Phosphate-induced autophagy counteracts vascular calcification by reducing matrix vesicle release. Kidney Int, 83, 1042-1051.

• Page 9 - (Shao, et al., 2014) – is this the correct reference to put here?
Authors' response: We are sorry for the wrong reference, and it has been removed accordingly.

• Page 10 – Elaborate on the mechanism by which autophagy regulates Ca^{2+} homeostasis in VSMCs. Is the exact mechanism known?
Authors' response: To the best of our knowledge, there is currently only one paper which has investigate Ca^{2+} homeostasis in mouse model with VSMC specific deletion of *Atg7* (*Atg7$^{fl/fl}$ SM22α-Cre^{+})* (Micheils *et al* 2015). This paper has been discussed in our manuscript, Page 9, line 12-24.
It has been shown that increased cytosolic concentrations of Ca^{2+} (e.g. Thapsigargin treatment) induces autophagosome formation both by TOR dependent and independent pathways (Williams, et al., 2008). However, this increase in autophagosome numbers does not leads to enhanced autophagy, rather causes a decline in autophagic clearance via lysosomes (Ganley, Wong, Gammoh, & Jiang, 2011). The reasons behind autophagy impairment under increased calcium concentrations remains to be elucidated. Interestingly autophagy is shown to regulate Ca^{2+} homeostasis in VSMCs. VSMC specific deletion of *Atg7* (*Atg7$^{fl/fl}$ SM22α-Cre^{+})* in mice causes an imbalance between Ca^{2+} uptake and Ca^{2+} release. The voltage-gated Ca^{2+} channels which promotes Ca^{2+} entry inside the cell from the extracellular space were more sensitive to depolarisation (open for Ca^{2+} entry from extracellular space) in autophagy defective VSMCs. On the contrary there was reduced expression of the plasma membrane Ca^{2+} ATPase required for removal of Ca^{2+} to extracellular space, leading to elevated basal levels of intracellular Ca^{2+} levels inside the cell (Michiels, Fransen, De Munck, De Meyer, & Martinet, 2015).

4. Autophagy in cardiovascular calcification
• Page 11 – "…the MVs released from osteogenic cells facilitate hydroxyapatite formation in the ECM" suggests that MVs in Figure 2 should be shown inside the cell as well as outside the cell.
Authors' response: We have revised our Figure 2 accordingly, and MVs are now shown inside the cell as well as outside.

• Page 11 – second paragraph also refers to Figure 2 and should be noted in the text. Perhaps adding extra detail in the figure that is discussed in text will help readers better visualise the factors inducing to osteogenic transition of VMSCs. For example, "High Pi increases PiT-1 expression, which leads to elevated levels of intracellular Pi. This further enhances Runx2 expression and the osteogenic transition of VSMCs", however the figure suggests it is extracellular.
Authors' response: We have revised Figure 2 accordingly.

• Page 12 – State that the Atg5 deficient mice are Atg5 deficient in macrophages.
Authors' response: This paragraph has been removed from our revised manuscript as suggested by Reviewer 1.

• Page 12 – second paragraph, define CKD.

Authors' response: We have defined CKD in our revised manuscript as chronic kidney disease Page 11, line 15.

• Page 12 – Reference (Giachelli et al., 2005) is not found in References.

Authors' response: We are sorry for our mistake. It should be Giachelli et al., 2009. This reference has been added, Page 11, line 17.

• Page 12 – Reference (Kendrick and Chonchol, 2011) is not found in References.

Authors' response: This reference has been added Page 11, line 18

• Page 13 - (Hsu, et al., 2015) is not in References

Authors' response: This reference has been added, Page 11, line 21

• Page 13- Define RO

Authors' response: RO has been defined as reactive oxygen species (ROS) Page11, line24.

• Page 14 – Typo in sentence: remove full stop after "Hyperglycemia, a known inducer of vascular calcification. Increases…"

Authors' response: Full stop has been removed, Page 12, line 22.

• Page 15 – Link between MVs and autophagic machinery should have it's own subheading and not fall within 'Autophagy in cardiovascular calcification'. It draws a nice summary of the origin of the calcification process and ties a lot of the sections together.

Authors' response: The Link between MVs and autophagic machinery is now in a separate section (section 5) in our revised manuscript, Page 13, para 3.

• Page 15 – second paragraph, Be specific. "VSMCs and macrophages are the primary source of these calcified MVs which are released into the collagen rich matrix in the intima" should read " In the context of atherosclerosis, VSMCs …" otherwise it sounds like only VSMCs and macrophages are the course of MVs out of all the cells that produce calcium and phosphate.

Authors' response: We thank the reviewer for the consideration of this point. We have modified our text as below (Page 13, line 17-19).

In the context of atherosclerosis, VSMCs and macrophages are the primary source of these calcified MVs which are released into the collagen rich matrix in the intima.

• Page 15 – second paragraph, the reference (Chistiakov, Myasoedova, Melnichenko, Grechko, & Orekhov, 2017) is written twice in a row but different format.

Authors' response: The repeat reference is removed (Page 13, line 20).

5. Mitophagy in vascular calcification

• Page 17 – Reference in text (Martin & Matthews, 1970) is not in reference list

Authors' response: This reference has been added (Page 23).

• Figure 3 – It is not clear if the mitochondria derived vesicles in the figure are the same ones that contain Ca2+ and Pi. The figure suggests that MVs with Ca and Pi appear later in the autophagosomes and do not come from mitochondria which is what is suggested in text.
Authors' response: We have revised Figure 3 to differentiate between MVs and mitochondria derived vesicles.

• Does the mitochondria need to be damaged to release mitochondria derived-vesicles or does the mitochondria release Ca2+ and Pi under normal conditions also?
Authors' response: We agree that mitochondria may release Ca^{2+} and Pi under normal conditions but damaged mitochondria accumulate and release more of them. Evidence towards this phenomenon can be seen in this recent study by Pei *et al* 2018. This reference is now cited (page 23) and discussed in the manuscript (Page 15, line 18-20).

Interestingly, Pei et al has demonstrated the release of mitochondrial electron dense granules from human dental pulp stem cells after osteogenic induction and their interaction with autolysosomes (Pei, et al., 2018).

Reference:
Pei, D. D., Sun, J. L., Zhu, C. H., Tian, F. C., Jiao, K., Anderson, M. R., Yiu, C., Huang, C., Jin, C. X., Bergeron, B. E., Chen, J. H., Tay, F. R., & Niu, L. N. (2018). Contribution of Mitophagy to Cell-Mediated Mineralization: Revisiting a 50-Year-Old Conundrum. *Adv Sci (Weinh), 5*, 1800873.

• Make legend for hydroxyapatite crystals and ECM
Authors' response: Legend for hydroxyapatite crystals and ECM has been added to revised Figure 3.

• There are no hydroxyapatite crystals being released out of the cell by the autolysosome as is suggested in text.
Authors' response: Figure 3 has been corrected with hydroxyapatite crystals present in autolysosomes.

• Refer to each a,b,c and d step of Figure in text where mentioned. For example (a) CPP enters cell should be referred to on page 16 so readers can follow easily.
Authors' response: Each a,b,c and d step of Figure are now referred in text where mentioned.

• Page 18 – '. Interestingly, overexpression of BCL2 Interacting Protein 3, BNIP3, which depolarises mitochondria and hence induces mitophagy (J. Zhang & Ney, 2009), reverses mitophagy and attenuates lactate induced calcification (Y. Zhu, et al., 2019)'. This sentence does not make sense. Are you suggesting that inhibiting mitophagy would inhibit calcification? Please clarify.
Authors' response: The sentence has been modified to state that enhancing mitophagy inhibits lactate induced calcification as demonstrated in the paper by Zhu et al 2019 (Page 16, line 10).
Interestingly, overexpression of BCL2 Interacting Protein 3, BNIP3, which depolarises

mitochondria and induces mitophagy (J. Zhang & Ney, 2009) and thus attenuates lactate-induced calcification (Y. Zhu, et al., 2019).

6. Pharmacological modulation of autophagy in vascular calcification

• Page 18 – Tease out more. Suggests what sort of studies are needed. This is in the conclusion anyway but feels a bit brief.

Authors' response: We have elaborated a bit more both the section 7 Pharmacological modulation of autophagy in vascular calcification and section 8 conclusions (Page 16-18).

Section 7 Pharmacological modulation of autophagy in vascular calcification, Page 17, line 14-21.

For example, the use of a targeted autophagy inducer such as peptide TAT Beclin (Shoji-Kawata, et al., 2013) or the modulation of master regulator of autophagosomes and lysosomal biogenesis transcription factor EB (TFeb) (Napolitano & Ballabio, 2016) in ECs and VSMCs could shed more light into the intricate involvement of the autophagy process with the progression of VC. High through-put chemical screening for novel autophagy modulators is ongoing for diseases like cancer, neurodegeneration and viral and bacterial diseases (Panda, et al., 2019). It would be interesting to investigate if these novel autophagy modulators have an effect on the process of VC.

Section 8 conclusions, Page 18, line4-10.

Precise understanding of the role of autophagy in VC can be achieved by making novel VC mice models with VSMC specific deletion or overexpression of autophagy genes. It's also crucial to understand the intimate interplay between Ca^{2+} flux, autophagy and the process of VC. *In vitro* and *in vivo* experiments are also required to establish the basal levels of autophagy and mitophagy during the entire length of the VC process. This will require the expression of autophagy/ mitophagy specific markers in VC mice models. This precise temporal information will lead to development of focused treatments in future.

7. Concluding remarks

• Page 19 – New examples are being introduced in this section which should be mentioned in section 6 (e.g. TAT Beclin and TFeb)

Authors' response: Discussion on TAT Beclin and TFeb has now been added to section 7 Pharmacological modulation of autophagy in vascular calcification (Page 17, line14-17).

• Page 19 – References (Shoji-Kawata et al., 2013) and (Napolitano and Ballabio, 2016) are not in references

Authors' response: These two references have now been added (Page 23, 24).

• Are there limitations for using the pharmacological modulators mentioned that could cause alternative side effects in patients if used as a potential therapy?

Authors' response: The side effects of Rapamycin as an immunosuppressant has been added in our revised manuscript (Page 17, line 11-12).

However, most of these studies are based on pharmacological modulators such as rapamycin and valporic acid, which may induce non-specific effects, for example rapamycin is an

immunosuppressant (Dumont & Su, 1996).

• Figures

All figures have different font, please be consistent.

Authors' response: All the fonts have been standardized for Figure 1, 2 and 3.

• Please fix all figures accordingly as stated in each section.

Authors' response: We have amended all figures accordingly.

• Table

Page 30 - (Hsu, et al., 2015) is not in References.

Authors' response: The reference has been added now (Page 21).

Autophagy as a novel therapeutic target in vascular calcification

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Abstract

The autophagy pathway is a key regulator of cellular metabolism and homeostasis, and plays a critical role in maintaining normal vascular cell function. It is well recognized that autophagy can regulate endothelial cell homeostasis, vascular smooth muscle cell (VSMC) phenotype transition, and calcium (Ca^{2+}) homeostasis in VSMCs. Emerging evidence has demonstrated that autophagy directly protects against vascular calcification (VC). Crosstalk between endosomes, dysfunctional mitochondria, autophagic vesicles and Ca^{2+} and phosphate (Pi) enriched matrix vesicles (MVs) may underpin the pathogenesis of VC. In this review, we summarize the current experimental evidence in understanding how autophagy maintains normal vascular cell function and its protective role against vascular calcification. We also discuss the underlying molecular and cellular mechanisms through which autophagy inhibits vascular calcification. Pharmacological modulation of autophagy may offer an exciting new strategy for the treatment of vascular calcification.

Key words

Autophagy, Vascular cell function, Phenotype transition, Matrix vesicles, Vascular calcification.

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Abbreviations

ALP, alkaline phosphatase; **Ca^{2+}**, Calcium; **CAVD**, calcific aortic valve disease; **CKD**, chronic kidney disease; **EC**s, endothelial cells; **ECM**, extracellular matrix; **LC3**, Light Chain 3; **TOR**, target of rapamycin; **MVs**, matrix vesicles; **PDGF**, platelet derived growth factor; **Pi**, phosphate; **ROS**, reactive oxygen species; **VC**, vascular calcification; **VSMCs**, vascular smooth muscle cells; **VICs**, valve interstitial cells;

1. Introduction

Autophagy is essential for health and longevity (Nakamura & Yoshimori, 2018). This catabolic pathway is initialised by a double membraned phagophore which engulfs misfolded proteins, damaged organelles or unwanted metabolites forming an autophagosome. The autophagosome fuses with single membraned acidic lysosomes, where the engulfed content is degraded into cellular building blocks, thereby, recycling the nutrients and providing a source of energy to the cells (Singh & Cuervo, 2011). Based on the membrane dynamics and the molecular machinery involved, three main types of autophagy have been identified so far, macroautophagy, chaperone-mediated autophagy and microautophagy (Cuervo & Wong, 2014; Feng, He, Yao, & Klionsky, 2014; W. W. Li, Li, & Bao, 2012). In this review we focus on macroautophagy (referred hereafter as autophagy) which could be both selective and non-selective. Non-selective autophagy degrades cytoplasmic contents, however selective autophagy can degrade specific substrates like mitochondria (Lazarou, et al., 2015), ribosomes (Kraft, Deplazes, Sohrmann, & Peter, 2008), bits of ER (Wilkinson, 2019), peroxisomes (Kim, Hailey, Mullen, & Lippincott-Schwartz, 2008), bacteria (Bauckman, Owusu-Boaitey, & Mysorekar, 2015) etc. using specific autophagy receptors. Most of the living cells perform low level of basal autophagy to maintain protein turnover and for recycling of damaged organelles, however autophagy pathway is up-regulated when the cells are challenged with stressors such as nutrient starvation (Shang, et al., 2011), hypoxia (Daskalaki, Gkikas, & Tavernarakis, 2018), reactive oxygen species (ROS) (Filomeni, De Zio, & Cecconi, 2015), protein aggregates (Menzies, Fleming, & Rubinsztein, 2015), infection (Levine & Kroemer, 2008) and increased phosphate levels (Dai, et al., 2013). In the last twenty years, autophagy has been extensively investigated as a common mechanism underpinning the development of important human diseases including cancer (White, 2015), neurodegeneration (Nixon, 2013), cardiomyopathy (Tong & Sadoshima, 2016), diabetes (Gonzalez, et al., 2011), liver disease (Rautou, et al.,

2010), autoimmune diseases (Yang, Goronzy, & Weyand, 2015) and infection (Deretic, Saitoh, & Akira, 2013). Crucially, the role of autophagy in vascular calcification, a significant risk factor for cardiovascular mortality, is just emerging. In this review, we discuss our present understanding of the important role of autophagy in the maintenance of vascular cell function and vascular calcification, and the emerging strategies to target autophagy for the treatment of vascular calcification.

2. The key regulators of autophagy

The autophagic molecular machinery was first elucidated in yeast followed by identification of homologues in humans (Itakura & Mizushima, 2010; Mizushima, Yoshimori, & Levine, 2010; Tsukada & Ohsumi, 1993). To date, there are more than 40 autophagy related (*ATG*) genes known. These genes orchestrate a highly dynamic autophagy pathway which gets activated during stress (Murrow & Debnath, 2013) (Fig 1). When nutrients are limited in the environment, the target of rapamycin TOR complex is inhibited, which results in the activation of ULK1/2 complex. This complex interacts with Atg13, FIP200, Atg101, leading to phagophore assembly (Cheong & Klionsky, 2008; Cheong, Nair, Geng, & Klionsky, 2008; Mao, et al., 2013) (Fig 1a). Phagophore formation is a vital step in the autophagy pathway as it leads to formation of the double membrane sequestering compartment – the autophagosome (Mizushima, Yoshimori, & Ohsumi, 2011; Suzuki, Kubota, Sekito, & Ohsumi, 2007). Several Atg proteins participate in this process including Beclin1, Vps34, Ambra1, Atg6, Atg14 and Atg38 (Z. Xie & Klionsky, 2007) (Fig 1a). After initiation of phagophore formation, the next step is its expansion and maturation. This is carried out by Atg12 and light chain 3 (LC3), the two Ubiquitin-like proteins. Atg12 forms a complex with Atg5 and Atg16L1 (also known as E3 enzyme) with the action of E1 and E2-like enzymes Atg7 and Atg10 (Nakatogawa, 2013) covalently attaches to phosphatidylethanolamine (PE) (Fig 1b) , a lipid, to from a LC3-PE complex on the surface of

the autophagosome, resulting in the formation of a mature autophagosome (Satoo, et al., 2009) (Fig 1b). The conjugation of PE with LC3 requires Atg7, an E1-like enzyme (Tanida, et al., 1999), and Atg3, an E2-like enzyme (Ichimura, et al., 2000). For the efficient expansion of the phagophore and recycling of LC3-PE, Atg4, a cysteine protease (Satoo, et al., 2009) is required. These mature autophagosomes filled with cargo marked for degradation can now fuse with the lysosome, forming a fusion compartment autolysosome (Fig 1c). Cargo engulfed by autophagosome require ubiquitination and is recognised by specific adaptor molecules including p62, NBR1, NDP52, VCP and optineurin (Shaid, Brandts, Serve, & Dikic, 2013). This flagged cargo then binds to LC3/GABARAP/GATE16 family on the internal membrane of the autophagosome (Fimia, Kroemer, & Piacentini, 2013). Inside autolysosomes the cargo is degraded by hydrolases present in the acidic lumen of the lysosomes (Fig 1c). The functional role of the primary autophagy molecules has been extensively reviewed previously (Dikic & Elazar, 2018; Kaur & Debnath, 2015; Mizushima, 2007). Furthermore, autophagy pathway could also be regulated by TOR independent pathways like inositol signalling pathways (Sarkar, et al., 2005), Ca^{2+}/calpain pathway (Williams, et al., 2008), cAMP pathway (Williams, et al., 2008) etc. It is also interesting to note that autophagy pathway, which is mainly a cellular degradation pathway is intimately linked to cell death and is different from other programmed cell death mechanisms like apoptosis, necrosis and necroptosis. Autophagy cell death is often seen associated with increased number of autophagosomes inside the cell (Yonekawa & Thorburn, 2013). However, there is a complex crosstalk between autophagy and apoptosis where the calpain-mediated cleavage of Atg5 can trigger cell death by apoptosis (Yousefi, et al., 2006). In this review we have only focused on autophagy as a cytoprotective pathway.

3. Autophagy in maintenance of vascular smooth muscle cell function

Vascular smooth muscle cells (VSMCs) are the principal cellular components of blood vessels. These cell types both play a key role in maintaining blood flow, vessel tone, and are crucial for restoring vascular homeostasis during mechanical shear stress, vascular injury and blood clots (Cahill & Redmond, 2016). Crucially, autophagy is essential for the maintenance of physiological vascular cell function (Grootaert, et al., 2015; Liao, et al., 2012; Vion, et al., 2017).

VSMCs are the most abundant cells in the medial layer of arteries, where they maintain vessel dilation and constriction, regulating blood pressure and distribution of oxygen and nutrients to surrounding cells (Lacolley, Regnault, Nicoletti, Li, & Michel, 2012). A defining feature of VSMCs is their heterogeneity, i.e. they can switch from a contractile (quiescent) phenotype to a proliferative, synthetic (osteogenic) phenotype. VSMCs with a contractile phenotype express markers including SM-α actin, calponin and SM22α, and have decreased mobility, reduced proliferation, and lower extracellular matrix (ECM) production (Fig 2). However, following phenotypic switching they start to express markers including matrix metalloproteinase, collagenase, alkaline phosphatase (ALP), vimentin, osteopontin and Runx2 (Iyemere, Proudfoot, Weissberg, & Shanahan, 2006) (Fig 2). The cells are now capable of moving to the intima, proliferating and enhancing ECM formation. These osteogenic VSMCs are also a crucial source of calcifying matrix vesicles (Naik, et al., 2012). This phenotypic switching is prompted by various biochemical and physical environmental cues and is seen in several conditions including atherosclerosis, diabetes, hypertension and aging (Durham, Speer, Scatena, Giachelli, & Shanahan, 2018; Lacolley, Regnault, & Avolio, 2018; Reddy, et al., 2016; Touyz, et al., 2018) (Fig 2).

PDGF (platelet derived growth factor) is a principal phenotype switching cytokine elevated during vascular injury and diseases including hypertension, atherosclerosis, and diabetes (Egan, Wainwright, Wadsworth, & Nixon, 2005; Gomez & Owens, 2012; Lacolley, et al., 2018). Studies in VSMCs have shown that PDGF stimulates cell phenotype switching, concomitantly activating autophagy (Salabei, et al., 2013) (Fig 2). The role of autophagy here is likely to be mediated through the degradation of proteins including SM-α actin, calponin and SM22α, required by VSMCs to maintain the contractile phenotype. However, genetic ablation of autophagy in PDGF-treated VSMCs is required to establish whether this phenotypic switching is entirely autophagy dependent. Further studies are also required to elucidate whether autophagy is crucial to VSMC phenotype switching in response to additional stimuli including prostacyclins, statins, amino acids and growth factors.

It has been shown that increased cytosolic concentrations of Ca^{2+} (e.g. thapsigargin treatment) induces autophagosome formation both by TOR dependent and independent pathways (Williams, et al., 2008). However, this increase in autophagosome numbers does not leads to enhanced autophagy, rather causes a decline in autophagic clearance via lysosomes (Ganley, Wong, Gammoh, & Jiang, 2011). The reasons behind autophagy impairment under increased calcium concentrations remains to be elucidated. Interestingly autophagy is shown to regulate Ca^{2+} homeostasis in VSMCs. VSMC specific deletion of *Atg7* (*Atg7$^{fl/fl}$ SM22α-Cre^{+})* in mice causes an imbalance between Ca^{2+} uptake and Ca^{2+} release. The voltage-gated Ca^{2+} channels which promotes Ca^{2+} entry inside the cell from the extracellular space were more sensitive to depolarisation (open for Ca^{2+} entry from extracellular space) in autophagy defective VSMCs. On the contrary there was reduced expression of the plasma membrane Ca^{2+} ATPase required for removal of Ca^{2+} to extracellular space, leading to elevated basal levels of intracellular Ca^{2+} levels inside the cell (Michiels, Fransen, De Munck, De Meyer, & Martinet, 2015). This study clearly shows the crucial role of autophagy in regulating Ca^{2+} flux in VSMCs which further

have consequences on contractile capacity of aorta. It would be interesting to investigate the calcification levels of these cells and also if restoring autophagy pharmaceutically could normalise the Ca^{2+} flux and the aortic contractibility.

4. Autophagy in cardiovascular calcification

Vascular calcification (VC) is a significant risk factor for cardiovascular mortality and morbidity in patients with chronic kidney disease, atherosclerosis and diabetes. Previously considered a passive process due to ageing, recent advances suggest that VC is an actively regulated cell mediated process that shares many similarities with bone formation (Doherty, et al., 2003). According to its location, three principal types of vascular calcification have been reported – intimal (associated with atherosclerosis), medial (also known as Mönckeberg's sclerosis or arteriosclerosis) and calcific aortic valve disease. The cells involved in vascular calcification include ECs, VSMCs, pericytes, calcifying vascular cells and valve interstitial cells, (Meng, et al., 2018; Pillai, et al., 2017; Yao, et al., 2013; D. Zhu, Mackenzie, Farquharson, & Macrae, 2012). These cells form a calcified matrix and undergo a bone-like osteogenic phenotypic transition in the presence of this calcifying environment. This drives apoptosis (Ewence, et al., 2008), matrix vesicle (MV) release (N. X. Chen, O'Neill, & Moe, 2018) and osteogenic differentiation in VSMCs (Liu, Lin, Ju, Chu, & Zhang, 2015) along with hydroxyapatite deposition in tissues and vessels (Lee, Morrisett, & Tung, 2012). Enriched with a concoction of calcifying enzymes, the MVs released from osteogenic cells facilitate hydroxyapatite formation in the ECM (Buchet, Pikula, Magne, & Mebarek, 2013).

Calcifying VSMCs express genes like transcription factor Msx2 (Andrade, Carmo, Farias-Silva, & Liberman, 2017; Zhou, et al., 2013), Runx2 (Speer, Li, Hiremath, & Giachelli, 2010) and phosphate transporter PiT-1 (X. Li & Giachelli, 2007). High Pi increases PiT-1 expression, which leads to elevated levels of intracellular Pi. This further enhances Runx2 expression and

the osteogenic transition of VSMCs (Fig 2). Tissue non-specific alkaline phosphatase (TNAP) expression is also central to the vascular calcification process. TNAP hydrolyses pyrophosphate (PPi), a calcification inhibitor, generating phosphate for hydroxyapatite formation in calcifying VSMCs (Sheen, et al., 2015). Furthermore, the ankylosis protein (ANK) and ecto-nucleotide pyrophosphatase/phosphodiesterases-1 (ENPP1) inhibit vascular calcification through enhancing extracellular PPi levels (Back, et al., 2018; Ho, Johnson, & Kingsley, 2000) and matrix Gla protein (MGP) inhibits vascular calcification possibly by functional inhibition of bone morphogenetic proteins (BMP-2 and BMP-4) in VSMCs (Barrett, O'Keeffe, Kavanagh, Walsh, & O'Connor, 2018; Bjorklund, et al., 2018).

Exciting new evidence suggests that the autophagy pathway may play a pivotal role in regulating the key events underpinning the progression of vascular calcification. Here we discuss current experimental evidence supporting the crucial role of autophagy in regulating vascular calcification.

4.1 Autophagy in CKD medial calcification

Hyperphosphatemia (high serum Pi levels) regulates VC in chronic kidney disease (CKD) patients and predisposes them to progressive VC (Giachelli, 2009). Increased Pi levels induce calcification of VSMCs and the surrounding ECM (Giachelli, 2009) and transforms the cells into an osteogenic phenotype which drives further calcification (Kendrick & Chonchol, 2011). Hyperphosphatemia enhances endothelial dysfunction through the regulation of autophagy. High concentrations of Pi inhibit TOR signalling *in vitro* and thus, enhance the autophagic flux. This flux offers the ECs protection from Pi-induced apoptosis (Hsu, et al., 2015). Further *in vivo* studies demonstrated increased, LC3 expression in the endothelial cells from CKD rats compared to sham-operated controls. Similar *in vitro* protective effects of autophagy has been shown in VSMCs whereby increased autophagy levels counteract VC induced by reactive

oxygen species (ROS) under high Pi levels (Dai et al., 2013). These observations are reinforced by recent *in vivo* experiments on DBA/2 mice which develop uremic media calcification when fed with high Pi diet. These mice show increased expression of the autophagy markers LC3-II, p62, Igfbp3, Atg16l1 in aortic VSMCs compared to control mice (Frauscher, et al., 2018). Treatment with rapamycin significantly reduced aortic calcification and release of pro-inflammatory cytokines including tumour necrosis factor alpha (TNF-α) and Interleukin 6 (IL-6), with enhanced survival noted in these mice (Frauscher et al., 2018).

These compelling observations suggest a regulatory role of autophagy pathway in the homeostasis of Ca^{2+} and Pi in the population of VSMCs modulated during CKD. Furthermore, these studies establish high Pi as a stress signal for TOR, a key regulator of autophagy.

4.2 Autophagy in diabetic medial calcification

Patients with type-II diabetes mellitus show extensive vascular calcification with disturbed vessel wall homeostasis characterised by endothelial dysfunction and phenotypic switching of VSMCs (Casella, Bielli, Mauriello, & Orlandi, 2015; Dhananjayan, Koundinya, Malati, & Kutala, 2016; Harper, et al., 2016). Recent studies have shown that autophagy induction inhibits both the endothelial dysfunction (Fetterman, et al., 2016; Y. Xie, et al., 2011) in endothelial cells from diabetic patients and phenotypic switching of VSMCs in diabetic vascular lesions (An, Li, Wei, Li, & Xu, 2018; Qiu, et al., 2018). These studies demonstrate a protective role of autophagy in diabetic vascular disorders.

Hyperglycemia, a known inducer of vascular calcification increases posttranslational modification of proteins by O-linked N-acetylglucosamine (O-GlcNAcylation) in diabetic arteries (Heath et al., 2014). Furthermore, they show that O-GlcNAcylation of AKT (protein kinase B) leads to AKT phosphorylation and activation, thereby promoting VC. AKT

activation promotes activation of TOR and thus suppresses autophagy. Inhibition of TOR by rapamycin suppressed this O-GlcNAcylation mediated VC, suggesting a novel mechanistic role of autophagy in abrogating hyperglycemia induced VC (Heath, et al., 2014).

The induction of autophagic flux through the application of tert-butyl hydroquinone (tBHQ) treatment has demonstrated an increase in aortic nuclear factor (erythroid-derived 2)-like 2, using a diabetic mouse model. Treatment with tBHQ provided atheroprotection by reducing both inflammation and the lipid content of atheroma plaques (Lazaro, et al., 2018). Selective uptake of lipid droplets by autophagosomes is an emerging pathway in cellular lipid metabolism (Zechner, Madeo, & Kratky, 2017) which will have crucial implications in metabolic disorders such as diabetes, and requires further investigation.

5. Link between MVs and autophagic machinery

MVs refer to nano (20-200 nm) spherical bodies which are known to bud from the plasma membrane (Fedde, 1992; Thouverey, Strzelecka-Kiliszek, Balcerzak, Buchet, & Pikula, 2009). They are made up of a lipid bilayer enriched with amorphous Ca^{2+} and Pi along with enzymes including TNAP, PHOSPHO 1, Na+/K+ ATPase, ENPP1, and Pit1 (Golub, 2011). MVs are typically found associated with small crystals of calcium phosphate hydroxyapatite mineral (Cui, Houston, Farquharson, & MacRae, 2016; Golub, 2009). In context of atherosclerosis, VSMCs and macrophages are the primary source of these calcified MVs which are released into the collagen rich matrix in the intima. The MVs promote atherosclerotic calcification, directly leading to the formation of calcified plaques (Chistiakov, Myasoedova, Melnichenko, Grechko, & Orekhov, 2017).

It has been shown that MV membranes are enriched in phosphatidylethanolamine (Golub, 2009, 2011), which is also a main constituent of the mature autophagosome membrane (Yin, Pascual,

& Klionsky, 2016). Furthermore, TEM images of MVs reveal that they may have a single, double or a multi-layered membrane structure especially in the context of VC in atherosclerosis (Perrotta & Perri, 2017). Moreover, it is possible that, precursors of calcification such as Ca^{2+} and Pi are formed or processed initially within a subcellular compartment such as endosomes, multi vesicular bodies, autophagosome or autolysosome as part of intracellular control of Ca^{2+} and Pi homeostasis in the cellular energy landscape. Indeed, there is recent evidence to support this view; during Ca^{2+} mediated DNA transfection, calcium phosphate precipitates (CPP), not the DNA, induce autophagosome formation in HEK293 cells (X. Chen, et al., 2014). The CPP-DNA complex enter the cells via endosomes which interact with LC3-positive autophagosomes once inside the cytoplasm (Fig 3a). These endosomes are galectin-3 positive, suggesting that they are damaged. Furthermore, these CPP-induced LC3 positive vesicles co-localise with ubiquitin and p62, a selective autophagy adaptor. Interestingly, these vesicles also colocalise with LAMP1, the lysosomal marker, completing the autophagy cycle. Annexin-V which is a major constituent of the MV is also known to have role in formation of mature autophagosomes and is seen present on the lysosomal membrane where it participates in Ca^{2+} signalling (Ghislat & Knecht, 2012). It is also interesting to note that autophagosomes from calcified mouse primary osteoblasts are packed with calcified hydroxyapatite (Nollet et al., 2014). It is possible that the acidic lysosomes have a role in solubilizing this hydroxyapatite when they fuse with these autophagosomes.

Recent proteomic analysis of MVs released from rat VICs by our laboratory shows expression of autophagic proteins including LAMP1, LAMP2 and LAMTOR1 (Cui, et al., 2016); LAMP-I and LAMP-II are lysosomal membrane proteins required for recycling via the autophagy pathway. LAMTOR1 is part of the regulator complex on the lysosomal membrane (Colaco & Jaattela, 2017). These data suggest that MVs may be entwined with the network of autophagic vesicles either at the stage of their formation or release during the process of vascular

calcification (Fig 3a and 3c). Indeed, the recent emergence of a new field of secretary autophagy (as opposed to degradative autophagy), whereby autophagic machinery participates in conventional and unconventional secretions via plasma membrane (Ponpuak, et al., 2015) may present novel mechanistic avenues to explore (Fig 3).

6. Mitophagy in vascular calcification

Mitochondria have the capacity to accumulate Ca^{2+} in an energy dependent manner and are a crucial regulator of cellular Ca^{2+} homeostasis (Glancy & Balaban, 2012). Excess Ca^{2+} intake by mitochondria triggers the opening of the permeability transition pores and the release of cytochrome C, resulting in cell death by apoptosis or necrosis (Izzo, Bravo-San Pedro, Sica, Kroemer, & Galluzzi, 2016). Using scanning electron microscopy, mitochondria-derived vesicles enriched with Ca^{2+} and Pi have been observed in calcifying skeletal cells including chondrocytes, osteoblasts and osteocytes (Martin & Matthews, 1970; Pei, et al., 2018; Sayegh, Solomon, & Davis, 1974; Sutfin, Holtrop, & Ogilvie, 1971). Indeed it has been proposed during the process of calcification mitochondria release intracellular Ca^{2+} in mitochondrial- derived vesicles (MDV), which are subsequently transported to the ECM where these vesicles deposit hydroxyapatite (Boonrungsiman, et al., 2012). These MDV enriched with Ca^{2+} could be engulfed by autophagosomes or directly taken up by lysosomes, where they could be either degraded or released outside the cell (Fig 3d). Interestingly, Pei *et al* has demonstrated the release of mitochondrial electron dense granules from human dental pulp stem cells after osteogenic induction and their interaction with autolysosomes (Pei, et al., 2018).

In vascular cells, oxidative stress, elevated Pi levels, inflammation, mitochondrial dysfunction and apoptosis are all intimately associated with the calcification process (Byon, et al., 2008; Giachelli, 2009; Madamanchi & Runge, 2007; Proudfoot, et al., 2001; Shanahan, 2007). In a healthy cell, dysfunctional mitochondria are cleared by autophagosomes via mitophagy; it is

not known whether the calcifying vascular cells perform enough mitophagy to remove these dysfunctional mitochondria or do they accumulate and become a source of mitochondria derived vesicles enriched with Ca^{2+}? Recent studies have provided exciting clues in this direction. β-Glycerophosphate (β-GP) a known inducer of VC (Bai, et al., 2015; Shioi, et al., 1995) leads to phenotypic transition of VSMCs. Interestingly metformin, (used to treat type 2 diabetes), has been shown to induce autophagy to restore β-GP-induced impairment of mitochondrial biogenesis and apoptosis in VSMCs, along with blocking the phenotypic transition of VSMCs (Ma, et al., 2019) (Fig 2). Furthermore, lactate, an inducer of calcification, suppresses both autophagic flux and mitophagy in VSMCs (Y. Zhu, et al., 2019). Interestingly, overexpression of BCL2 Interacting Protein 3, BNIP3, which depolarises mitochondria and induces mitophagy (J. Zhang & Ney, 2009) and thus attenuates lactate-induced calcification (Y. Zhu, et al., 2019). These studies suggest that mitophagy is defective during the progression of vascular calcification. Defining the role of autophagy/mitophagy in restoring mitochondrial homeostasis during the phenotypic switching of VSMCs will provide important therapeutic intervention in vascular calcification disorders.

7. Pharmacological modulation of autophagy in vascular calcification

Several studies have investigated whether modulating autophagy by pharmacological agents can prevent vascular calcification (**Table 1**). Treatment with spermidine reduces lipid accumulation and necrotic core formation in atherosclerotic plaques of $ApoE^{-/-}$ mice, this reduction is specific to VSMCs (Michiels, Kurdi, Timmermans, De Meyer, & Martinet, 2016). Spermidine, a polyamine, is a known inducer of longevity, enhancing autophagy and suppressing necrosis in various models of aging (Eisenberg, et al., 2009). Treating mice with spermine and spermidine reverses age-related cardiac deterioration by inhibiting age-related myocardial morphology alterations, myocardial fibrosis, and cell apoptosis (H. Zhang, et al.,

2017). However, the direct effect of these compounds on vascular calcification remains to be elucidated. Furthermore, treatment with estrogen attenuates arterial calcification by blocking the osteoblastic differentiation of VSMCs and this is via induction of the autophagy pathway (Peng, et al., 2017) (Fig 2). This protective effect of estrogen treatment is enhanced by the addition of rapamycin (Peng, et al., 2017) (Fig 2). Valporic acid, an autophagy inducer, has also been shown to inhibit VSMC calcification *in vitro* (Dai, et al., 2013). Additionally, rapamycin has been reported to inhibit vascular calcification in the DBA/2 diabetic mouse model (Frauscher, et al., 2018). Pharmacological modulation of autophagy may therefore have therapeutic efficacy for ameliorating disorders associated with VC and preventing their onset. However, most of these studies are based on pharmacological modulators such as rapamycin and valporic acid, which may induce non-specific effects, for example rapamycin is an immunosuppressant (Dumont & Su, 1996). Tissue/cell-specific ablation of the key regulators of autophagy is therefore required to delineate the specific effects of autophagy. For example, the use of a targeted autophagy inducer such as peptide TAT Beclin (Shoji-Kawata, et al., 2013) or the modulation of master regulator of autophagosomes and lysosomal biogenesis transcription factor EB (TFeb) (Napolitano & Ballabio, 2016) in ECs and VSMCs could shed more light into the intricate involvement of the autophagy process with the progression of VC. High through-put chemical screening for novel autophagy modulators is ongoing for diseases like cancer, neurodegeneration and viral and bacterial diseases (Panda, et al., 2019). It would be interesting to investigate if these novel autophagy modulators have an effect on the process of VC.

8. Concluding remarks

Our knowledge of the role of autophagy in cardiovascular calcification is relatively limited at this time. Nonetheless, there is crucial growing evidence that autophagy may protect

cardiovascular tissue from calcification. It would be interesting to investigate whether membranes from autophagic vesicles contribute to the formation of MVs or if a selective form of autophagy is involved in recycling amorphous Ca^{2+} and Pi or hydroxyapatite (Fig 3a, b, c). Precise understanding of the role of autophagy in VC can be achieved by making novel VC mice models with VSMC specific deletion or overexpression of autophagy genes. It's also crucial to understand the intimate interplay between Ca^{2+} flux, autophagy and the process of VC. *In vitro* and *in vivo* experiments are also required to establish the basal levels of autophagy and mitophagy during the entire length of the VC process. This will require the expression of autophagy/ mitophagy specific markers in VC mice models. This precise temporal information will lead to development of focused treatments in future. Furthermore, the development of specific therapeutic autophagy agents, and novel reliable methods for monitoring and measuring autophagy in patients is required. In conclusion, targeting the autophagic molecular machinery is an attractive therapeutic strategy to inhibit progression or induce regression of vascular calcification.

Disclosure statement

The authors declare that they have no conflict of interest.

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Table 1: Effect of autophagy inducers and inhibitors in VC.

Model	Inducer (+)/ Inhibitor (-) of autophagy	Effect	Reference
ECs, *Apoe*$^{-/-}$; *Atg5*$^{flox/flox}$; *VE-cadherin-cre mice*	Wortmannin (-), *Atg5* shRNA (-)	Failure of EC alignment under high shear stress.	(Vion, et al., 2017)
ECs	*Atg3* siRNA (-)	Impaired endothelial nitric oxide synthase phosphorylation, higher accumulation of ROS and inflammatory cytokines like MCP-1 and IL-8 in response to shear stress	(Bharath, et al., 2014)
VSMCs	PDGF (+)	Induces phenotype switching	(Salabei, et al., 2013)
Cultured rat aortic rings, Bovine aortic smooth muscle cells	Valporic acid (+)	Ameliorate Pi- induced VC	(Dai, et al., 2013)
HMEC-1 cells	High Pi (+)	Blocks Pi induced apoptosis	(Hsu, et al., 2015)
VSMCs *Atg7fl/fl SM22α-Cre*$^{+}$	*ATG7 KO* (-)	Elevated intracellular levels of Ca^{2+}	(Michiels, et al., 2015)
VSMCs, calcified arteries	Estrogen (+)	Inhibits the osteoblastic differentiation of VSMCs	(Peng, et al., 2017)
DBA/2 mice model for uremic media calcification	Rapamycin (+)	Reduced aortic calcification, reduced release of proinflammatory	(Frauscher, et al., 2018)

		cytokines, enhanced survival	
Diabetic arteries	Rapamycin (+)	Suppressed calcification	(Heath, et al., 2014)
Diabetic mouse Aorta	tBHQ (+)	Atheroprotective, reduction in inflammation and lipid content of plaques	(Zechner, et al., 2017)
VSMCs	Metformin (+)	Restores mitochondrial biogenesis.	(W. Q. Ma, et al., 2019)
VSMCs	Overexpression of BNIP3 (+)	Attenuates lactate induced calcification and enhances mitophagy	(Y. Zhu, et al., 2019)
VSMCs *ApoE*$^{-/-}$ mice	Spermidine (+)	Reduced lipid accumulation and necrotic core formation in atherosclerotic plaques	(Michiels, et al., 2016)

Figure legends

Figure 1: Overview of key molecular players in the autophagy pathway. **a)** Phagophore formation is triggered under cellular stress which engulfs cellular debris including damaged mitochondria, bits and pieces of ER, ribosomes etc. **b)** This phagophore matures into a double membrane autophagosome decorated with ATG8/LC3-PE. **c)** Mature autophagosomes fuse with single membrane lysosomes with variety of hydrolases in their lumen which degrade the content of the autophagosome. **d)** This fusion compartment is called autolysosome which returns the recycled building blocks to the cell.

Figure 2: Role of autophagy in the phenotypic transition of VSMCs during the progression of VC. a) Basal levels of autophagy are required by the VSMCs for maintaining both the contractile and the osteogenic phenotype. **b)** Increased concentrations of PDGF and intracellular Pi induces cytoprotective autophagy to counter ROS and apoptosis associated with an osteogenic phenotype**. c)** Inducing autophagy using estrogen, rapamycin, metformin prevents osteogenic phenotypic switching.

Figure 3: A possible crosstalk among endosomes, dysfunctional mitochondria, autophagic vesicles and MVs in VC. a) CPP or hydroxyapatite enters the cells via endocytosis and these endosomes fuse with autophagosomes and are passed on to autolysosomes. **b)** Hydroxyapatite may be degraded inside the autolysosomes. **c)** Or is passed on to autolysosomes packed in nano MVs, these autolysosomes fuse with plasma membrane and release of MVs to the ECM, where they aid in hydroxyapatite formation. **d)** Mitochondria derived-vesicles as a source of calcification could be engulfed by autophagosomes or directly taken up by lysosomes where either they could be degraded or released outside the cell via autolysosomes.

Fig. 1

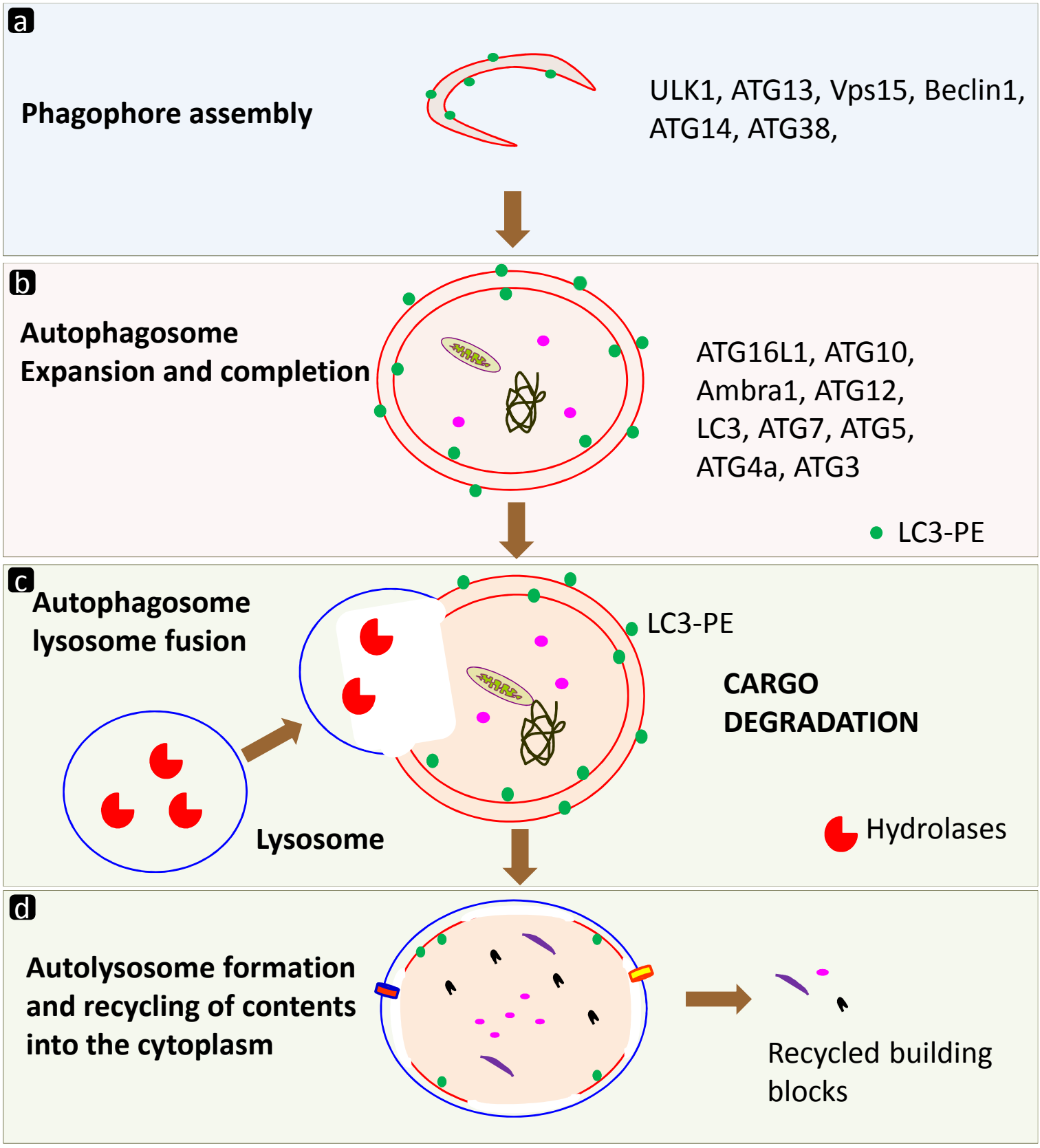

a
Phagophore assembly
ULK1, ATG13, Vps15, Beclin1,
ATG14, ATG38,
b
Autophagosome
Expansion and completion
ATG16L1, ATG10,
Ambra1, ATG12,
LC3, ATG7, ATG5,
ATG4a, ATG3
LC3-PE
c
Autophagosome
lysosome fusion
LC3-PE
CARGO
DEGRADATION
Lysosome
Hydrolases
d
Autolysosome formation
and recycling of contents
into the cytoplasm
Recycled building
blocks

Fig. 2

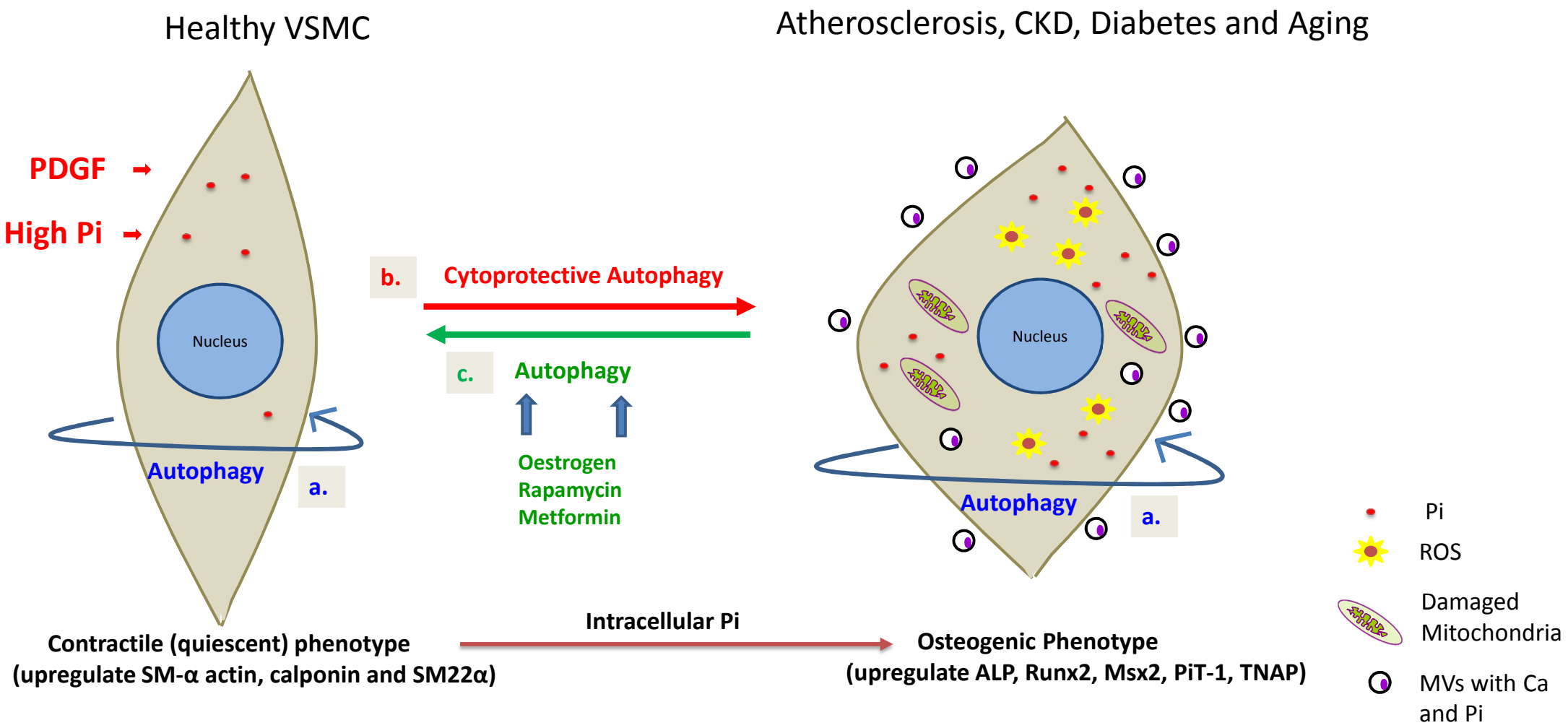
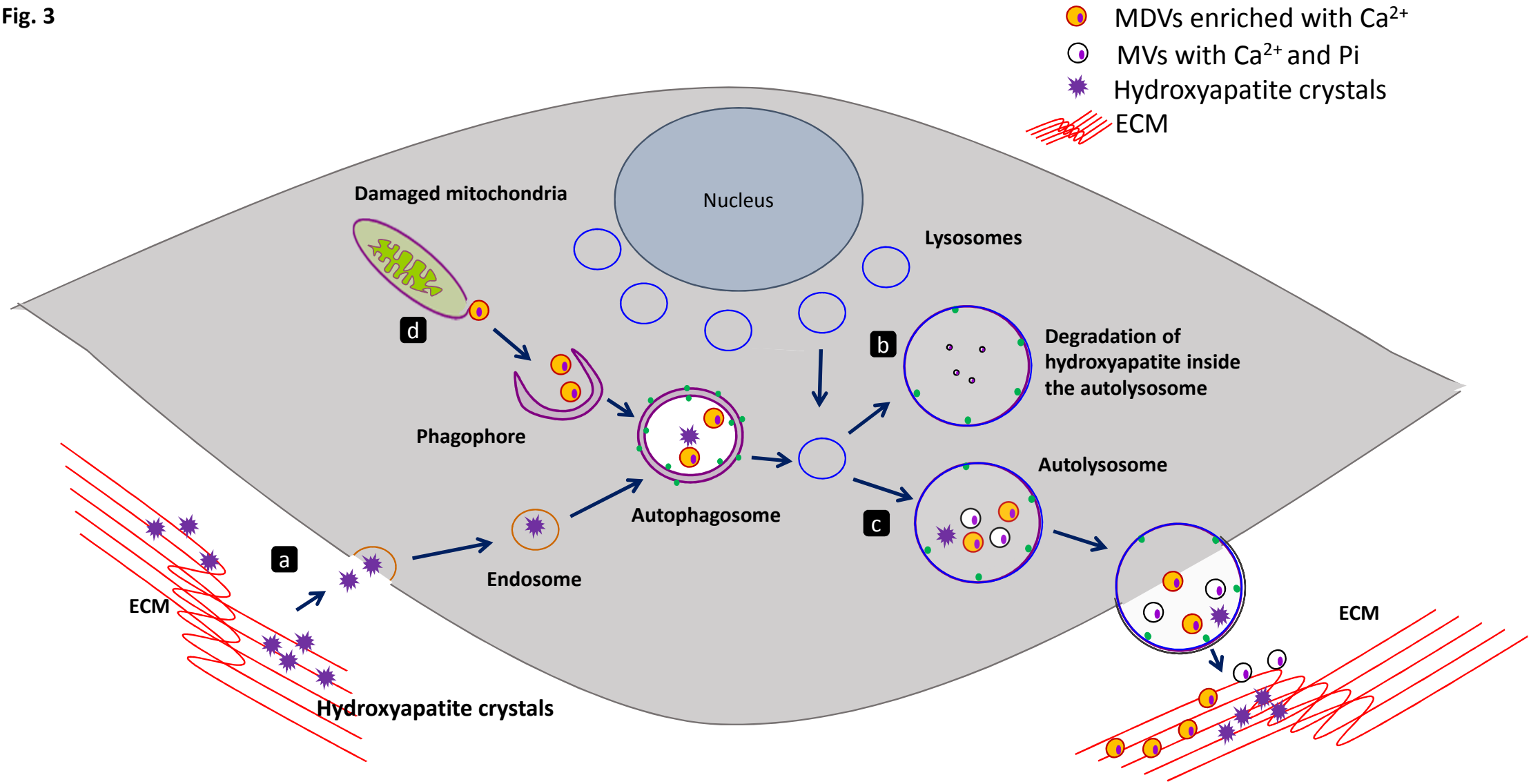

Healthy VSMC
Atherosclerosis, CKD, Diabetes and Aging
PDGF
High Pi
Nucleus
Autophagy
a.
b.
Cytoprotective Autophagy
c.
Autophagy
Oestrogen
Rapamycin
Metformin
Nucleus
Autophagy
a.
Pi
ROS
Damaged Mitochondria
MVs with Ca and Pi
Intracellular Pi
Contractile (quiescent) phenotype
(upregulate SM-α actin, calponin and SM22α)
Osteogenic Phenotype
(upregulate ALP, Runx2, Msx2, PiT-1, TNAP)

Fig. 3


MDVs enriched with Ca^{2+}
MVs with Ca^{2+} and Pi
Hydroxyapatite crystals
ECM
Damaged mitochondria
Nucleus
Lysosomes
d
Phagophore
b
Degradation of hydroxyapatite inside the autolysosome
Autolysosome
Autophagosome
c
a
Endosome
ECM
Hydroxyapatite crystals
ECM