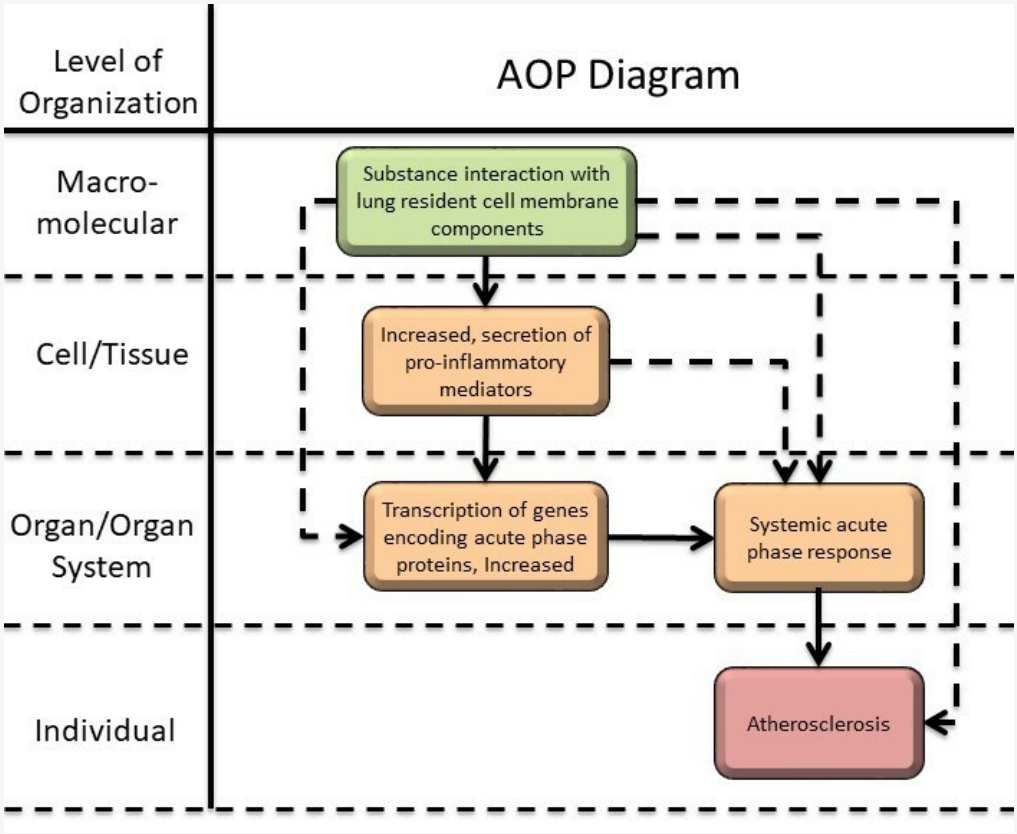
AOP ID and Title:

AOP 237: Substance interaction with lung resident cell membrane components leading to atherosclerosis
Short Title: Interaction with lung cells leading to atherosclerosis

Graphical Representation



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Abstract

AOP237 describes key events initiated with the interaction of substances with the membrane components of the pulmonary cells, and leading to atherosclerosis in humans. Atherosclerosis is defined as the thickening of the wall of an artery due to plaque deposition, and this condition can lead to severe events as myocardial infarction and stroke. This AOP presents the induction of acute phase response as a pathway for atherosclerosis progression. The interaction between a substance and the lung resident cell membrane components is the molecular initiating event (MIE; Event 1495) for this AOP; this interaction leads to an increased secretion of proinflammatory mediators [Key event (KE)1;

Event 1496]. The release of proinflammatory factors triggers an increase in transcription of genes encoding acute phase proteins (KE2; Event 1438), leading to systemic acute phase response (KE3;Event 1439) once the acute phase proteins are translated and released into the systemic circulation. A continuous acute phase response leads to atherosclerosis, the adverse outcome (AO) of this AOP (Event 1443).

AOP 237 mainly focus on particles or particulate matter as stressors, however other compounds or inflammatory conditions that induce acute phase response, can be consider stressors and lead to atherosclerosis. In addition, most of the evidence is based on animal studies (mice) as a model for the human system, however the adverse outcome of the present AOP, atherosclerosis, is only applicable to humans. The AOP presents the biological plausibility, evidence and quantitative understanding for the relationship between KEs. In addition, evidence that KE2, KE3 and KE4 occur after the MIE is presented as non-adjacent relationships. This AOP presents a mechanism of substance-induced acute phase response leading to atherosclerosis, and it can be used for regulatory purposes and health-based risk assessments of inhalable materials.

Background

Cardiovascular disease (CVD) is the leading cause of death worldwide, being responsible for 32% of all deaths in 2019 (WHO; http://www.who.int). The term CVD covers all diseases of the cardiovascular system, including atherosclerosis, which is manifested as increased plaque deposition or build-up in the arteries. Although, atherosclerosis is not a cause of death, it can lead to fatal conditions as stroke and myocardial infarction. Atherosclerosis is normally an asymptotic disease and is initiated by a biological, chemical or physical insult to the artery walls. This leads to the expression of cell adhesion molecules on the endothelial lining of the arteries, which facilitates the activation, recruitment, and migration of monocytes through the endothelial monolayer (Cybulsky et al., 2001; Hansson & Libby, 2006). Inside the intima layer, the monocytes differentiate into macrophages and internalize fatty deposits (mainly oxidized low-density lipoprotein). This results in them transforming into foam cells, which is a major component of the atherosclerotic fatty streaks. The fatty streaks reduce the elasticity of the artery walls and the foam cells promote a pro-inflammatory environment by secretion of cytokines and reactive oxidative species. In addition, foam cells also induce the recruitment of smooth muscle cells to the intima. Added together, these changes lead to the formation of plaques on the artery walls. A fibrous cap of collagen and vascular smooth muscle cells protects the necrotic core and stabilizes the plaque (Libby, 2012; Virmani et al., 2005). However, blood clots can be formed if the plaque ruptures. These may travel with the bloodstream and obstruct the blood flow of smaller vessels, e.g. the coronary arteries, which ultimately can lead to myocardial infarction.

Inhalation of particulate matter, chemicals and pathogens have been related to increased pulmonary inflammation. Whereas a normal immune reaction is crucial for effective elimination of threats to the body, chronic and unresolved inflammation has been linked to both adverse pulmonary and adverse systemic effects in humans. In concordance with this, various retrospective and prospective epidemiological studies have linked pulmonary exposure to respirable air particulates with increased the risk of developing CVD (Clancy, Goodman, Sinclair, & Dockery, 2002; Dockery et al., 1993; Pope et al., 2004; Pope et al., 1995). Inhalation of particles has been proposed to affect the cardiovascular system in several different ways, including through disruption of vasomotor function and through acceleration of plaque progression in atherosclerosis (Cao et al., 2014; Moller et al., 2016).

Acute phase response is characterized by the change in plasma concentration of acute phase proteins (APP), along with other physiological changes during inflammatory conditions (Gabay & Kushner, 1999; Mantovani & Garlanda, 2023). Serum amyloid A (SAA) and C-reactive protein (CRP) are the major acute phase proteins in humans and are considered risk factors for CVDs (Table 1 presents acute phase response characteristics in humans and mice). In particular, SAA restricts the transport of cholesterol to the liver, allowing the accumulation of cholesterol in arteries and the formation of foam cells.

Table 1. Selected differences in APR between humans and mice.

Characteristic	Humans	Mice
Number of identified genes involved in acute phase response	61	62
Major acute phase proteins	CRP, SAA	Haptoglobin, SAA, serum amyloid P
Moderate and minor acute phase proteins	Haptoglobin, fibrinogen, α_1 acid glycoprotein	CRP, fibrinogen
SAA isoforms	*Saa1, Saa2* and *Saa4*	*Saa1, Saa2, Saa3* and *Saa4*

References: (Cray, 2012; Gabay & Kushner, 1999; NCBI, 2023; Tannock et al., 2018).

Atherosclerosis is a disease influenced by multiple factors including high levels of lipoproteins in blood, elevated blood pressure, smoking, obesity, type 2 diabetes, diet, and physical activity (Herrington, Lacey, Sherliker, Armitage, & Lewington, 2016; Libby et al., 2019; Raitakari, Pahkala, & Magnussen, 2022). Inflammation is also involved in atherosclerosis, providing pathways via which risk factors might cause the development and advancement of atherosclerotic plaques (Libby, 2021a, 2021b). Therefore, although inflammation and acute phase response are not the only causes of atherosclerosis, the early key events (KE1, KE2 and KE3) can be used to evaluate the particle-

induced risk of developing atherosclerosis.

For the development of AOP 237, the MIE and KE1 from AOP 173 have been used (AOP 173: Substance interaction with the pulmonary resident cell membrane components leading to pulmonary fibrosis). The information presented in AOP 173 has not been modified for AOP 237.

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Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1495	Substance interaction with the lung resident cell membrane components	Interaction with the lung cell membrane
	KE	1496	Increased, secretion of proinflammatory mediators	Increased proinflammatory mediators
2	KE	1438	Transcription of genes encoding acute phase proteins, Increased	Increased transcription of genes encoding acute phase proteins
3	KE	1439	Systemic acute phase response	Systemic acute phase response
7	AO	1443	Atherosclerosis	Atherosclerosis

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components	adjacent	Increased, secretion of proinflammatory mediators	High	Low
Increased, secretion of proinflammatory mediators	adjacent	Transcription of genes encoding acute phase proteins, Increased	High	Moderate
Transcription of genes encoding acute phase proteins, Increased	adjacent	Systemic acute phase response	High	Moderate
Systemic acute phase response	adjacent	Atherosclerosis	High	High
Substance interaction with the lung resident cell membrane components	non-adjacent	Transcription of genes encoding acute phase proteins, Increased	High	Moderate
Substance interaction with the lung resident cell membrane components	non-adjacent	Systemic acute phase response	High	Moderate
Increased, secretion of proinflammatory mediators	non-adjacent	Systemic acute phase response	High	Moderate
Substance interaction with the lung resident cell membrane components	non-adjacent	Atherosclerosis	High	Moderate

Stressors

Name	Evidence
Lipopolysaccharide	Not Specified
Graphene oxide nanoparticles	Not Specified

Name	Evidence
Carbon nanotubes	Not Specified
Insoluble nano-sized particles	Not Specified
Virus	Not Specified

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI

Sex Applicability

Sex	Evidence
Male	High
Female	High

This AOP is applicable to adult humans of both sexes. Although atherosclerosis is a condition that begins during childhood and progresses through life, its clinical manifestation is mostly observed in older individuals (Raitakari et al., 2022).

The AOP is applicable to all stressors that can be inhaled and, therefore, interact with the pulmonary cells and induce pulmonary inflammation.

Essentiality of the Key Events

For the development of AOP 237, the molecular initiating event (MIE) and key event (KE) 1 from AOP 173 have been reused (AOP 173: Substance interaction with the pulmonary resident cell membrane components leading to pulmonary fibrosis). The information presented in AOP 173 has not been modified for AOP 237.

	Defining question	High	Moderate	Low
Support for essentiality of KEs	What is the impact on downstream KEs and/or the AO if an upstream KE is modified or prevented?	Direct evidence from specifically designed experimental studies illustrating prevention or impact on downstream KEs and/or the AO if upstream KEs are blocked or modified	Indirect evidence that modification of one or more upstream KEs is associated with a corresponding (increase or decrease) in the magnitude or frequency of downstream KEs	No or contradictory experimental evidence of the essentiality of any of the KEs.

MIE: Substance interaction with the lung resident cell membrane components (Event 1495)	**Moderate.** Stressors have a dose-response relationship with transcription of genes encoding acute phase proteins (KE2) and systemic acute phase response (KE3) (Bengtson et al., 2017; Di Ianni et al., 2020; Monse et al., 2018; Poulsen et al., 2017; Saber et al., 2013). Knockout of toll-like receptor 4 (*Tlr4)* prevents the lipopolysaccharide-induced increase of cytokine/chemokines mRNA levels in lung and liver tissues (KE1) and prevents lipopolysaccharide-induced systemic acute phase response (KE3) in mice (Danielsen et al., 2021). Knockout of toll-like receptor 2 (*Tlr2)* prevents the multiwalled carbon nanotubes-induced increase of *Saa1* mRNA levels in liver tissue (KE2) and serum amyloid A (SAA)1 levels in plasma (KE3) in mice (Danielsen et al., 2021).
KE1: Increased, secretion of proinflammatory mediators (Event 1496)	**High.** Interleukin (IL) 6 gene disruption (IL-6$^{-/-}$) reduces the liver mRNA levels (KE2) and serum levels (KE3) of the acute phase proteins haptoglobin, α1-acid glycoprotein and SAA in mice (Kopf et al., 1994). Blockage of IL-6 receptors reduced SAA1 mRNA, while blockage of IL-1β and tumor necrosis factor α receptors partially reduces the expression of SAA1 mRNA (KE2), in hepatic cell lines (Hagihara et al., 2004). Administration of monoclonal antibodies for IL-1β reduces blood levels of C-reactive protein (CRP) (KE2 and KE3), and decreased the incidence rates of recurrent cardiovascular events (AO), in patients with a history of myocardial infarction (Ridker et al., 2017)
KE2: Transcription of genes encoding acute phase proteins, Increased (Event 1438)	**High.** Gene transcription is necessary for the synthesis of proteins (KE3) (Alberts, 2017). Suppression of SAA3 and double knockout of SAA1/SAA2 reduces atherosclerotic plaque area (AO), in ApoE$^{-/-}$ mice (Thompson et al., 2018).
KE3: Systemic acute phase response (Event 1439)	**High.** Elevated levels of SAA induce plaque progression (AO) (Christophersen et al., 2021; Dong et al., 2011; Thompson et al., 2018). CRP and SAA levels are predictive of risk of cardiovascular disease (Pai et al., 2004; Ridker, Hennekens, Buring, & Rifai, 2000).
AO: Atherosclerosis (Event 1443)	**N/A.** This is the AO and it is essential for the AOP.

Uncertainties or Inconsistencies

- Physicochemical characteristics of nanomaterials such as size, surface area, surface functionalization, shape, composition, among others, affect the magnitude and duration of acute phase response in mice (Bengtson et al., 2017; Gutierrez et al., 2023; Poulsen et al., 2017). In animal models, both inflammatory and acute phase response are predicted by the total surface area of the retained, insoluble particles (Cosnier et al., 2021; Gutierrez et al., 2023).
- C-reactive protein (CRP) and serum amyloid A (SAA) are risk factors for cardiovascular disease (Ridker et al., 2000). However, Mendelian randomization studies have shown that CRP genotypes are not associated with risk of coronary heart disease and that genetically elevated levels of CRP are not associated with coronary heart disease risk (Collaboration et al., 2011; Elliott et al., 2009).
- In mice studies, it is possible to measure both Saa gene expression and SAA protein levels, however the dynamic range for *Saa* gene expression is larger. In humans, measuring gene expression of acute phase proteins is not

very common, as a tissue sample is needed, while measuring acute phase protein in blood is more common.
- It is suggested that acute phase proteins are mainly produced in the liver (Gabay & Kushner, 1999), however in mice the liver has little upregulation of *Saa* genes after exposure to ultrafine carbon particles or diesel exhaust particle. On the other hand, the lung shows a marked expression of *Saa3* mRNA (Saber et al., 2009; Saber et al., 2013).
- A level of inconsistency between the results from human studies exists. It has been observed that in most controlled human studies, an increase in CRP and/or SAA was observed after exposure to particulate matter (Baumann et al., 2018; Haase et al., 2022; Monse et al., 2018; Monse et al., 2021; Walker et al., 2022; Wyatt, Devlin, Rappold, Case, & Diaz-Sanchez, 2020). However, in other studies the exposure did not induce acute phase response (Andersen, Saber, Clausen, et al., 2018; Andersen, Saber, Pedersen, et al., 2018), maybe due to low levels of exposure (Andersen et al., 2019) or limited statistical power.

Weight of Evidence Summary

Biological plausibility of each KER

Please also refer to AOP173: Substance interaction with the pulmonary resident cell membrane components leading to pulmonary fibrosis, which shares MIE and KE1 with the present AOP.

	Defining question	**High**	**Moderate**	**Low**
Support for Biological Plausibility of KERs	Is there a mechanistic (i.e., structural or functional) relationship between KEup and KEdown consistent with established biological knowledge?	Extensive understanding based on extensive previous documentation and broad acceptance - Established mechanistic basis	The KER is plausible based on analogy to accepted biological relationships but scientific understanding is not completely established.	There is empirical support for a statistical association between KEs (See 3.), but the structural or functional relationship between them is not understood.
MIE => KE1: Interaction with the lung cell membrane leads to Increased proinflammatory mediators (Relationship 1702)	Biological Plausibility of the MIE => KE1 is**High**. Rationale: There is extensive evidence showing that interaction of stressors with the respiratory system induces the release of proinflammatory markers (Behzadi et al., 2017; Denholm & Phan, 1990; Dostert et al., 2008; Mossman & Churg, 1998).			
KE1 => KE2: Increased proinflammatory mediators leads to Increased transcription of genes encoding acute phase proteins (Relationship 2053)	Biological Plausibility of the KE1 => KE2 is**High**. Rationale: Acute phase proteins are induced by pro-inflammatory cytokines. These cytokines are produced at sites of inflammation mainly by monocytes and macrophages (Gabay & Kushner, 1999; Mantovani & Garlanda, 2023; Uhlar & Whitehead, 1999; Venteclef, Jakobsson, Steffensen, & Treuter, 2011).			
KE2 => KE3: Increased transcription of genes encoding acute phase proteins leads to Systemic acute phase response (Relationship 1589)	Biological Plausibility of the KE2 => KE3 is**High**. Rationale: After gene expression of acute phase proteins in tissues mRNA is translated and folded into proteins (Alberts, 2017). These proteins are then release to the systemic circulation (Van Eeden, Leipsic, Paul Man, & Sin, 2012).			

KE3 => AO: Systemic acute phase response leads to Atherosclerosis (Relationship 2860)	Biological Plausibility of the KE3 => KE2 is**High**. Rationale: During acute phase response, serum amyloid A (SAA), one of the major acute phase proteins, replaces apolipoprotein A-1 from high density lipoprotein (HDL). This replacement obstructs the reverse transport of cholesterol to the liver, allowing the accumulation of cholesterol in cells (Lindhorst, Young, Bagshaw, Hyland, & Kisilevsky, 1997; McGillicuddy et al., 2009; Meek, Urieli-Shoval, & Benditt, 1994).
Non-adjacent MIE => KE2: Interaction with the lung cell membrane leads to Increased transcription of genes encoding acute phase proteins (Relationship 2958)	Biological Plausibility of the MIE => KE2 is**High**. Rationale: After cells sense pathogens, tissue damage or dysmetabolism, production of acute phase proteins is triggered by cellular pattern-recognition molecules, through a cytokine cascade (Mantovani & Garlanda, 2023). There is extensive evidence that nanomaterials induce the expression of acute phase response genes in mice (Bengtson et al., 2017; Di Ianni et al., 2020; Erdely, Liston, et al., 2011; Gutierrez et al., 2023; Hadrup et al., 2019; Halappanavar et al., 2015; Poulsen, Saber, Mortensen, et al., 2015; Saber et al., 2013).
Non-adjacent MIE => KE3: Interaction with the lung cell membrane leads to Systemic acute phase response (Relationship 2959)	Biological Plausibility of the MIE => KE3 is**High**. Rationale: Pulmonary inflammation occurs when stressors interact with the airways (Moldoveanu et al., 2009) and acute phase response is induced during inflammatory conditions (Gabay & Kushner, 1999). There is plenty of evidence showing that inhalation or instillation of stressors induces systemic acute phase response in humans and mice mice (Baumann et al., 2016; Bendtsen et al., 2019; Bengtson et al., 2017; Bourdon et al., 2012; Erdely, Liston, et al., 2011; Kim, Chen, Boyce, & Christiani, 2005; Monse et al., 2018; Monse et al., 2021; Poulsen et al., 2017; Poulsen, Saber, Williams, et al., 2015; Westberg et al., 2016).
Non-adjacent KE1 => KE3: Increased proinflammatory mediators leads to Systemic APR (Relantionship 3052)	Biological Plausibility of the KE1 => KE3 is**High**. Rationale: Pro-inflammatory cytokines induce the release of acute phase proteins. These proteins are released from inflammatory sites to the systemic circulation (Gabay & Kushner, 1999; Mantovani & Garlanda, 2023).
Non-adjacent MIE => AO: Interaction with the lung cell membrane leads to Atherosclerosis (Relantionship 2960)	Biological Plausibility of the MIE => AO is**Moderate**. Rationale: There is evidence that the interaction of the lungs with stressor induces atherosclerotic plaque progression; however, the mechanistic relationship has not been clarified (Christophersen et al., 2021; Erdely, Hulderman, et al., 2011; M. R. Miller et al., 2013; M. R. Miller & Newby, 2020; Van Eeden et al., 2012).

Empirical support for each KER

Please also refer to AOP173: Substance interaction with the pulmonary resident cell membrane components leading to pulmonary fibrosis, which shares MIE and KE1 with the present AOP.

	Defining question	**High**	**Moderate**	**Low**

Empirical Support	Does KEup occur at lower doses and earlier time points than KE down and at the same dose of prototypical stressor, is the incidence of KEup > than that for KEdown? Are there inconsistencies in empirical support across taxa, species and prototypical stressor that don't align with expected pattern for hypothesised AOP?	Multiple studies showing dependent change in both events following exposure to a wide range of specific prototypical stressors. (Extensive evidence for temporal, dose-response and incidence concordance) and no or few critical data gaps or conflicting data	Demonstrated dependent change in both events following exposure to a small number of specific prototypical stressors and some evidence inconsistent with expected pattern that can be explained by factors such as experimental design, technical considerations, differences among laboratories, etc.	Limited or no studies reporting dependent change in both events following exposure to a specific prototypical stressor (i.e., endpoints never measured in the same study or not at all); and/or significant inconsistencies in empirical support across taxa and species that don't align with expected pattern for hypothesised AOP
MIE => KE1: Interaction with the lung cell membrane leads to Increased proinflammatory mediators (Relationship 1702)	Empirical Support of the MIE => KE1 is**Moderate**. Rationale: There are limited *in vitro* studies which show a temporal and dose-dependent relationship between these two events (Chan et al., 2018; Denholm & Phan, 1990; Roy, Singh, Das, Tripathi, & Dwivedi, 2014).			
KE1 => KE2: Increased proinflammatory mediators leads to Increased transcription of genes encoding acute phase proteins (Relationship 2053)	Empirical Support of the KE1 => KE2 is**High**. Rationale: There are several studies showing a dose concordance and temporal concordance between KEs (Bendtsen et al., 2019; Di Ianni et al., 2020; Kyjovska et al., 2015; Saber et al., 2012; Saber et al., 2013; Wallin et al., 2017).			
KE2 => KE3: Increased transcription of genes encoding acute phase proteins leads to Systemic acute phase response (Relationship 1589)	Empirical Support of the KE2 => KE3 is**High**. Rationale: There are studies showing a dose concordance and temporal concordance between KE (Bengtson et al., 2017; Gutierrez et al., 2023; Poulsen et al., 2017). However, there are inconsistencies between gene expression and translation of acute phase proteins.			
KE3 => AO: Systemic acute phase response leads to Atherosclerosis (Relationship 2860)	Empirical Support of the KE3 => AO is**Moderate**. Rationale: There is a limited number of animal studies showing the relationship between the KEs, in addition of epidemiological studies showing association between the KEs (Christophersen et al., 2021; Dong et al., 2011; Pai et al., 2004; Rivera et al., 2013; Thompson et al., 2015; Thompson et al., 2018).			

Non-adjacent MIE => KE2: Interaction with the lung cell membrane leads to Increased transcription of genes encoding acute phase proteins (Relationship 2958)	Empirical Support of the MIE => KE2 is**Moderate**. Rationale: There are several studies showing a dose concordance and temporal concordance in animal studies. However, in the case of nanomaterials it has been shown that physicochemical characteristics affect the magnitude and duration of the expression of acute phase proteins in mice (Bengtson et al., 2017; Bourdon et al., 2012; Gutierrez et al., 2023; Kyjovska et al., 2015; Poulsen et al., 2017; Saber et al., 2013; Wallin et al., 2017).
Non-adjacent MIE => KE3: Interaction with the lung cell membrane leads to Systemic acute phase response (Relationship 2959)	Empirical Support of the MIE => KE3 is**Moderate**. Rationale: There are plenty of studies showing a dose concordance and temporal concordance in animal and controlled human studies (Brand et al., 2014; Erdely, Liston, et al., 2011; Kim et al., 2005; Monse et al., 2018; Monse et al., 2021; Poulsen et al., 2017; Walker et al., 2022; Wyatt et al., 2020). However, it has been observed that systemic acute phase response is not always observed after exposure.
Non-adjacent KE1 => KE3: Increased proinflammatory mediators leads to Systemic APR (Relantionship 3052)	Empirical Support of the KE1 => KE3 is**Moderate**. Rationale: There are several studies showing a dose concordance and temporal concordance. However, there are inconsistencies between changes in blood levels of pro-inflammatory mediators and systemic APR (Baumann et al., 2016; Kim et al., 2005; Monse et al., 2018; Monse et al., 2021; Poulsen et al., 2017).
Non-adjacent MIE => AO: Interaction with the lung cell membrane leads to Atherosclerosis (Relantionship 2960)	Empirical Support of the MIE => AO is**Moderate.** Rationale: There are several studies showing the relationship between the key events (Christophersen et al., 2021; Li et al., 2007; Mikkelsen et al., 2011; M. R. Miller et al., 2013).

Quantitative Consideration

The table below presents the quantitative understanding of every KER.

It is important to clarify that when assessing stressors in mice studies, it is possible to measure the gene expression of acute phase proteins (KE2) in different tissues, whereas in humans this is not likely as a tissue sample would be required. On the other hand, in humans it is much more common and easier to measure systemic acute phase response (KE3) through a blood sample. In mice, it has been shown that *Saa3* mRNA in lung tissue and blood levels of serum amyloid A (SAA)3 are correlated (Gutierrez et al., 2023). In addition, SAA levels in mice and humans seem to be in level in magnitude after exposure to zinc oxide nanoparticles (Gutierrez et al., 2023). This suggest that systemic acute phase response in humans can be estimated from mice studies.

Saa3 mRNA in lung tissue is also correlated to pulmonary inflammation measured as neutrophil numbers in broncheoalveolar lavage fluid (i.e. indirect marker of the release of pro-inflammatory factors because the release of inflammatory mediators) in mice after pulmonary exposure to nanomaterials. Both of these endpoints can be estimated by calculating the dosed surface area (specific surface area multiplied by dose level) (Gutierrez et al., 2023).

Finally, the relative risk of people developing a cardiovascular disease can be calculated from blood levels of acute phase proteins in epidemiological studies.

KER	**Quantitative understanding**

MIE => KE1: Interaction with the lung cell membrane leads to Increased proinflammatory mediators (Relationship 1702)	The quantitative understanding of MIE => KE1 is **Low**. Rationale: The quantitative prediction of the release of proinflammatory factors can be made from the interaction of the stressors with the pulmonary system. In the case of some stressors (nanomaterials) it is possible to make a prediction using the dosed surface area of the materials and neutrophil numbers in broncheoalveolar lavage (BALF) as an indirect marker of the release of pro-inflammatory factors (Gutierrez et al., 2023; Oberdorster, Ferin, Gelein, Soderholm, & Finkelstein, 1992; Oberdorster, Ferin, & Lehnert, 1994; Schmid & Stoeger, 2016; Stoeger et al., 2006).
KE1 => KE2: Increased proinflammatory mediators leads to Increased transcription of genes encoding acute phase proteins (Relationship 2053)	The quantitative understanding is of KE1 => KE2 is **Moderate**. Rationale: In mice, the gene expression o f *Saa* after exposure to metal oxide nanomaterials can be estimated using an indirect marker of the release of pro-inflammatory factors (neutrophil numbers in BALF) (Gutierrez et al., 2023).
KE2 => KE3: Increased transcription of genes encoding acute phase proteins leads to Systemic acute phase response (Relationship 1589)	The quantitative understanding of KE2 => KE3 is **Moderate**. Rationale: In mice, the systemic levels of SAA after exposure to metal oxide nanomaterials can be estimated from the gene expression in lung tissue (Gutierrez et al., 2023).
KE3 => AO: Systemic acute phase response leads to Atherosclerosis (Relationship 2860)	The quantitative understanding is of KE3 => AO is **High**. Rationale: The risk of developing a cardiovascular disease at population level can be calculated from blood levels of acute phase proteins (KER 2860).
Non-adjacent MIE => KE2: Interaction with the lung cell membrane leads to Increased transcription of genes encoding acute phase proteins (Relationship 2958)	The quantitative understanding of MIE => KE2 is **Moderate.** Rationale: In mice, the gene expression o f *Saa* after exposure to metal oxide nanomaterials can be estimated from the dosed surface area (Gutierrez et al., 2023).
Non-adjacent MIE => KE3: Interaction with the lung cell membrane leads to Systemic acute phase response (Relationship 2959)	The quantitative understanding of MIE => KE3 is **Moderate**. Rationale: In mice, the blood levels of SAA after exposure to metal oxide nanomaterials can be estimated from the dosed surface area (Gutierrez et al., 2023).

Non-adjacent KE1 => KE3: Increased proinflammatory mediators leads to Systemic APR (Relantionship 3052)	The quantitative understanding of KE1 => KE3 is **Moderate**. Rationale: In mice, the blood levels of SAA after exposure to metal oxide nanomaterials and multiwalled carbon nanotubes can be estimated from neutrophil numbers in BALF (Gutierrez et al., 2023; Poulsen et al., 2017).
Non-adjacent MIE => A O : Interaction with the lung cell membrane leads to Atherosclerosis (Relantionship 2960)	The quantitative understanding of MIE => AO is **Moderate**. Rationale: Epidemiological studies have shown the risk ratios of having a cardiovascular event per increase or decrease of exposure to particulate matter (Beelen et al., 2014; Cesaroni et al., 2014; Clancy et al., 2002; K. A. Miller et al., 2007)

Considerations for Potential Applications of the AOP (optional)

Particle-induced acute phase response can be regarded as a critical effect linking particle-exposure to cardiovascular disease. Dose-response relationships can be used to establish no-observed-adverse-effect levels (NOAEL) for regulatory purposes and occupational exposure limits for inhalable materials can be determined through health-based risk assessments. This approach was taken by the Danish National Research Centre for the Working Environment at request of the Danish Working Environment Authority and an occupational exposure limit for zinc oxide was proposed based on the induction of acute phase response as the critical effect (the report can be found in: Dokumentation for helbredsbaserede grænseværdier for kemiske stoffer i arbejdsmiljøet (nfa.dk)).

As mentioned previously, not all KE can easily be measured in humans, therefore animal studies can be used to measure early KEs and perform a risk assessment of different stressors. Additionally, physicochemical properties, such as specific surface area and dissolution, are important predictors of particle-induced acute phase response that can be used for hazard assessment (Gutierrez et al., 2023).

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Appendix 1

List of MIEs in this AOP

Event: 1495: Substance interaction with the lung resident cell membrane components

Short Name: Interaction with the lung cell membrane

Key Event Component

Process	Object	Action
pattern recognition receptor signaling pathway		increased
toll-like receptor signaling pathway	Toll-like receptor	increased
toll-like receptor 4 signaling pathway	Toll-like receptor 4	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:173 - Substance interaction with the pulmonary resident cell membrane components leading to pulmonary fibrosis	MolecularInitiatingEvent
Aop:451 - Interaction with lung resident cell membrane components leads to lung cancer	MolecularInitiatingEvent
Aop:237 - Substance interaction with lung resident cell membrane components leading to atherosclerosis	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adults	High

Sex Applicability

Sex	Evidence
Male	High

Human, mouse, rat.

Although the expression of DAMPs following exposure to pro-fibrotic substances is not assessed across species, it is known that alarmins are released after trauma or injury, and their release is important for initiating the inflammatory response in all species including humans. The immediate acute inflammatory response involving DAMP signalling is also observed in human idiopathic pulmonary fibrosis (IPF); however, anti-inflammatory drugs have proven ineffective for treating IPF. Danger signalling axis including uric acid, adenosine triphosphate and IL-33/ST2 has been proven to promote lung fibrosis in animals.

Key Event Description

The human lung consists of approximately 40 different resident cell types that play different roles during homeostasis, injury, repair and disease states (Franks et al., 2008; Luettich et al., 2021). Of these, resident airway epithelial cells, alveolar/interstitial macrophages and dendritic cells are well characterised for their ability to sense the danger upon interaction with harmful substances and relay the message to mount the necessary immune/inflammatory response. The resident macrophages are present in all tissues, and in a steady state, macrophages contribute to epithelial integrity, survey the tissue for invading pathogens or chemicals and maintain an immunosuppressive environment. Their main function is to clear the incoming irritants and microbes. They are named differently based on the tissue type and their specific functions (Kierdorf et al., 2015).

Substance interactions:

The chemicals or pathogens interact with cellular membrane to gain access to the organisms' interior. A predominant interaction mechanism involves the recognition of innate immune response agonists by pattern recognition receptors (PRRs) present on resident cells such as epithelial and alveolar macrophages. PRRs are also present on other immune and parenchymal cells. PRRs can be activated by two classes of ligands. Pathogen associated molecular patterns (PAMPs) are microbial molecules derived from invading pathogens. PAMPs will not be discussed further as pathogens are not the focus for the AOP presented here. The other class of ligands are called danger associated molecular patterns (DAMPs) that include cellular fragments, nucleic acids, small molecules, proteins and even cytokines released from injured or dying cells (Bianchi, 2007). Most fibrogenic stressors discussed in this AOP act via DAMPs-driven PRR activation. High aspect ratio (HAR) materials such as asbestos or carbon nanotubes (CNTs) pierce the cellular membrane of epithelial cells or resident macrophages resulting in cell injury or non-programmed cellular death. Alveolar macrophages trying to engulf HAR fibres that are long and stiff undergo frustrated phagocytosis because of their inability to engulf the piercing fibres and subsequently lead to cell injury (Boyles et al., 2015; Brown et al., 2007; Donaldson K et al., 2010; Dörger et al., 2001; Mossman and Churg, 1998). The cellular debris from injured or dying cell then serves as ligands for PRRs (Nakayama, 2018), leading to cell activation. In case of pro-fibrotic insoluble particles such as silica, coal dust and nanomaterials (NMs), the particle adsorbed opsonins such as immunoglobulins, complement proteins, or serum proteins act as ligands to the receptors on the macrophage cell surface (Behzadi et al., 2017). The tissue response to these materials resembles that observed following foreign body invasion in lungs.

Toll-like receptors (TLRs) are highly conserved PRRs that are associated with fibrogenic stressors (Desai et al., 2018). Inhibition of TLR-4 is protective against bleomycin-induced fibrosis (Li et al., 2015). However, the exact role and mechanisms by which TLRs mediate lung fibrosis are yet to be uncovered and some studies have shown TLRs to be protective against lung fibrosis (Desai et al., 2018). Asbestos and silica crystals are suggested to engage scavenger receptors present on the macrophages. Mice deficient in class A scavenger macrophage receptor with collagenous structure (MARCO) are shown to induce reduced fibrogenic response following chrysotile asbestos exposure; although, the direct binding of MARCO by asbestos is not investigated in the study (Murthy et al., 2015). In case of soluble substances such as bleomycin, paraquat (Dinis-Oliveira et al., 2008) (N,N'-dimethyl-4, 4′-bipyridinium dichloride) and other soluble fibrogenic chemicals, direct damage of lung epithelial cells and resulting cellular debris or secreted cytokines (DAMPs) serve as triggers for downstream cascading pro-inflammatory events, tissue injury and fibrosis. Engagement of PRRs and consequent cell activation is observed in various organisms including flies and mammals (Denholm and Phan, 1990; Matzinger, 2002).

How it is Measured or Detected

Detection of DAMPs or homeostasis-altering molecular processes:

Cellular interaction with substances or particles can be measured by assessing the release of DAMPs from stressed, injured or dying cells - indicative of binding of PRRs on the cell surface. Release of DAMPs is reflective of substance interaction with resident cells and their activation, a key step in the process of inflammation.

The release of DAMPs can be measured by the techniques listed in the published literature (Nikota et al., 2017; Rabolli et al., 2014; Suwara et al., 2014).

Targeted enzyme-linked immunosorbent assays (ELISA) (routinely used and recommended):

ELISA – permits quantitative measurement of antigens in biological samples. For example, in a cytokine ELISA (sandwich ELISA), an antibody (capture antibody) specific to a cytokine is immobilised on microtitre wells (96-well, 386-well, etc.). Experimental samples or samples containing a known amount of the specific recombinant cytokine are then reacted with the immobilised antibody. Following removal of unbound antibody by thorough washing, plates are reacted with the secondary antibody (detection antibody) that is conjugated to an enzyme such as horseradish peroxidase, which when bound, will form a sandwich with the capture antibody and the cytokine (Amsen and De Visser, 2009). The secondary antibody can be conjugated to biotin, which is then detected by addition of streptavidin linked to horseradish peroxidase. A chromogenic substrate can also be added, which is the most commonly used method. Chromogenic substrate is chemically converted by the enzyme coupled to the detection antibody, resulting in colour change. The amount of colour detected is directly proportional to the amount of cytokine in the sample that is bound to the capture antibody. The results are read using a spectrophotometer and compared to the levels of cytokine in control samples where cytokine is not expected to be secreted or to the samples containing known recombinant cytokine levels.

Interleukin (IL)-1α and -1β is activated or secreted into the cytosol following stimulus (Di Paolo and Shayakhmetov,

2016). Targeted ELISA can be used to quantify IL-1α or IL-1β that is released in the culture supernatant of the cells exposed to toxicants, in bronchoalveolar lavage fluid and serum of exposed animals. The assay is also applicable to human serum, cerebrospinal fluid, and peritoneal fluids.

Similarly, other alarmins can also be quantified by ELISA. Western blot is another method that can be used to quantify the release of various alarmins using specific antibodies. ELISA or real-time reverse transcription-polymerase chain reaction (qRT-PCR) assays can also be used to quantify the expression of genes or proteins that are regulated by the receptor binding – e.g. downstream of TLR binding.

Frustrated phagocytosis and cellular uptake of NMs:

In vitro, interaction of NMs with the cellular membrane is investigated by assessing their uptake by lysosomes (Chen et al., 2013; Nel et al., 2009; Varela et al., 2012). Immunohistochemistry methods targeting lysosome specific proteins are regularly employed for this purpose. In co-localisation experiments, lysosomal marker Lysosomal-associated membrane protein 1 (LAMP1) antibody is used to detect particle co-localisation with lysosomes. A combination of Cytoviva hyperspectral microscope and immunolocalisation (Decan et al., 2016) or confocal microscopy to visualise co-localisation of fluorescence labelled nanoparticles with lysosomal markers have been used.

Frustrated phagocytosis is assessed using microscopic techniques such as time-lapse microscopy, backscatter electron microscopy and others (Donaldson et al., 2010; Murphy et al., 2012; Padmore et al., 2017; Pascolo et al., 2013; Schinwald et al., 2012). In addition, MIE 1668 of AOP303 notes other indirect methods for measuring frustrated phagocytosis.

Cellular co-culture models of the pulmonary epithelium:

Complex co-culture systems, such as those containing epithelial cells and immune cells, better model the environment of the lung epithelium and can be used to study the interaction of potentially pro-fibrotic fibres and particles with resident lung cells. This type of model has been used, alongside electron microscopy, to study lung cell interactions with CNTs following 24 h *in vitro* exposure (Clift *et al.,* 2014). More recently, the EpiAlveolar model, which contains primary human alveolar epithelial cells, endothelial cells, as well as fibroblasts was assessed for its ability to predict fibrosis induced by CNTs (Barasova *et al.,* 2020). Using laser scanning, fluorescence, and enhanced darkfield microscopy, CNT interaction with the resident cells of the model was shown, and this interaction induced the formation of holes in the epithelial model (Barasova *et al.,* 2020). While new co-culture models are a better recapitulation of the native lung environment as compared to traditional mono-cultures, the increased complexity necessitates enhanced expertise in tissue culture techniques, and can make them less practical as compared to submerged mono culture methods.

Ex vivo model of the lung – Precision cut lung slices (PCLS):

Even closer to the *in vivo* condition than co-culture models, PCLS techniques capture the native lung architecture, cell-cell communication and cellularity of the lung. Advancement in culturing and cryopreservation techniques has increased accessibility and use of PCLS for longer term studies (Bai *et al.*, 2016, Neuhaus *et al.*, 2017). These slices can be cultured *ex vivo* for up to a week with minimal reduction in viability, and the technique has recently been assessed for its applicability to assess nanomaterial induced fibrosis *ex vivo* (Rahman *et al.,* 2020). Using multi-walled carbon nanotubes (MWCNTs) and darkfield microscopy, interaction between the nanofibers and the lung epithelium could be determined. The main downside of this technique is the animal requirement, which precludes their use in a first-pass screening context for the MIE.

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List of Key Events in the AOP

Event: 1496: Increased, secretion of proinflammatory mediators

Short Name: Increased proinflammatory mediators

Key Event Component

Process	Object	Action
cytokine production involved in inflammatory response	Cytokine	increased
chemokine secretion	Chemokine	increased
complement activation		increased
	Interleukin	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:173 - Substance interaction with the pulmonary resident cell membrane components leading to pulmonary fibrosis	KeyEvent

AOP ID and Name	Event Type
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
Aop:382 - Angiotensin II type 1 receptor (AT1R) agonism leading to lung fibrosis	KeyEvent
Aop:392 - Decreased fibrinolysis and activated bradykinin system leading to hyperinflammation	KeyEvent
Aop:409 - Frustrated phagocytosis leads to malignant mesothelioma	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:39 - Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response	KeyEvent
Aop:319 - Binding to ACE2 leading to lung fibrosis	KeyEvent
Aop:451 - Interaction with lung resident cell membrane components leads to lung cancer	KeyEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to hyperinflammation (via cell death)	KeyEvent
Aop:237 - Substance interaction with lung resident cell membrane components leading to atherosclerosis	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rats	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adults	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Human, mouse, rat

Cytokines are the common pro-inflammatory mediators secreted following inflammogenic stimuli. Cytokines can be defined as a diverse group of signaling protein molecules. They are secreted by different cell types in different tissues and in all mammalian species, irrespective of gender, age or sex. A lot of literature is available to support cross species, gender and developmental stage application for this KE. The challenge is the specificity; most cytokines exhibit redundant functions and many are pleotropic.

Key Event Description

Pro-inflammatory mediators are the chemical and biological molecules that initiate and regulate inflammatory reactions. Pro-inflammatory mediators are secreted following exposure to an inflammogen in a gender/sex or developmental stage independent manner. They are secreted during inflammation in all species. Different types of pro-inflammatory mediators are secreted during innate or adaptive immune responses across various species (Mestas

and Hughes, 2004). Cell-derived pro-inflammatory mediators include cytokines, chemokines, and growth factors. Blood derived pro-inflammatory mediators include vasoactive amines, complement activation products and others. These modulators can be grouped based on the cell type that secrete them, their cellular localisation and also based on the type of immune response they trigger. For example, members of the interleukin (IL) family including IL-2, IL-4, IL-7, IL-9, IL-15, IL-21, IL-3, IL-5 and Granulocyte-macrophage colony stimulating factor (GM-CSF) are involved in the adaptive immune responses. The pro-inflammatory cytokines include IL-1 family (IL-1α, IL-1β, IL-1rα, IL-18, IL-36α, IL-36β, IL-36γ, IL-36Rα, IL-37), IL-6 family, Tumor necrosis factor (TNF) family, IL-17, and Interferon gamma (IFN-γ) (Turner et al., 2014). While IL-4 and IL-5 are considered T helper (Th) cell type 2 response,IFN-γ is suggested to be Th1 type response.

Different types of pro-inflammatory mediators are secreted during innate or adaptive immune responses across various species (Mestas and Hughes, 2004). However, IL-1 family cytokines, IL-4, IL-5, IL-6, TNF-α, IFN-γ are the commonly measured mediators in experimental animals and in humans. Similar gene expression patterns involving inflammation and matrix remodelling are observed in human patients of pulmonary fibrosis and mouse lungs exposed to bleomycin (Kaminski, 2002).

Literature evidence for its perturbation:

Several studies show increased proinflammatory mediators in rodent lungs and bronchoalveolar lavage fluid, and in cell culture supernatants following exposure to a variety of carbon nanotube (CNT) types and other materials. Poland et al., 2008 showed that long and thin CNTs (>5 µm) can elicit asbestos-like pathogenicity through the continual release of pro-inflammatory cytokines and reactive oxygen species. Exposure to crystalline silica induces release of inflammatory cytokines (TNF-α, IL-1, IL-6), transcription factors (Nuclear factor kappa B [NFκB], Activator protein-1 [AP-1]) and kinase signalling pathways in mice that contain NF-κB luciferase reporter (Hubbard et al., 2002). Boyles et al., 2015 found that lung responses to long multi-walled carbon nanotubes (MWCNTs) included high expression levels of pro-inflammatory mediators Monocyte chemoattractant protein 1 (MCP-1), Transforming growth factor beta 1 (TGF-β1), and TNF-α (Boyles et al., 2015). Bleomycin administration in rodents induces lung inflammation and increased expression of pro-inflammatory mediators (Park et al., 2019). Inflammation induced by bleomycin, paraquat and CNTs is characterised by the altered expression of pro-inflammatory mediators. A large number of nanomaterials induce expression of cytokines and chemokines in lungs of rodents exposed via inhalation (Halappanavar et al., 2011; Husain et al., 2015a). Similarities are observed in gene programs involving pro-inflammatory event is observed in both humans and experimental mice (Zuo et al., 2002).

How it is Measured or Detected

The selection of pro-inflammatory mediators for investigation varies based on the expertise of the lab, cell types studied and the availability of the specific antibodies.

Real-time reverse transcription-polymerase chain reaction (qRT-PCR) – will measure the abundance of cytokine mRNA in a given sample. The method involves three steps: conversion of RNA into cDNA by reverse transcription method, amplification of cDNA using the PCR, and the real-time detection and quantification of amplified products (amplicons) (Nolan et al., 2006). Amplicons are detected using fluorescence, increase in which is directly proportional to the amplified PCR product. The number of cycles required per sample to reach a certain threshold of fluorescence (set by the user – usually set in the linear phase of the amplification, and the observed difference in samples to cross the set threshold reflects the initial amount available for amplification) is used to quantify the relative amount in the samples. The amplified products are detected by the DNA intercalating minor groove-binding fluorophore SYBR green, which produces a signal when incorporated into double-stranded amplicons. Since the cDNA is single stranded, the dye does not bind enhancing the specificity of the results. There are other methods such as nested fluorescent probes for detection, but SYBR green is widely used. RT-PCR primers specific to several pro-inflammatory mediators in several species including mouse, rat and humans, are readily available commercially.

Enzyme-linked immunosorbent assays (ELISA) – permit quantitative measurement of antigens in biological samples. The method is the same as described for the MIE. Both ELISA and qRT-PCR assays are used *in vivo* and are readily applicable to *in vitro* cell culture models, where cell culture supernatants or whole cell homogenates are used for ELISA or mRNA assays. Both assays are straight forward, quantitative and require relatively a small amount of input sample.

Apart from assaying single protein or gene at a time, cytokine bead arrays or cytokine PCR arrays can also be used to detect a whole panel of inflammatory mediators in a multiplex method (Husain et al., 2015b). This method is quantitative and especially advantageous when the sample amount available for testing is scarce. Lastly, immunohistochemistry can also be used to detect specific immune cell types producing the pro-inflammatory mediators and its downstream effectors in any given tissue (Costa et al., 2017). Immunohistochemistry results can be used as weight of evidence; however, the technique is not quantitative and depending on the specific antibodies used, the assay sensitivity may also become an issue (Amsen and De Visser, 2009).

Cell models - of varying complexity have been used to assess the expression of pro-inflammatory mediators. Two dimensional submerged monocultures of the main fibrotic effector cells – lung epithelial cells, macrophages, and fibroblasts – have routinely been used *in vitro* due to the large literature base, and ease of use, but do not adequately mimic the *in vivo* condition (Sharma *et al.,* 2016; Sundarakrishnan *et al.,* 2018). Recently, the EpiAlveolar *in vitro* lung model (containing epithelial cells, endothelial cells, and fibroblasts) was used to predict the fibrotic potential of MWCNTs, and researchers noted increases in the pro-inflammatory molecules TNF-α, IL-1β, and the pro-fibrotic TGF-β using ELISA (Barasova *et al.,* 2020). A similar, but less complicated co-culture model of immortalized human alveolar epithelial cells and idiopathic pulmonary fibrosis patient derived fibroblasts was used to assess pro-fibrotic

signalling, and noted enhanced secretion of Platelet derived growth factor (PDGF) and Basic fibroblast growth factor (bFGF), as well as evidence for epithelial to mesenchymal transition of epithelial cells in this system (Prasad et al., 2014). Models such as these better capitulate the *in vivo* pulmonary alveolar capillary, but have lower reproducibility as compared to traditional submerged mono-culture experiments.

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Event: 1438: Transcription of genes encoding acute phase proteins, Increased

Short Name: Increased transcription of genes encoding acute phase proteins

Key Event Component

Process	Object	Action
acute-phase response	Acute phase proteins	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:237 - Substance interaction with lung resident cell membrane components leading to atherosclerosis	KeyEvent

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
lung

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

- Taxonomic applicability: Acute phase response is part of the immune response and is observed in vertebrate species (Cray et al., 2009).
- Life stage applicability: This key event is applicable to all life stages.
- Sex applicability: This key event is applicable to male and female sexes.

Key Event Description

Acute phase proteins (APPs) are proteins that have an increase in plasma concentration of at least 25% during an acute phase response (Gabay & Kushner, 1999; Mantovani & Garlanda, 2023). In humans, the major APPs are C reactive protein (CRP) and serum amyloid A (SAA), while in mice the major APPs are SAA, haptoglobin and serum amyloid P (Cray, Zaias, & Altman, 2009; Gabay & Kushner, 1999).

It is widely accepted than APPs are mainly produced in the liver, while several other tissues have been shown to express APPs. In humans, APP genes have been shown to be expressed in the adrenal gland, adipose tissue, appendix, gall bladder, heart, kidney, liver, lung, placenta, prostate, salivary gland, skin, small intestine, stomach,

thymus, thyroid, trachea and uterus (de Dios et al., 2018; NCBI, 2023; Schrödl et al., 2016; Urieli-Shoval, Cohen, Eisenberg, & Matzner, 1998; Venteclef, Jakobsson, Steffensen, & Treuter, 2011). In mice, APPs have been shown to be expressed in the adrenal gland, bladder, central nervous system, colon, duodenum, genital fat pad, heart, kidney, large intestine, limbs, liver, lung, mammary gland, ovary, placenta, small intestine, spleen, subcutaneous fat pad, testis and thymus (Kalmovarin et al., 1991; NCBI, 2023; Saber et al., 2013).

Table 1 presents a list of acute phase response genes in humans and mice according the National Center for Biotechnology Information (NCBI): Table 1.

It is important to note that humans and mice express four SAA isoforms (*Saa1, Saa2, Saa3* and *Saa4*), while *Saa3* is a pseudogene in humans (Shridas & Tannock, 2019). CRP is expressed in humans and mice, although only moderately expressed in mice (Pepys & Hirschfield, 2003).

It has been shown that in mice, the *Saa3* isoform is the most differentially expressed APP gene in lung tissue and it is not highly expressed in the liver, while *Saa1* gene is the most differentially expressed in liver tissue after exposure to particles (Halappanavar et al., 2011; Poulsen et al., 2017; Saber et al., 2014).

How it is Measured or Detected

Gene expression of acute phase proteins (APPs) can be measured from tissue samples using quantitative Polymerase Chain Reaction (PCR). This technique allows the amplification of selected fragments of DNA or cDNA by using primers (i.e. known end-portions of the selected DNA). By repeated cycles of transcription, DNA is amplified. The use of fluorescent probes to quantify the expression the targeted DNA, as the binding of the probe to this DNA emits a fluorescent signal. Sequences of RNA can be quantify using PCR, by first synthetizing DNA from a RNA sample, resulting in cDNA. This technique is called reverse transcriptase PCR (Nelson, Nelson, Lehninger, & Cox, 2017).

Other techniques for evaluating the expression of several APPs at the same time are microarray analysis and total RNA sequencing (Halappanavar et al., 2011; Nelson et al., 2017).

To evaluate this key event in mice, gene expression of *Saa3* can be quantified in lung tissue and *Saa1* gene in liver tissue after exposure to a stressor (Halappanavar et al., 2011; Poulsen et al., 2017; Saber et al., 2014).

In humans, it is not common to measure gene expression as a tissue sample is required, however gene expression of *crp*, *saa1*, *saa2* and *saa4* can be measured from samples taken during surgery (Calero et al., 2014).

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Event: 1439: Systemic acute phase response

Short Name: Systemic acute phase response

Key Event Component

Process	Object	Action
acute-phase response	Acute phase proteins	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:237 - Substance interaction with lung resident cell membrane components leading to atherosclerosis	KeyEvent

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term

blood

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

- Taxonomic applicability: Acute phase response is part of the immune response and is observed in vertebrates (Cray et al., 2009).
- Life stages applicability: This key event is applicable to all life stages.
- Sex applicability: This key event is applicable to male and females sexes.

Key Event Description

Acute phase response (APR) is the systemic response to acute and chronic inflammatory states, for example bacterial infection, trauma and infarction. These changes include variations in plasma concentration of proteins, along with other physiological changes. Proteins are considered acute phase proteins (APPs) when their plasma concentration changes at least 25% (Gabay & Kushner, 1999).

APPs that increase their concentration during APR are called positive APP, while negative APP are decreased during APR (Gabay & Kushner, 1999). In humans, the two major APPs are C-reactive protein (CRP) and serum amyloid A (SAA), whose concentration can increase more than 1000-fold during an acute phase response (Gabay & Kushner, 1999). In mice, the major APPs (i.e. the APPs whose plasma concentration increases the most during APR) are SAA, haptoglobin and serum amyloid P (Cray, Zaias, & Altman, 2009).

How it is Measured or Detected

Systemic acute phase response is assessed by measuring acute phase proteins (APPs) concentrations in blood plasma or serum.

In mice, serum amyloid A (SAA) isoforms are measured using enzyme-linked immunosorbent assay (ELISA) assays or Western blot. (Christophersen et al., 2021; Gutierrez et al., 2023; Hadrup et al., 2019; Halappanavar et al., 2011; Poulsen et al., 2017). ELISA assays allows the measurement of proteins in a sample by adsorbing the desired proteins (antigens) to an inert surface. Using antibodies specific to the protein and an enzyme that catalyzes the reaction, a colorimetric signal is produced and measured (Nelson, Nelson, Lehninger, & Cox, 2017). In the case of Western blot, also called immunoblot assay, proteins in a sample are first separated by gel electrophoresis. Following, the proteins are transferred to a membrane and treated with antibodies, obtaining a coloured precipitate along the band of the desired protein (Nelson et al., 2017).

In humans, most often C-reactive protein (CRP) and SAA are measured. These proteins are measured by immunoassays detecting single or multiple proteins (Adetona et al., 2017; Andersen et al., 2019; Baumann et al., 2018; Meier et al., 2014; Monse et al., 2018; Walker et al., 2022; Wyatt, Devlin, Rappold, Case, & Diaz-Sanchez, 2020). In addition, CRP is measured by turbidimetric (Barregard et al., 2006; Kim, Chen, Boyce, & Christiani, 2005; Sikkeland et al., 2018) and nephelometric assays (Brand et al., 2014).

For both techniques, a blood sample (serum or plasma) is mixed with a suspension of latex beads coated with CRP antibodies. When CRP binds to the beads, a complex is formed that produces the scattering of light. The amount of light scattered is proportional to the amount of complexes formed. While in nephelometry the amount of light scaterred is measure, in turbidimetry the amount of light that passes through the suspension is measured. The measurements are later converted to CRP concentration using a calibration curve (Drieghe, Alsaadi, Tugirimana, & Delanghe, 2014; Hamilton, 2014).

CRP is used in the clinical setting as a marker of systemic inflammation.

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List of Adverse Outcomes in this AOP

Event: 1443: Atherosclerosis

Short Name: Atherosclerosis

Key Event Component

Process	Object	Action
Atherosclerosis		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:237 - Substance interaction with lung resident cell membrane components leading to atherosclerosis	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

- Taxonomic applicability: Human.
- Life stages applicability: All life stages. Although atherosclerosis is mostly observed in adult humans, this condition begins early in life, and progresses through adulthood (McGill, McMahan, & Gidding, 2008; McMahan et al., 2005). Children with chronic inflammation diseases have shown to develop atherosclerosis in early childhood. (Tyrrell et al., 2010; Yamamura et al., 2014)
- Sex applicability: Unspecific, atherosclerosis is manifested in males and females (Libby, 2021).

Key Event Description

Atherosclerosis is defined as the thickening of the arterial wall towards the lumen. The thickening, called atherosclerotic plaques, is composed of macrophages, smooth muscle cells, lymphocytes, lipids (including cholesterol), and connective tissue. This thickening reduces the space in the blood vessels which is available for the blood flow (Widmaier, Raff, Strang, & Vander, 2016).

Atherosclerosis is initiated with an endothelial injury that allows the translocation of low density lipoprotein (LDL) molecules to the intima layer of the artery, where they become oxidized (oxLDL). The endothelial cells release chemokines and adhesion molecules that recruits blood monocytes to the injury site, where monocytes cross to the sub-endothelial space. Monocytes then differentiate into macrophages and take up oxLDL, thus becoming laden with lipoproteins (“foam cells”). The lipid accumulation and foam cell formation continues over time, and the migration of smooth muscle cells from the media layer to the intima space helps establishing an atherosclerotic plaque with the release extracellular matrix molecules (Libby et al., 2019).

How it is Measured or Detected

Current *in vitro* models have been used to evaluate different parts of the atherosclerosis development, for example: expression of adhesion molecules, adhesion of monocytes to endothelial cells, monocytes migration and foam cell formation (Chen et al., 2021).

In animal models, the induction and/or progression of atherosclerosis after exposure to a stressor can be studied. Examples of these are the *ApoE*$^{-/-}$ and *LdLr*$^{-/-}$ mouse models and Watanabe rabbit model, where the development of atherosclerotic can be assessed (Gistera, Ketelhuth, Malin, & Hansson, 2022).

In humans, atheroscleorosis is diagnosed by clinicians. Techniques that allow direct visualization of atherosclerotic

plaques include ultrasonography, computed tomography angiography, magnetic resonance imaging, and optical coherence tomography (Libby et al., 2019). These techniques can measure the intima thickness of arteries, along with detection of calcified components (Poyrazoglu, Vurdem, Arslan, & Uytun, 2016; van der Meer et al., 2004). Techniques that allow the evaluation of atherosclerosis without direct visualization of plaques include angiography, aortic pulse wave velocity and the ankle-arm systolic blood pressure index (Libby et al., 2019; Rodondi et al., 2010; van der Meer et al., 2004). Finally, nonspecific, inflammatory markers are also used to evaluate atherosclerosis. These include blood levels of interleukin 6 (IL-6), C-reactive protein and tumor necrosis factor α (TNF-α) (Rodondi et al., 2010).

Regulatory Significance of the AO

Atherosclerosis is the principal cause of cardiovascular diseases including myocardial infarction, stroke and angina pectoris (Frostegard, 2013; Jebari-Benslaiman et al., 2022; Libby et al., 2019). In turn, cardiovascular diseases are the principal cause of deaths worldwide and measures have been made by many countries to control risk factors and prevent this disease (Vaduganathan, Mensah, Turco, Fuster, & Roth, 2022). It is pertinent to remark that ambient (outdoor) and indoor particulate matter are risk factors for cardiovascular and the World Health Organization (WHO) has estimated that 6.7 million annual premature deaths are associated with these risk factors (Vaduganathan et al., 2022; WHO, 2023).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1702: Interaction with the lung cell membrane leads to Increased proinflammatory mediators

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the pulmonary resident cell membrane components leading to pulmonary fibrosis	adjacent	Moderate	Moderate
Interaction with lung resident cell membrane components leads to lung cancer	adjacent	Moderate	Moderate
Substance interaction with lung resident cell membrane components leading to atherosclerosis	adjacent	High	Low

Key Event Relationship Description

Innate immune response is the first line of defence in any organism against invading infectious pathogens and toxic substances. It involves tissue triggered startle response to cellular stress and is described by a complex set of interactions between the toxic stimuli, soluble macromolecules and cells (reviewed in Nathan, 2002). The process culminates in a functional change defined as inflammation, purpose of which is to resolve infection and promote healing. In lungs, the interaction of toxic substances with resident cells results in cellular stress, death or necrosis (Pouwels et al., 2016) leading to release of intracellular components such as alarmins (Damage associated molecular patterns [DAMPs], Interleukin (IL)-1α, High mobility group box 1 [HMGB1]). Released alarmins (danger sensors) bind cell surface receptors such as Interleukin 1 Receptor 1 (IL-1R1), Toll Like Receptors (TLRs) or others leading to activation of innate immune response signalling.

For example, binding of IL-1α to IL-1R1 can release Nuclear factor kappa B (NF-κB) resulting in its translocation to nucleus and transactivation of pro-inflammatory genes including cytokines, growth factors and acute phase genes. The signalling also stimulates secretion of a variety of pro-inflammatory mediators. Overexpression of IL-1α in cells induces increased secretion of pro-inflammatory mediators. Products of necrotic cells are shown to stimulate the immune system in an IL-1R1-dependent manner (Chen et al., 2007).

The secreted alarmins activate resident cells pre-stationed in the tissues such as mast cells or macrophages leading to propagation of the already initiated immune response by releasing more eicosanoids, cytokines, chemokines and other pro-inflammatory mediators. Thus, secreted mediators signal the recruitment of neutrophils, which are the first cell types to be recruited in acute inflammatory conditions. Neutrophil influx in sterile inflammation is driven mainly by IL-1α (Rider P, 2011). IL-1 mediated signalling regulates neutrophil influx in silica-induced acute lung inflammation (Hornung et al., 2008). IL-1 signalling also mediates neutrophil influx in other tissues and organs including liver and peritoneum. Other types of cells including macrophages, eosinophils, and lymphocytes are also recruited in a signal-specific manner. Recruitment of leukocytes induces critical cytokines associated with the T helper type 2 immune response, including Tumor necrosis factor alpha (TNF-α), IL-1β, and IL-13.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility of this relationship is high. There is a mechanistic relationship between the MIE (Event 1495) and KE1 (Event 1496) which has been evidenced in a number of both *in vitro* and *in vivo* model systems in response to stressors such as, asbestos, silica, bleomycin, carbon nanotubes, and metal oxide nanoparticles (NPs) (Behzadi *et al.,* 2017; Denholm & Phan 1990; Dostert et al., 2008; Mossman & Churg 1998).

Increased expression of IL-1α or IL-1β following lung exposure to multi-walled carbon nanotubes (MWCNTs), bleomycin, micro silica particles, silica crystals, and polyhexamethylene guanidine phosphate has been shown to be associated with neutrophil influx in rodents (Gasse et al., 2007; Girtsman et al., 2014; Hornung et al., 2008; Nikota et al., 2017; Rabolli et al., 2014; Suwara et al., 2014). Inhibition of IL-1 function by knocking out the expression of IL-1R1 using IL-1R1 knockout mice or via treatment with IL-1α or IL-1β neutralising antibodies results in complete abrogation of lung neutrophilic influx following exposure to MWCNTs (Nikota et al, 2017), cigarette smoke (CS) (Halappanavar et al., 2013), silica crystals (Rabolli et al., 2014) and bleomycin (Gasse et al., 2007). IL1-R1, Myeloid differentiation primary response protein (Myd88) or the IL-33/St2 signaling are involved in pulmonary fibrosis induced by bleomycin (Gasse et al., 2007; Xu et al., 2016).

Empirical Evidence

Empirical support for this KER is moderate. There are limited *in vitro* studies, which show a temporal and dose-dependent relationship between these two events, using the upregulation of specific surface receptors as a proxy for direct membrane interaction (Chan et al., 2018; Denholm & Phan, 1990; Roy et al., 2014). There are also studies that provide general support for the idea that an interaction with the lung resident cell membrane components leads to increased, secretion of pro-inflammatory and pro-fibrotic mediators (Table 1).

Dose-Response Evidence:

There are a few studies which provide evidence for a dose-response relationship in this KER. An *in vitro* study demonstrated a concentration-response relationship, in which silica exposure induced increases in pro-inflammatory cytokines through scavenger receptors in cultured bone marrow-derived murine mast cells. Cells were exposed to 6.25, 12.5, 25 or 50 $\mu g/cm^2$ silica dioxide (SiO_2) for 24 h. Macrophage scavenger receptor (MSR2) expression increased over time at 50 $\mu g/cm^2$ and in a concentration-dependent relationship. Moreover, Tumor necrosis factor alpha (TNF-α), IL-13 and Monocyte chemoattractant protein-1 (MCP-1) increased in a concentration-dependent manner (Brown et al., 2007). This provides indications that at higher concentrations of the stressor, the interaction with the lung resident cell membrane components (Event 1495) leads to an increased secretion of pro-inflammatory mediators (Event 1496).

Temporal Evidence:

In vitro and *in vivo* studies have demonstrated temporal concordance of the KEs.

TLR4 signal pathway was evaluated in differentiated macrophages exposed to silica at 2.5$\mu g/cm^2$. After 16 and 24 h, the mRNA expression level of TLR4 increased. Moreover, the protein expression level of TLR-4 and related MyD88/Toll-interleukin-1 receptor domain containing adaptor protein (TIRAP) pathway increased at 24 h. Release of IL-1β, IL-6, IL-10, and TNF-α was induced by silica exposure at 24 h. Pre-treatment with resatorvid (TAK-242), an inhibitor of TLR4 signaling, suppressed the release of the cytokines (Chan et al., 2018).

Macrophages exposed to zinc oxide (ZnO) NPs at 2.5μg/mL for 24 h increased the expression level of TLR6 and MyD88, TNF receptor-associated factor (TRAF), and IL-1 receptor-associated kinase (IRAK). At 24 h, they also observed an increase in the mRNA and protein levels of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. These results demonstrated that ZnO NPs induced pro-inflammatory mediators by TLR stimulation and Mitogen-activated protein kinases (MAPKs) activation (Roy et al., 2014).

The pro-inflammatory IL-1β induced granulocyte migration and can be produced as a result of cellular detection of pathogen associated molecular patterns (PAMPs). Mice exposed to 2.5 mg/mouse of silica by instillation showed an increase of mRNA expression of pro-IL-1β in bronchoalveolar lavage fluid (BALF) at 6, 12, and 24 h post-exposure in a time-dependent manner. At early time points (1 h, 3 h, 6 h), there was an increase in the release of an alarmin (IL-1α) which indicates that the alarmin was released due to cell damage leading to cytokine production and an inflammatory reaction. Moreover, at 24 h, the levels of mature IL-1β and neutrophil accumulation in BALF increased. Neutralization or deletion of IL-1α reduced the observed responses (Rabolli et al., 2014).

Epithelial damage can lead to the release of alarmins. In this stead, conditioned media from primary human bronchial epithelial cells (PBECs) exposed to thapsigargin was able to induce a pro-inflammatory response in primary human lung fibroblasts. PBECs were exposed to thapsigargin (a tumor promoter in mammalian cells) 20 μM for 2 h. After that, the cell culture medium was replaced, and cells were incubated for 24 h. At this time, the medium was recovered and used to culture lung fibroblast for 5 h. This conditioned media from epithelial cell damage contains the alarmin IL-1α, which induced increased gene expression of IL-6, IL-8, MCP-1, and Granulocyte-macrophage colony-stimulating factor (GM-CSF) in fibroblasts. These responses were reduced with anti-IL-1α treatment (Suwara et al., 2014).

Heijink et al. 2015 conducted a similar strategy to identify the relationship between DAMPs and pro-inflammatory mediator release after exposure to CS. Neutrophils treated with CS bubbled for 1 min, released high levels of HMGB1 as a consequence of necrotic cell death. The cell-free supernatant, which contains HMGB1, was used to culture human bronchial epithelial cells, and after 24 h it promoted the production of the C-X-C motif chemokine ligand (CXCL)8 or IL-8 by lung epithelial cells. Pharmacological inhibitors, such as 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (OxPAPC) and Receptor for advanced glycation endproducts (RAGE) antagonist peptide (RAP), reduced the effect of CXCL8 release.

HMGB1 and Heat shock protein 70 (HSP-70) can be released by damaged hepatocytes. In a study, mice were treated with acetaminophen 350 mg/Kg for 3 and 6 h. At these time points, the liver perfusate was obtained and an increase in HSP-70 and HMGB1 protein levels was observed. RAW 264.7 cells (a macrophage cell line) treated with the liver perfusate exhibited increased mRNA expression levels of MCP-1 and IL-1β (Martin-Murphy et al. 2010).

Female mice were intratracheally administered with bleomycin at 5 mg/kg to represent idiopathic pulmonary fibrosis. IL-33, a molecule that can act as a DAMP, increased in lungs after 3 and 7 days of treatment. In serum, at 7-, 14- and 28-days post-exposure, IL-4 and IL-13 increased. It was concluded that IL-33/ST2 signaling pathway is involved in pulmonary fibrosis by bleomycin (Xu et al., 2016).

Uncertainties and Inconsistencies

Attenuation or complete abrogation of KE1 (Event 1496) and KE2 (Event 1497) following inflammogenic stimuli is observed in rodents lacking functional IL-1R1 or other cell surface receptors that engage innate immune response

upon stimulation. However, following exposure to MWCNTs, it has been shown that absence of IL-1R1 signalling is compensated for eventually and neutrophil influx is observed at a later post-exposure time point (Nikota et al., 2017). In another study, acute neutrophilic inflammation induced by MWCNTs was suppressed at 24 h in mice deficient in IL-1R1 signalling; however, these mice showed exacerbated neutrophilic influx and fibrotic response at 28 days post-exposure (Girtsman et al., 2014). The early defence mechanisms involving DAMPs is fundamental for survival, which may necessitate activation of compensatory signaling pathways. As a result, inhibition of a single biological pathway mediated by an individual cell surface receptor may not be sufficient to completely abrogate the lung inflammatory response. Forced suppression of pro-inflammatory and immune responses early after exposure to substances that cannot be effectively cleared from lungs, may enhance the injury and initiate other pathways leading to exacerbated response.

Quantitative Understanding of the Linkage

A majority of the *in vivo* studies are conducted with only one dose and thus, it is difficult to derive quantitative dose-response relationships based on the existing data. However, it is clear from the studies referenced above that greater concentrations or doses of pro-fibrotic substances result in higher release of alarmins, and consequently, higher pro-inflammatory signalling. The above studies also demonstrate strong temporal relationships between the individual KEs.

Response-response relationship

One study has demonstrated a response-response relationship for this KER.

Human intervertebral disc cells were treated with 0, 0.5, 1, or 2 mg/ml of recombinant HMGB1 for 24 h. Protein levels were determined in cell medium supernatant by enzyme-linked immunosorbent assay (ELISA). HMGB1 stimulates the expression of IL-6 and Matrix metalloproteinase 1 (MMP-1) in a response-response relationship. A strong correlation was observed by Spearman’s rank correlation coefficient between HMGB1 treatment and IL-6 or MMP-1 levels (Shah et al., 2019).

Other reports have studied both KEs, but they do not indicate if the response-response relationship was linear or not (coefficient or correlation is not shown) (Chakraborty et al., 2017; Fukuda et al. 2017; Kim et al., 2020, Piazza et al., 2013; Yang et al., 2012;).

Time-scale

Some studies have described how long after a change in the MIE (Event 1495; interaction substance and components), KE1 (Event 1496; pro-inflammatory mediators are secreted) is impacted (Table 2).

Table 2. Time-scale related studies relevant to the MIE (Event 1495) - KE1 (Event 1496) relationship.

Reference	*In vitro*/*in vivo*/population study	Design	MIE (Event 1495)	KE1 (Event 1496)
			Timepoint	Timepoint
Xu et al., 2016	*In vivo*	40 Female Kunming strain mice Bleomycin was intratracheally administered 5 mg/Kg. Days post-exposure	IL-33 3, 7 days	IL-4, IL-13 7, 14, and 28 days
Roy et al., 2014	*In vitro*	Primary mice macrophages exposed to 2.5 mg/ml ZnO for 24 hrs.	Increased TLR6 expression 0.5, 3, 6, 12, and 24 h	Increased IL-6, TNF-α 24 h
Rabollli et al., 2014	*In vivo*	Female C57BL/6 mice Exposed to silica 2.5 mg/mouse by instillation	Increased the release of IL-1α 1, 3, and 6 h	Increased mRNA expression of pro-IL-1β 6, 12, and 24 h

Known Feedforward/Feedback loops influencing this KER

Pancreatic cancer cells stimulated with S100 calcium-binding protein A8 (S100A8) and S100 calcium-binding protein A9 (S100A9) released pro-inflammatory cytokines IL-8, TNF-α, and Fibroblast growth factor (FGF). Cancer cell-derived conditioned media and the individual cytokines (TNF-α and Transforming growth factor beta [TGF-β]) induced the protein expression of S100A8 and S100A9 in HL-60 monocytic cell line and primary human monocytes (Nedjadi et al.

2018).

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Relationship: 2053: Increased proinflammatory mediators leads to Increased transcription of genes encoding acute phase proteins

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with lung resident cell membrane components leading to atherosclerosis	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Acute phase response is present in vertebrate species (Cray, Zaias, & Altman, 2009). In addition, serum amyloid A, one of the major acute phase proteins, has been conserved in mammals throughout evolution and has been described

in humans, mice, dogs, horses, among others (Uhlar & Whitehead, 1999).

Key Event Relationship Description

This KER presents the association between the secretion of pro-inflammatory mediators (Key event 1496) and transcription of genes encoding acute phase proteins (f. ex. *Saa1*, *Saa2* and *Saa3*) (Key event 1438) in different tissues, mainly lung and liver. Pro-inflammatory mediators are the secondary messengers that initiate and regulate inflammatory reactions. They are secreted during inflammation in all species. Acute phase proteins are proteins that have an increase in plasma concentration of at least 25% during an acute phase response (Gabay & Kushner, 1999). Acute phase proteins are induced by pro-inflammatory mediators (f. ex. IL-6, TNF-α and IL-1β) and their genes are expressed mainly in the liver, but also in several other tissues (Gabay & Kushner, 1999; Urieli-Shoval et al., 1998). The evidence of the KER presented is based on *in vitro* studies, animal studies (mice) and human studies.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility is high. It is known that acute phase proteins are induced by pro-inflammatory cytokines, primary interleukin (IL)-6, IL-1β, and tumor necrosis factor α (TNF-α). These cytokines are produced at sites of inflammation, mainly by monocytes and macrophages (Gabay & Kushner, 1999; Mantovani & Garlanda, 2023; Uhlar & Whitehead, 1999; Venteclef, Jakobsson, Steffensen, & Treuter, 2011). Following cytokine release, signaling cascades and transcription factors are activated, regulating the expression of acute phase reaction genes (Venteclef et al., 2011).

In this KER, pulmonary inflammation has been considered as an indirect marker of the release of pro-inflammatory factors because the release of inflammatory mediators (i.e. cytokines and chemokines) recruits immune cells to inflammation sites (Janeway, Murphy, Travers, & Walport, 2008). In mice, pulmonary inflammation is commonly assessed as the number or fraction of neutrophils in the broncheoalveolar lavage fluid (BALF) (Van Hoecke, Job, Saelens, & Roose, 2017).

Empirical Evidence

- Interleukin (IL)-1 (IL-1α and IL-1β, 10 ng/mL each) and IL-6 (500 units/mL), both in presence of 1 μM dexamethasone, increased the relative levels of serum amyloid A (SAA) mRNA in cultured human adult aortic smooth muscle cells (Meek, Urieli-Shoval, & Benditt, 1994).
- Human hepatoma cells exposed to IL-6, IL-1β and tumor necrosis factor α (TNF-α) for 20 h showed a reduced synthesis of albumin and increased synthesis of the acute phase proteins C3 and ceruloplasmin. In addition, mice exposed to IL-1β and TNF-α showed an increase of *Saa* mRNA in liver tissue (Ramadori, Van Damme, Rieder, & Meyer zum Buschenfelde, 1988).
- After pulmonary exposure to lipopolysaccharide (LPS) (300 μg/mL), lung tissue from female C57BL/6 mice showed upregulation of several cytokines and chemokines genes and upregulation of the acute phase proteins genes *Saa* and α_1-protease inhibitor (Jeyaseelan, Chu, Young, & Worthen, 2004).
- Mice presenting IL-6 gene disruption (IL-$6^{-/-}$) shown a reduced response in liver mRNA levels of acute phase proteins haptoglobin, α1-acid glycoprotein and SAA, after challenged by turpentine, LPS and bacterial infection (Kopf et al., 1994).
- After repeated instillation of carbon black nanoparticles, female C57BL/6BomTac mice showed increased expression of chemokine genes along with increased *Saa3* gene expression in lung tissue. In addition, dose-response relationships with several cytokine proteins were identified in lung tissue (Jackson et al., 2012).
- Intratracheal instillation of titanium dioxide in female C57BL/6 mice showed that 28 days after exposure, several genes of cytokines, chemokines and acute phase proteins were upregulated. Additionally, there were significant increases in inflammatory mediators in lung tissue (Husain et al., 2013).

The table below presents evidence of the KER using neutrophil numbers in broncheoalveolar lavage fluid (BALF) as indirect evidence of the release of pro-inflammatory mediators (Key event 1496), while the transcription of genes encoding acute phase proteins was measured in tissues (Key event 1438).

Species	Stressor	Secretion of pro-inflammatory mediators	Transcription of genes encoding acute phase proteins	Reference
Mouse	Ultrafine carbon particles	No significant increase in polymorphonuclear cells (includes neutrophils) in BALF.	Yes, increased *S a a 3* gene expression at 24 h.	(Andre et al., 2006)
Mouse	Diesel exhaust particles	Yes, significant increase of neutrophils in BALF.	Yes, increased expression of *Saa3 genes* in lung tissue. No expression of *Sap, Saa1 or Saa3* genes on liver tissue.	(Saber et al., 2005, 2009, 2013)

Species	Stressor	Secretion of pro-inflammatory mediators	Transcription of genes encoding acute phase proteins	Reference
Mouse	Titanium dioxide nanoparticles	Yes, significant increased numbers of neutrophils in BALF.	Yes, increased expression of *Saa1* and *Saa3* genes in lung tissue	(Halappanavar et al., 2011; Hougaard et al., 2010)
Mouse	Carbon black nanoparticles	Yes, significant increase of neutrophil number 1, 3 and 28 days after exposure.	Yes, significant *Saa1, Saa2* and *Saa3* gene expression increase in lung tissue, at days 1, 3 and 28 after exposure. *Saa3* gene expression increase in liver tissue at day 1 after exposure.	(Bourdon, Halappanavar, et al., 2012; Bourdon, Saber, et al., 2012)
Mouse	Titanium dioxide nanoparticles	Yes, increased neutrophil numbers in BALF 1 and 3 days after exposure to 54 µg, and 1, 3 and 28 days after exposure to 162 µg.	Yes, increased mRNA expression of *Saa3* in lung issue at days 1, 3 and 28 after exposure with 162 µg, and at day 3 with 54 µg.	(Saber et al., 2012, 2013)
Mouse	Carbon black nanoparticles	Yes, increased neutrophil numbers in BALF 1, 3 and 28 days after exposure to 54 and 162 µg, and 1 and 3 days after exposure to 18 µg.	Yes, increased mRNA expression of *Saa3* in lung issue at days 1, 3 and 28 after exposure with 54 µg and 162 µg, and at days 1 and 3 with 18 µg.	(Saber et al., 2012, 2013)
Mouse	Diesel exhaust particles	Yes, increased neutrophil numbers in BALF 3 days after exposure to 54 µg, and 1 and 3 days after exposure to 162 µg.	Yes, increased *Saa3* gene expression after 1, 3 and 28 days with 162 µg, at day 28 with 54 µg, and at day 3 with 18 µg.	(Kyjovska et al., 2015)
Mouse	Multiwalled carbon nanotubes (referred as CNT_{small})	Yes, increased neutrophil numbers in BALF 1, 3 and 28 days after exposure to 18, 54 and 162 µg.	Yes, increased differential expression of acute phase response genes in lung and liver tissue.	(Poulsen, Saber, Mortensen, et al., 2015; Poulsen, Saber, Williams, et al., 2015)
Mouse	Multiwalled carbon nanotubes (referred as CNT_{large})	Yes, increased neutrophil numbers in BALF 1, 3 and 28 days after exposure to 18, 54 and 162 µg.	Yes, increased differential expression of acute phase response genes in lung and liver tissue.	(Poulsen, Saber, Mortensen, et al., 2015; Poulsen, Saber, Williams, et al., 2015)
Mouse	Sanding dust from epoxy composite containing carbon nanotubes	Yes, increased neutrophil numbers in BALF 1 and 3 days after exposure to 54, 162 and 486 µg, and 28 days after exposure to 486 µg.	Yes, significant increase in *Saa1* mRNA expression in liver tissue (only assessed 1 days after exposure to 486 µg).	(Saber et al., 2016)
Mouse	Sanding dust from epoxy composite without carbon nanotubes	Yes, increased neutrophil numbers in BALF 1 day after exposure to 54, 162 and 486 µg, 3 days after exposure to 162 and 486 µg, and 28 days after exposure to 486 µg.	Yes, significant increase in *Saa1* mRNA expression in liver tissue (only assessed 1 days after exposure to 486 µg).	(Saber et al., 2016)
Mouse	Carbon nanotubes	Yes, increased neutrophil numbers in BALF 1, 3 and 28 after exposure to 18, 54 and 162 µg.	Yes, significant increase in *Saa1* mRNA expression in liver tissue (only assessed 1 days after exposure to 162 µg).	(Saber et al., 2016)
Mouse	Graphene oxide	Yes, increased neutrophil numbers in BALF 1 and 3 days after exposure to 18, 54 and 162 µg.	Yes, increased mRNA expression of *Saa3* in lung tissue, at all dose 1 and 3 days after exposure. Increased gene expression of *Saa1* in liver tissue 1 day after exposure to 18 µg, and 3 days after exposure to 162 µg.	(Bengtson et al., 2017)
Mouse	Reduced graphene oxide	Yes, increased neutrophil numbers 1 and 3 days after exposure to 162, and 90 days after exposure to 18, 54 and 162 µg.	Yes, increased mRNA expression of *Saa3* in lung tissue, 3 days after exposure to 162 µg. No changes in gene expression of *Saa1* in liver tissue.	(Bengtson et al., 2017)

Species	Stressor	Secretion of pro-inflammatory mediators	Transcription of genes encoding acute phase proteins	Reference
Mouse	Carbon black	Yes, increased neutrophil numbers in BALF 1, 3, 28 and 90 days after exposure.	Yes, increased mRNA expression of *Saa3* in lung tissue 1, 3, 28 and 90 days after exposure. Increased gene expression of *Saa1* in liver tissue 1 day after exposure.	(Bengtson et al., 2017)
Mouse	Unmodified rutile (TiO2)	Yes, increased neutrophil numbers in BALF 1 and 3 days after exposure to 54 and 162 µg, and 28 days after exposure to 162 µ.	Yes, increased expression of *Saa3* mRNA in lung tissue 1, 3 and 28 days after exposure to 162 µg. Increased expression of *Saa1* in liver tissue 1 day after exposure to 162 µg and 3 days after exposure to 54 and 162 µg.	(Wallin et al., 2017)
Mouse	Surface modified rutile (TiO2)	Yes, increased neutrophil numbers in BALF 1, 3 and 28 days after exposure to 54 and 162 µg.	Yes, increased expression of *Saa3* mRNA in lung tissue 1, and 28 days after exposure to 54 µg, and 1, 3 and 28 days after exposure to 162 µg. Increased expression of *Saa1* in liver tissue 1 day after exposure to 162 µg.	(Wallin et al., 2017)
Mouse	Particulate matter from non-commercial airfield	Yes, increased neutrophil numbers in BALF 1 day after exposure to 18 and 54 µg.	Yes, increased expression of *Saa3* mRNA in lung tissue and *Saa1* mRNA in liver tissue after 1 day of exposure to 54 µg. No effect after 28 and 90 days.	(Bendtsen et al., 2019)
Mouse	Particulate matter from commercial airport	Yes, increased neutrophil numbers in BALF 1 day after exposure to 18 and 54 µg.	Yes, increased expression of *Saa3* mRNA in lung tissue after 1 day of exposure to 18 and 54 µg. No effect after 28 and 90 days.	(Bendtsen et al., 2019)
Mouse	Diesel exhaust particles	Yes, increased neutrophil numbers in BALF 1 day after exposure to 54 and 162 µg, and 28 days after exposure to 162 µg.	Yes, increased expression of *Saa3* mRNA in lung tissue after 1 day of exposure to 54 and 162 µg, and increased expression of *Saa1* mRNA in liver tissue 1 day after exposure to 162 µg. No effect after 28 days.	(Bendtsen et al., 2019)
Mouse	Carbon black	Yes, increased neutrophil in BALF after 1, 28 and 90 days of exposure.	Yes, increased expression of *Saa3* mRNA in lung tissue at day 1 and day 90.	(Bendtsen et al., 2019)
Mouse	Coated zinc oxide nanoparticles	Yes, increased neutrophil numbers in BALF 1 and 3 days after exposure to 2 µg, and 28 days after exposure to 0.2 and 0.7 µg.	Yes, increase on *Saa3* mRNA in lung tissue 1 day after exposure to 0.7 and 2 µg. No effect 3 and 28 days after exposure.	(Hadrup et al., 2019)
Mouse	Surface modified hallosytes	Yes, increased neutrophil numbers in BALF 1 and 3 days after exposure to 54 µg, and 28 days after exposure to 6 and 54 µg.	Yes, increase *Saa3* mRNA expression in lung tissue 1 and 3 days after exposure to 54 µg. No effect on *Saa1* mRNA expression on liver tissue.	(Barfod et al., 2020)
Mouse	Carbon black	Yes, increased neutrophil numbers in BALF 1, 3 and 28 days after exposure to 162 µg.	Yes, increase *Saa3* mRNA expression in lung tissue 1, 3 and 28 days after exposure. No effect on *Saa1* mRNA expression on liver tissue.	(Barfod et al., 2020)
Mouse	Nanofil9 (Organomodified nanoclay)	Yes, increased neutrophil numbers in BALF 1 day after exposure to 54 µg, and 3 days after exposure to 18 and 54 µg.	Yes, increased *Saa3* mRNA expression in lung tissue 1 day after exposure to all doses, and 3 days after exposure to 6 and 18 µg.	(Di Ianni et al., 2020)

Species	Stressor	Secretion of pro-inflammatory mediators	Transcription of genes encoding acute phase proteins	Reference
Mouse	NanofilSE3000 (Organomodified nanoclay)	Yes, increased neutrophil numbers in BALF 1 day after exposure to 54 and 162 µg, and 3 days after exposure to 162 µg.	Yes, increased *S a a 3* mRNA expression in lung tissue 1 day after exposure to 54 and 162 µg, and 3 days after exposure to 54 µg.	(Di Ianni et al., 2020)
Mouse	Bentonite	Yes, increased neutrophil numbers in BALF 1 and 3 days after exposure to 18, 54 and 162 µg.	Yes, increased *S a a 3* mRNA expression in lung tissue 1 and 3 days after exposure to all doses, and 28 days after exposure to 162 µg.	(Di Ianni et al., 2020)
Mouse	Carbon black	Yes, increased neutrophil numbers in BALF 1 and 3 days after exposure to 18, 54 and 162 µg, and 28 day after exposure to 162 µg.	Yes, increased *S a a 3* mRNA expression in lung tissue 1 and 3 days after exposure to all doses, and 28 days after exposure to 54 and 162 µg.	(Di Ianni et al., 2020)

Additional evidence can be found in the following link:Additional evidence KER 2053_1 and Additional evidence KER 2053_2.

Uncertainties and Inconsistencies

The table below presents inconsistencies for this KER, where secretion of pro-inflammatory mediators has been observed after exposure to a stressor, while systemic acute phase response was not observed, or viceversa. Secretion of pro-inflammatory mediators was measured as change in concentration of pro-inflammatory markers in blood or increase neutrophil numbers in bronchoalveolar lavage fluid (BALF), while the transcription of genes encoding acute phase proteins was measured in tissues.

Species	Stressor	Secretion of pro-inflammatory mediators	Transcription of genes encoding acute phase proteins	Reference
Mouse	Carbon black	No significant increase of neutrophils in BALF.	Yes, increased expression of *Saa3* gene in lung tissue. No expression of *Sap, Saa1 or Saa3* genes on liver tissue.	(Saber et al., 2005, 2009, 2013)
Mouse	Uncoated zinc oxide nanoparticles	No increase of neutrophil numbers in BALF after exposure.	Yes, increase on *Saa3* mRNA in lung tissue 1 day after exposure to 2 µg. No effect 3 and 28 days after exposure.	(Hadrup et al., 2019)
Mouse	Unmodified hallosytes	Yes, increased neutrophil numbers in BALF 28 days after exposure to 18 µg.	No effect on *Saa3* mRNA expression in lung tissue nor *Saa1* mRNA expression in liver tissue.	(Barfod et al., 2020)
Mouse	Aluminum oxide	Yes, increased neutrophil numbers in BALF 1 and 28 days after exposure to 54 µg.	No change in *Saa3* mRNA expression in lung tissue.	(Gutierrez et al., 2023)

Quantitative Understanding of the Linkage

Response-response relationship

Neutrophil number in brochoalveolar lavage fluid (indirect measure of the secretion of proinflammatory mediators (Key event 1496) correlates with the expression of *Saa3* mRNA levels in lung tissue (Key event 1438), in female C57BL/6J mice 1 and 28 days after intratracheal instillation of metal oxide nanomaterials (Figure 1). The Pearson's correlation coefficient was 0.82 ($p<0.001$) between log-transformed neutrophil numbers in brochoalveolar lavage fluid and log-transformed *Saa3* mRNA levels in lung tissue (Gutierrez et al., 2023).

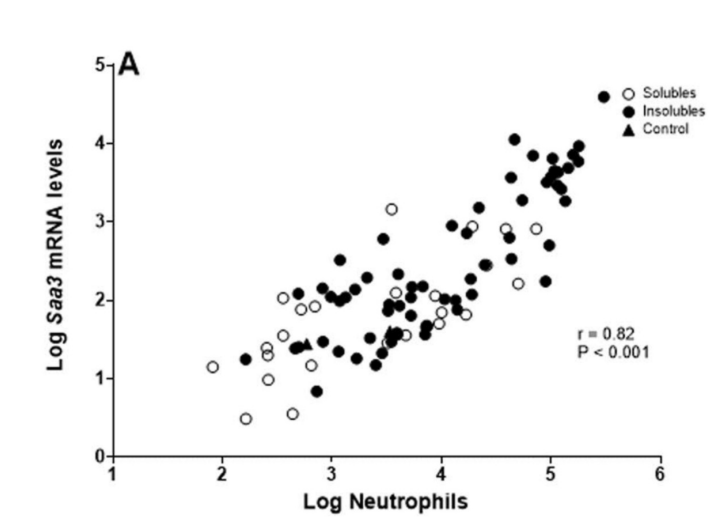

Figure 1. Correlations between neutrophil numbers and *Saa3* mRNA levels in lung tissue, including data from 1 and 28 days after exposure to nanomaterials. Reproduced from Gutierrez et al. (2023).

Time-scale

It has been shown that pro-inflammatory mediators concentrations increase before the expression of genes enconding acute phase proteins:

- Upregulation of cytokine genes [Interleukin (IL)-1α, IL-1β, IL-6 and tumor necrosis factor α] was shown to peak around 2h after pulmonary exposure to lipopolysaccharide in female C57BL/6J mice, while upregulation serum amyloid A genes showed their highest upregulation at 8-12h after exposure (Jeyaseelan et al., 2004).

Known Feedforward/Feedback loops influencing this KER

Some acute phase proteins (f. ex. C-reactive protein, serum amyloid A and complement components) have pro-inflammatory functions, including induction of inflammatory cytokines, chemotaxis and activation of immune cells. On the other hand, other acute phase proteins present anti-inflammatory functions (f. ex. Haptoglobin and fibrinogen) as antioxidative and tissue repair inducer (Gabay & Kushner, 1999).

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Relationship: 1589: Increased transcription of genes encoding acute phase proteins leads to Systemic acute phase response

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with lung resident cell membrane components leading to atherosclerosis	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Acute phase response is present in vertebrate species (Cray, Zaias, & Altman, 2009). In addition, serum amyloid A has been conserved in mammals throughout evolution and has been described in humans, mice, dogs, horses, among others (Uhlar & Whitehead, 1999).

Key Event Relationship Description

This KER presents the association between the increased transcription of genes encoding acute phase proteins(Key event 1438) in different tissues and the induction of systemic acute phase response(Key event 1439). Acute phase proteins are expressed in the liver, and in several other tissues including lung (Gabay & Kushner, 1999; Hadrup et al., 2020; NCBI, 2023; Saber et al., 2014; Urieli-Shoval et al., 1998). During acute phase response several changes occur, including variations in plasma concentration of acute phase proteins. The two major acute phase proteins are C-reactive protein and serum amyloid A (Gabay & Kushner, 1999). The evidence of the KER presented is based on animal studies (mice).

Evidence Supporting this KER

Biological Plausibility

The biological plausibility is high. After gene expression of acute phase proteins in tissues during inflammatory conditions, mRNA is translated and folded into proteins (Alberts, 2017). These proteins are then release to the systemic circulation (Van Eeden, Leipsic, Paul Man, & Sin, 2012).

Empirical Evidence

The table below presents evidence for this KER. The transcription of genes encoding acute phase proteins was measured in tissues (Key event 1438), while systemic acute phase response is measured as the concentration of acute phase proteins in blood plasma or serum (Key event 1439).

Species	Stressor	Transcription of genes encoding acute phase proteins	Systemic acute phase response	Reference
Mouse	Carbon black nanoparticles	Yes, significant *Saa1, Saa2* and *Saa3* gene expression increase in lung tissue, at days 1, 3 and 28 after exposure. *Saa3* gene expression increase in liver tissue at day 1 after exposure.	Yes, significant increase of plasma serum amyloid A (SAA) at 1 and 28 days after exposure.	(Bourdon et al., 2012)
Mouse	Multiwalled carbon nanotubes (referred as CNT_{small})	Yes, increased differential expression of acute phase response genes in liver tissue 1 and 3 days after exposure to 162 µg. Increased differential expression of acute phase response genes in lung tissue 3 days after exposure to 18 and 162 µg, and 1 and 3 days after exposure to 54 µg.	Yes, increased plasma SAA3 1, 3 and 28 days after exposure to 162 µg, and 3 days after exposure to 18 and 54 µg.	(Poulsen, Saber, Mortensen, et al., 2015; Poulsen, Saber, Williams, et al., 2015)
Mouse	Multiwalled carbon nanotubes (referred as CNT_{large})	Yes, increased differential expression of acute phase response genes in liver tissue 1 and 3 days after exposure to 162 µg. Increased differential expression of acute phase response genes in lung tissue 1 and 3 days after exposure to 54 and 162 µg.	Yes, increased plasma SAA3 1 and 3 days after exposure to 162 µg, and 3 days after exposure to 54 µg.	(Poulsen, Saber, Mortensen, et al., 2015; Poulsen, Saber, Williams, et al., 2015)
Mouse	Graphene oxide	Yes, increased mRNA expression of *Saa3* in lung tissue, at all dose 1 and 3 days after exposure. Increased gene expression of *Saa1* in liver tissue 1 day after exposure to 18 µg, and 3 days after exposure to 162 µg.	Yes, increased SAA3 plasma levels 3 days after exposure to 54 and 162 µg.	(Bengtson et al., 2017)
Mouse	Multiwalled carbon nanotubes	Yes, increased *Saa1* mRNA expression in liver tissue 1 day after exposure to 18 and 54 µg. Increase in *Saa1* mRNA levels in liver tissue 28 days exposure to 54 µg. Increased *Saa3* mRNA expression in lung tissue 1 day after exposure to 6, 18 and 54 µg. Increase in *Saa3* mRNA levels in lung tissue 28 days after 18 and 56 µg.	Yes, increased SAA1/2 and SAA3 plasma levels 1 day after exposure. No change in SAA1/2 and SAA3 28 and 92 days after exposure.	(Poulsen et al., 2017)

Species	Stressor	Transcription of genes encoding acute phase proteins	Systemic acute phase response	Reference
Mouse	Particulate matter from non-commercial airfield	Yes, increased expression of *Saa3* mRNA in lung tissue and *Saa1* mRNA in liver tissue after 1 day of exposure to 54 µg. No effect after 28 and 90 days.	Yes, increased plasma SAA3 levels after exposure to 54 µg after 3 days.	(Bendtsen et al., 2019)
Mouse	Diesel exhaust particles	Yes, increased expression of *Saa3* mRNA in lung tissue after 1 day of exposure to 54 and 162 µg, and increased expression of *Saa1* mRNA in liver tissue 1 day after exposure to 162 µg. No effect after 28 days.	Yes, increased plasma SAA3 levels after exposure to 54 µg, at 3 days.	(Bendtsen et al., 2019)
Mouse	Copper oxide	Yes, increased *Saa3* mRNA expression in lung tissue 1 day after exposure to 2 and 6 µg. Increased *Saa1* mRNA expression in liver tissue 1 day after exposure to 6 µg.	Yes, increased plasma SAA1/2 level after exposure to 6 µg, 1 day after exposure.	(Gutierrez et al., 2023)
Mouse	Tin dioxide	Yes, increased *Saa3* mRNA expression in lung tissue and *Saa1* mRNA expression in liver tissue, 1 day after exposure to 162 µg.	Yes, increased plasma SAA3 after exposure to 162 µg, 1 day after exposure.	(Gutierrez et al., 2023)
Mouse	Titanium dioxide	Yes, increased *Saa3* mRNA expression in lung tissue and *Saa1* mRNA expression in liver tissue 1 day after exposure.	Yes, increased plasma SAA3 and SAA1/2 after exposure to 162 µg, 1 day after exposure.	(Gutierrez et al., 2023)
Mouse	Carbon black	Yes, increased *Saa3* mRNA expression in lung tissue 1 and 28 days after exposure. Increased *Saa1* mRNA expression in liver tissue 1 day after exposure	Yes, increased plasma SAA3 and SAA1/2 after exposure to 162 µg, 1 day after exposure.	(Gutierrez et al., 2023)
Mouse	Singlewalled carbon nanotubes	Yes, increased SAA1, SAP and haptoglobin gene expression in liver tissue, 1 day after exposure.	Yes, increase serum CRP, haptoglobin and SAP 1 day after exposure.	(Erdely et al., 2011)
Mouse	Multiwalled carbon nanotubes	Yes, increased SAA1, SAP and haptoglobin gene expression in liver tissue, 1 day after exposure.	Yes, increase serum CRP, haptoglobin and SAP 1 day after exposure. No changes after 28 days.	(Erdely et al., 2011)
Mouse	Serum amyloid A	Yes, significantly increase of *Saa3* mRNA levels in lung tissue and *Saa1* mRNA levels in liver tissue.	Yes, increased levels of endogenous serum SAA3.	(Christophersen et al., 2021)

Uncertainties and Inconsistencies

Although it is suggested that acute phase proteins are mainly produced in the liver (Gabay & Kushner, 1999), it has been shown that in mice, the liver has little upregulation of *Saa* genes after exposure to ultrafine carbon particles or diesel exhaust particle. In contrast, in the lung there is a marked expression of *Saa3* mRNA (Saber et al., 2009, 2013).

Some studies show that the increase of *Saa* gene expression in lung or liver tissue does not translate into an increase in plasma SAA concentration (Bendtsen et al., 2019; Bengtson et al., 2017; Hadrup et al., 2019). This might be due to a protein concentration below the methods detection levels (Hadrup et al., 2019), while measuring gene expression provides a larger dynamic range.

The table below presents inconsistencies for this KER, where transcription of genes encoding acute phase proteins has been observed, while systemic acute phase response was not observed. The transcription of genes encoding acute phase proteins was measured in tissues, while systemic acute phase response is measured as the concentration of acute phase proteins in blood plasma or serum.

Species	Stressor	Transcription of genes encoding acute phase proteins	Systemic acute phase response	Reference
Mouse	Reduced graphene oxide	Yes, increased mRNA expression of *Saa3* in lung tissue, 3 days after exposure to 162 µg. No changes in gene expression of *Saa1* in liver tissue.	No, no change in serum amyloid A (SAA)3 plasma concentration 3 days after exposure.	(Bengtson et al., 2017)
Mouse	Particulate matter from commercial airport	Yes, increased expression of *Saa3* mRNA in lung tissue after 1 day of exposure to 18 and 54 µg. No effect after 28 and 90 days.	No change in plasma SAA3.	(Bendtsen et al., 2019)
Mouse	Carbon black	Yes, increased expression of *Saa3* mRNA in lung tissue at day 1 and day 90.	No change in plasma SAA3.	(Bendtsen et al., 2019)
Mouse	Uncoated zinc oxide nanoparticles	Yes, increase on *Saa3* mRNA in lung tissue 1 day after exposure to 2 µg. No effect 3 and 28 days after exposure.	No effect on plasma SAA3.	(Hadrup et al., 2019)
Mouse	Coated zinc oxide nanoparticles	Yes, increase on *Saa3* mRNA in lung tissue 1 day after exposure to 0.7 and 2 µg. No effect 3 and 28 days after exposure.	No effect on plasma SAA3.	(Hadrup et al., 2019)
Mouse	Zinc oxide	Yes, increased *Saa1* mRNA expression in liver tissue 1 day after exposure to 0.7 µg. No change in *Saa3* mRNA expression in lung tissue.	No change in plasma SAA3 or SAA1/2 levels.	(Gutierrez et al., 2023)

Quantitative Understanding of the Linkage

Response-response relationship

The expression of *Saa3* mRNA levels in lung tissue (Key event 1438) correlates with concentration of SAA3 plasma protein levels (Key event 1439), in female C57BL/6J mice 1 day after intratracheal instillation of metal oxide nanomaterials (Figure 1). The Pearson's correlation coefficient was 0.89 ($p<0.001$) between log-transformed *Saa3* mRNA levels in lung tissue and log-transformed SAA3 plasma protein levels (Gutierrez et al., 2023).

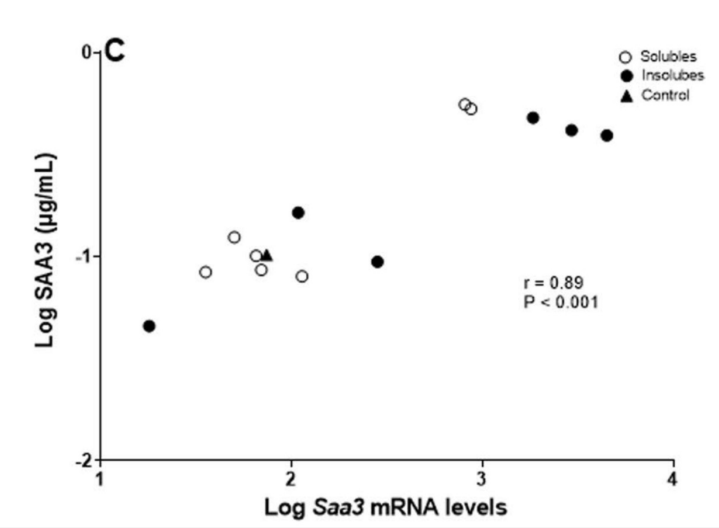

Figure 1. Correlations between *Saa3* mRNA levels in lung tissue and SAA3 plasma protein levels in mice, 1 day after exposure to nanomaterials. Reproduced from Gutierrez et al. (2023).

Time-scale

After exposure to titanium dioxide nanoparticles in mice, expression of *Saa1* mRNA in the liver is short lasting, while expression of *Saa3* mRNA in lung tissue is longer lasting, as it has been observed 28 days after exposure (Wallin et al., 2017).

After exposure to multiwalled carbon nanotubes, it has been observed that expression of *Saa1* and *Saa3* in liver and lung tissue can be elevated 28 days after exposure, however in most cases there is no increase in plasma SAA1/2 nor SAA3 levels past day 1 after exposure (Poulsen et al., 2017).

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Relationship: 2860: Systemic acute phase response leads to Atherosclerosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with lung resident cell membrane components leading to atherosclerosis	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Although atherosclerosis is mostly observed in adult humans, this condition begins early in life, and progresses through adulthood (McGill, McMahan, & Gidding, 2008; McMahan et al., 2005). Children with chronic inflammation diseases have shown to develop atherosclerosis in early childhood. (Tyrrell et al., 2010; Yamamura et al., 2014). In addition, atherosclerosis is manifested in males and females (Libby, 2021).

Key Event Relationship Description

This KER presents the association between systemic acute phase response (Key event 1439) and atherosclerosis (Key event 1443) as the adverse outcome. Acute phase response is the systemic response to acute and chronic inflammatory states, that includes changes in plasma concentration of acute phase proteins (Gabay & Kushner, 1999). Atherosclerosis is defined as the thickening of the arterial wall towards the lumen(Widmaier et al., 2016). The relationship between the key events is explained through the acute phase protein serum amyloid A. The evidence for the KER is based on in vitro studies, animal studies (mice) and human epidemiological studies.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility is high. During acute phase response, serum amyloid A (SAA), one of the major acute phase proteins, replaces apolipoprotein A-1 from high density lipoprotein (HDL). This replacement obstructs the reverse transport of cholesterol to the liver, allowing the accumulation of cholesterol in cells, denominated foam cells (Lindhorst, Young, Bagshaw, Hyland, & Kisilevsky, 1997; McGillicuddy et al., 2009; Meek, Urieli-Shoval, & Benditt, 1994). Foam cells are early markers of atherosclerotic lesions (Libby et al., 2019), and it has been shown that macrophages have a higher uptake of HDL containing SAA than HDL alone (Lindhorst et al., 1997).

The two major human acute phase response, SAA and C-reactive protein (CRP), have been shown to be correlated in humans (Baumann et al., 2018; Monse et al., 2018; Ridker, Hennekens, Buring, & Rifai, 2000), and both are predictors of future cardiovascular event risks (Ridker et al., 2000).

Empirical Evidence

- Increasing concentrations of SAA (0 – 2 µM) induced a dose-response relationship of foam cells in RAW264.7 cells (Lee et al., 2013).
- In mouse model of periodontal disease, ApoE$^{-/-}$ mice were infected with a polymicrobial consortium (*Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia*). 16-weeks after infection, the infected mice presented elevated levels of serum amyloid A (SAA) in comparison to control mice, in addition of increased plaque progression (Rivera et al., 2013).
- Male ApoE$^{-/-}$ mice overexpressing SAA1 presented higher levels of plasma SAA and an increase in atherosclerotic lesions (plaques) than non-SAA1 overexpressing ApoE$^{-/-}$ mice (Dong et al., 2011).
- After one injection of adenoviral vector enconding human SAA1, ApoE$^{-/-}$ mice presented elevated and transient levels of human SAA along with an increase in atherosclerotic lesions (Thompson et al., 2015).
- Overexpression of SAA3 led to increased levels of SAA3 and atherosclerosis lesions in ApoE$^{-/-}$ mice in comparison to control mice. In addition, when SAA3 was suppressed in ApoE$^{-/-}$ × SAA1.1/2.1-DKO (ApoE$^{-/-}$ mice deficient in SAA1 and SAA2), there was a significant decrease in atherosclerotic lesions (Thompson et al., 2018).
- Intratracheal instillation of human serum amyloid A once a week for 10 weeks in ApoE$^{-/-}$ mice (on Western-type diet) induced an increase in plasma SAA3 and atherosclerotic plaque progression (Christophersen et al., 2021).
- SAA was moderately associated with angiographic coronary artery disease in women (21-86 years old) suspected on having myocardial ischemia (Johnson et al., 2004).
- High levels of C-reactive protein (CRP) were associated with an increased risk of coronary heart disease in men and women (Pai et al., 2004).

Uncertainties and Inconsistencies

Mendelian randomization studies have shown that C-reactive protein (CRP) genotypes are not associated with risk of coronary heart disease and that genetically elevated levels of CRP are not associated with coronary heart disease risk (Collaboration et al., 2011; Elliott et al., 2009).

Quantitative Understanding of the Linkage

Response-response relationship

The concentration of blood C-reactive protein (CRP) and serum amyloid A (SAA) (Key event 1439) is associated with the risk of nonfatal myocardial infarction or fatal coronary heart disease (i.e. acute events due to the progression of atherosclerosis – Key event 1443) (Pai et al., 2004; Ridker et al., 2000).

The association can be calculated from prospective, epidemiological studies. This approach was used by the Dutch Expert Committee on Occupational Safety (DECOS) when establishing a health-based occupational exposure limit for diesel engine exhaust based on risk of lung cancer (https://www.healthcouncil.nl/documents/advisory-reports/2019/03/13/diesel-engine-exhaust).

The Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS) are prospective cohort investigations respectively involving 121,700 female U.S. registered nurses who were 30 to 55 years old at baseline in 1976 and 51,529 U.S. male health professionals who were 40 to 75 years old at baseline in 1986 (Pai et al., 2004). In the NHS, among women without cardiovascular disease or cancer before 1990, 249 women had a nonfatal myocardial infarction or fatal coronary heart disease between the date of blood drawing and follow-up in June 1998. In the HPFS, 266 men had a nonfatal myocardial infarction or fatal coronary heart disease between the date of blood drawing and the return of a follow-up questionnaire in year 2000.

In the NHS and HPFS studies, the associations between CRP in blood and risk of nonfatal myocardial infarction or fatal coronary heart disease for women and men were reported in Pai et al. (2004) (Pai et al., 2004), whereas the association for both SAA and CRP in NHS was reported in Ridker et al. (2000) (Ridker et al., 2000).

The dose-response relationships are shown in Figure 1. Here, plasma levels of CRP and SAA were closely associated with future risk of coronary heart disease (CHD).

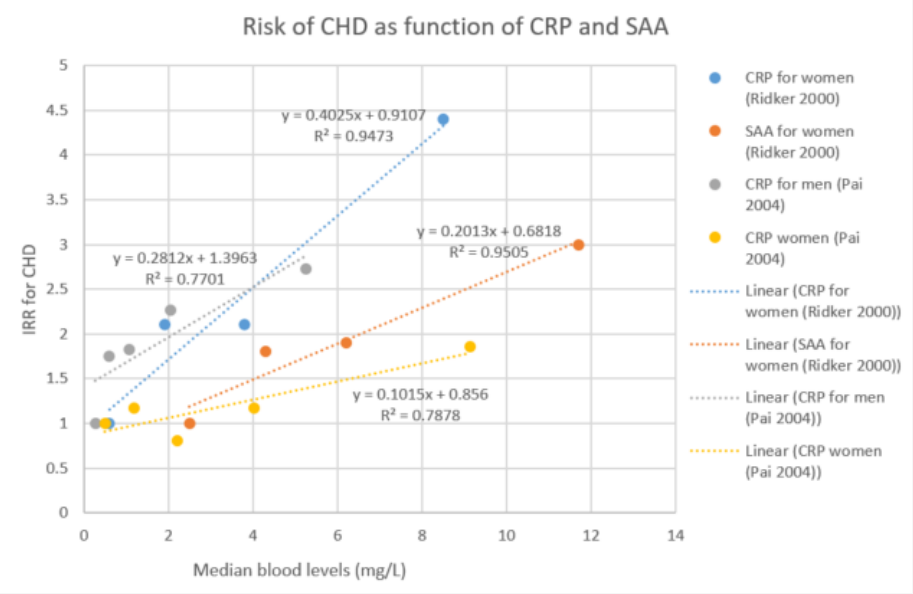

Figure 1. Association between the relative risk (RR) of CHD in NHS as function of quartiles of serum levels of CRP and SAA from Ridker et al. (Ridker et al., 2000) and quintiles of CRP from the NHS and the HPFS studies from Pai et al. (Pai et al., 2004). The trend lines are linear associations, as these gave the highest R^2 values.

According to the Danish Heart Foundation (https://hjerteforeningen.dk/alt-om-dit-hjerte/noegletal/), when a person reaches the age of 55 years, the lifetime risk of a cardiovascular event is 67% in men and 66% in women. Each year, 56,379 Danes are diagnosed with a cardiovascular disease, from which, 15,087 were diagnosed with are apoplexy and 16,050 with ischemic heart disease. As these diagnoses are regarded as manifestations of plaque progression, it means that 55% of the cardiovascular diagnoses are relate to plaque progression. The lifetime risk of these diseases is thus calculated as 0.66x0.55 (lifetime risk x %cardiovascular diseases) = 0.363 = 36%.

Based on this the lifetime risk, the relative risk of 1:100 excess cardiovascular disease was calculated as

RR= (1 + 36)/36= 1.02778

The relative risk of 1:1000 excess cardiovascular disease was calculated as

RR= (1+360)/360= 1.00278

If the relative risk of 1.02778 excess is used in the equations obtained in Figure 1 and presented in the next table, it is observed that in the studies by Ridker et. al and Pai et al., 6-54% increases in blood levels of CRP or SAA were associated with 1% increased risk of cardiovascular disease.

Biomarker	Equation of increased IRR	Increase of biomarker associated with 1% increased risk[(1)]	Baseline levels	Increase of biomarker in % of baseline level associated 1% increased risk
CRP women (Ridker et al., 2000)	ΔIRR = 0.4025 CRP (mg/L)	0.07 mg/L	0.6 mg/L	0.07/0.6= 12%
SAA women (Ridker et al., 2000)	ΔIRR= 0.2013 SAA (mg/L)	0.138 mg/L	2.5 mg/L	0.138/2.5=6%
CRP women (Pai et al., 2004)	ΔIRR= 0.1015 CRP (mg/L	0.27 mg/L	0.5 mg/L	0.27/0.5=54%
CRP men (Pai et al., 2004)	ΔIRR= 0.2812 CRP (mg/L)	0.099 mg/L	0.27 mg/L	0.099/0.27=37%

[(1)] The biomarker level is calculated as 0.02778/slope. For example, for CRP level in women CRP = 0.02778/0.4025 = 0.07 mg/L.

Known modulating factors

Modulating factor	Specification	Effects on the KER	References
Life style	High body mass index	Increased level of serum amyloid A (SAA) and C reactive protein (CRP), therefore increased risk of atherosclerosis.	(Johnson et al., 2004)
Life style	Smoking	Increased level of CRP, therefore increased risk of atherosclerosis.	(Johnson et al., 2004; Willeit et al., 2000)
Medication	Intake of non-steroidal anti-inflammatory drugs	Reduction of CRP and other pro-inflammatory markers, decrease risk of atherosclerosis.	(Libby et al., 2019)
Medical conditions	Chronic inflammatory diseases	Increased level of acute phase proteins, therefore increased risk of atherosclerosis.	(Gabay & Kushner, 1999)
Medical conditions	Infectious diseases	Increased levels of CRP, therefore increased risk of atherosclerosis.	(Willeit et al., 2000)

Known Feedforward/Feedback loops influencing this KER

Atherosclerosis is an inflammatory condition (Balci, 2011; Ross, 1999), therefore there are increased levels of pro-inflammatory factors, including acute phase proteins, than can sustain the progression of atherosclerosis (Kobiyama & Ley, 2018).

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List of Non Adjacent Key Event Relationships

Relationship: 2958: Interaction with the lung cell membrane leads to Increased transcription of genes encoding acute phase proteins

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with lung resident cell membrane components leading to atherosclerosis	non-adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

The expression of *Saa* mRNA in lung and liver tissue has been shown in mice after pulmonary exposure to a variety of nanomaterials (see Empirical evidence), and in humans in different tissues as lung, liver and arteries (Meek et al., 1994; Urieli-Shoval et al., 1998).

Key Event Relationship Description

This KER presents the association between the interaction of stressors with the lung resident cell membrane components (Key event 1495) and transcription of genes encoding acute phase proteins (Key event 1438) in different tissues, mainly lungs and liver. The lungs consist of many different cell types. Some of these cell types are capable of detecting danger when in contact with stressors and transmit the signal to initiate the required inflammatory or immunological response (Franks et al., 2008; Hiemstra et al., 2015). Acute phase proteins are induced by pro-inflammatory mediators and may be expressed lung, liver, and several other tissues (Gabay & Kushner, 1999; NCBI, 2023; Urieli-Shoval et al., 1998). The evidence of the KER presented is based on animal studies (mice).

Evidence Supporting this KER

Biological Plausibility

The biological plausibility is high. After cells sense pathogens, tissue damage or dysmetabolism, production of acute phase proteins (Key event 1438) is triggered by cellular pattern-recognition molecules, through a cytokine cascade (Mantovani & Garlanda, 2023). In the lungs, this cytokine cascade is produced by epithelial cells and resident macrophages (Key event 1495) (Moldoveanu et al., 2009).

Empirical Evidence

Any substance that is inhaled will interact with a component of the respiratory system, including cells. Any study that shows that inhalation exposure leads to transcription of genes encoding acute phase protein is considered evidence for this KER, even if the specific interaction between the substance and the respiratory system has not been investigated.

The table below presents evidence for this KER. Exposure through the respiratory system (inhalation or intratracheal instillation) of stressors was considered as interaction with lung resident cell membrane components (Key event 1495), while the transcription of genes encoding acute phase proteins was measured in tissues (Key event 1438).

Species	Stressor	Substance interaction with lung residents cell membrane components	Transcription of genes encoding acute phase proteins	Reference
Mouse	Ultrafine carbon particles	Yes, inhalation of 380 ug/m^3 for 4 or 24 h.	Yes, increased *S a a 3* gene expression at 24 h.	(Andre et al., 2006)
Mouse	Diesel exhaust particles	Yes, inhalation of 20 mg/m^3.	Yes, Increased expression of *Saa3* in lung tissue. No expression of *Sap, Saa1 or Saa3* genes on liver tissue.	(Saber et al., 2009, 2013)
Mouse	Carbon black	Yes, inhalation of 20 mg/m^3.	Yes, increased expression of *Saa3* in lung tissue. No expression of *Sap, Saa1 or Saa3* genes on liver tissue.	(Saber et al., 2009, 2013)
Mouse	Titanium dioxide nanoparticles	Yes, inhalation of 42.4 mg/m^3.	Yes, increased expression of *Saa1* and *Saa3* in lung tissue	(Halappanavar et al., 2011)
Mouse	Carbon black nanoparticles	Yes, intratracheal instillation of 162 µg.	Yes, significant *Saa1, Saa2* and *Saa3* gene expression increase in lung tissue, at days 1, 3 and 28 after exposure. *S a a 3* gene expression increase in liver tissue at day 1 after exposure.	(Bourdon et al., 2012)
Mouse	Titanium dioxide nanoparticles	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, genes biological processes related to acute phase response genes were enriched at day 1 post-exposure. There was also an increase in gene expression of *Saa1*, *Saa2* and *S a a 3* in lung tissue after 1 day.	(Husain et al., 2013)
Mouse	Titanium dioxide nanoparticles	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, increased mRNA expression of *Saa3* in lung tissue at days 1, 3 and 28 after exposure with 162 µg, and at day 3 with 54 µg.	(Saber et al., 2013)
Mouse	Carbon black nanoparticles	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, increased mRNA expression of *Saa3* in lung issue at days 1, 3 and 28 after exposure with 54 µg and 162 µg, and at days 1 and 3 with 18 µg.	(Saber et al., 2013)
Mouse	Multiwalled carbon nanotubes	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, increased mRNA expression of *Saa3* in lung issue at days 1, 3 and 28 with all doses.	(Saber et al., 2013)
Mouse	Singlewalled carbon nanotubes	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, increased mRNA expression of *Saa3* in lung issue at days 1, 3 and 28 after exposure with 54 µg and 162 µg, and at days 1 and 3 with 18 µg.	(Saber et al., 2013)
Mouse	Titanium dioxide	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, gene pathways related to acute phase response were significantly altered 1 day after exposure.	(Halappanavar et al., 2015)
Mouse	Diesel exhaust particles	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, increased *Saa3* gene expression after 1, 3 and 28 days with 162 µg, at day 28 with 54 µg, and at day 3 with 18 µg.	(Kyjovska et al., 2015)
Mouse	Multiwalled carbon nanotubes (referred as CNT_{small})	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, increased differential expression of acute phase response genes in lung and liver tissue.	(Poulsen, Saber, Mortensen, et al., 2015; Poulsen, Saber, Williams, et al., 2015)

Species	Stressor	Substance interaction with lung residents cell membrane components	Transcription of genes encoding acute phase proteins	Reference
Mouse	Multiwalled carbon nanotubes (referred as CNT_{large})	Yes, 18, 54 and 162 µg. intratracheal instillation of	Yes, increased differential expression of acute phase response genes in lung and liver tissue.	(Poulsen, Saber, Mortensen, et al., 2015; Poulsen, Saber, Williams, et al., 2015)
Mouse	Sanding dust from epoxy composite containing carbon nanotubes	Yes, intratracheal instillation of 486 µg.	Yes, significant increase in *Saa1* mRNA expression in liver tissue.	(Saber et al., 2016)
Mouse	Sanding dust from epoxy composite without carbon nanotubes	Yes, intratracheal instillation of 486 µg.	Yes, significant increase in *Saa1* mRNA expression in liver tissue.	(Saber et al., 2016)
Mouse	Carbon nanotubes	Yes, intratracheal instillation of 162 µg.	Yes, significant increase in *Saa1* mRNA expression in liver tissue.	(Saber et al., 2016)
Mouse	Graphene oxide	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, increased mRNA expression of *Saa3* in lung tissue, at all dose 1 and 3 days after exposure. Increased gene expression of *Saa1* in liver tissue 1 day after exposure to 18 µg, and 3 days after exposure to 162 µg.	(Bengtson et al., 2017)
Mouse	Reduced graphene oxide	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, increased mRNA expression of *Saa3* in lung tissue, 3 days after exposure to 162 µg. No changes in gene expression of *Saa1* in liver tissue.	(Bengtson et al., 2017)
Mouse	Carbon black	Yes, intratracheal instillation of 162 µg.	Yes, increased mRNA expression of *Saa3* in lung tissue 1, 3, 28 and 90 days after exposure. Increased gene expression of *Saa1* in liver tissue 1 day after exposure.	(Bengtson et al., 2017)
Mouse	Multiwalled carbon nanotubes	Yes, intratracheal instillation of 6, 18 and 54 µg.	Yes, increased *Saa1* mRNA expression in liver tissue, 1 day after exposure to 18 and 54 µg. Increase in *Saa1* mRNA levels in liver tissue 28 days after exposure to 54 µg. Increased *Saa3* mRNA expression in lung tissue 1 day after exposure to 6, 18 and 54 µg. Increase in *Saa3* mRNA levels in lung tissue 28 days after exposure to 18 and 54 µg.	(Poulsen et al., 2017)
Mouse	Unmodified rutile (TiO2)	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, increased expression of *Saa3* mRNA in lung tissue 1, 3 and 28 days after exposure to 162 µg. Increased expression of *Saa1* in liver tissue 1 day after exposure to 162 µg and 3 days after exposure to 54 and 162 µg.	(Wallin et al., 2017)
Mouse	Surface modified rutile (TiO2)	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, increased expression of *Saa3* mRNA in lung tissue 1, and 28 days after exposure to 54 µg, and 1, 3 and 28 days after exposure to 162 µg. Increased expression of *Saa1* in liver tissue 1 day after exposure to 162 µg.	(Wallin et al., 2017)
Mouse	Particulate matter from non-commercial airfield	Yes, intratracheal instillation of 6, 18 and 54 µg.	Yes, increased expression of *Saa3* mRNA in lung tissue and *Saa1* mRNA in liver tissue after 1 day of exposure to 54 µg. No effect after 28 and 90 days.	(Bendtsen et al., 2019)

Species	Stressor	Substance interaction with lung residents cell membrane components	Transcription of genes encoding acute phase proteins	Reference
Mouse	Particulate matter from commercial airport	Yes, intratracheal instillation of 6, 18 and 54 µg.	Yes, increased expression of *Saa3* mRNA in lung tissue after 1 day of exposure to 18 and 54 µg. No effect after 28 and 90 days.	(Bendtsen et al., 2019)
Mouse	Diesel exhaust particles	Yes, intratracheal instillation of 18, 54 and 54 µg.	Yes, increased expression of *Saa3* mRNA in lung tissue after 1 day of exposure to 54 and 162 µg, and increased expression of *Saa1* mRNA in liver tissue 1 day after exposure to 162 µg. No effect after 28 days.	(Bendtsen et al., 2019)
Mouse	Carbon black	Yes, intratracheal instillation of 54 µg.	Yes, increased expression of *Saa3* mRNA in lung tissue at day 1 and day 90.	(Bendtsen et al., 2019)
Mouse	Uncoated zinc oxide nanoparticles	Yes, intratracheal instillation of 0.2, 0.7 and 2 µg.	Yes, increase on *Saa3* mRNA in lung tissue 1 day after exposure to 2 µg. No effect 3 and 28 days after exposure.	(Hadrup et al., 2019)
Mouse	Coated zinc oxide nanoparticles	Yes, intratracheal instillation of 0.2, 0.7 and 2 µg.	Yes, increase on *Saa3* mRNA in lung tissue 1 day after exposure to 0.7 and 2 µg. No effect 3 and 28 days after exposure.	(Hadrup et al., 2019)
Mouse	Surface modified hallosytes	Yes, intratracheal instillation of 6, 18 and 54 µg.	Yes, increase *Saa3* mRNA expression in lung tissue 1 and 3 days after exposure to 54 µg. No effect on *Saa1* mRNA expression on liver tissue.	(Barfod et al., 2020)
Mouse	Carbon black	Yes, intratracheal instillation of 162 µg.	Yes, increase *Saa3* mRNA expression in lung tissue 1, 3 and 28 days after exposure. No effect on *Saa1* mRNA expression on liver tissue.	(Barfod et al., 2020)

Additional evidence can be found in the following links:Additional evidence KER2958_1, Additional evidence KER2958_2 and Additional evidence KER2958_3.

Uncertainties and Inconsistencies

Although it is suggested that acute phase proteins are mainly produced in the liver(Gabay & Kushner, 1999), it has been shown that in mice, the liver has little upregulation of *Saa* genes after exposure to ultrafine carbon particles or diesel exhaust particle, while it is in the lung where there is a marked expression of *Saa3* mRNA (Saber et al., 2009, 2013).

In the case of nanomaterials, it has been shown that physicochemical characteristics as size, surface area, surface functionalization, shape, composition, among others, affect the magnitude and duration of the expression of acute phase proteins in mice (Barfod et al., 2020; Bengtson et al., 2017; Danielsen et al., 2020; Gutierrez et al., 2023; Hadrup et al., 2019; Poulsen et al., 2017; Wallin et al., 2017).

In humans, measuring gene expression of acute phase proteins is not very common as a tissue sample is needed, while measuring acute phase protein in blood in more common. However, *Saa* mRNA has been shown expressed in different tissues including lung, liver and arteries (Meek et al., 1994; Urieli-Shoval et al., 1998)

The table below presents inconsistencies for this KER, where substance interaction with lung resident cell membrane components has occurred, while transcription of genes encoding acute phase proteins was not observed. Exposure through the respiratory system (intratracheal instillation) of stressors was considered as interaction with lung resident cell membrane components, while the transcription of genes encoding acute phase proteins was measured in tissues.

Species	Stressor	Substance interaction with lung residents cell membrane components	Transcription of genes encoding acute phase proteins	Reference
Mouse	Unmodified hallosytes	Yes, intratracheal instillation of 6, 18 and 54 µg.	No effect on *Saa3* mRNA expression in lung tissue nor *S a a 1* mRNA expression in liver tissue.	(Barfod et al., 2020)

Species	Stressor	Substance interaction with lung residents cell membrane components	Transcription of genes encoding acute phase proteins	Reference
Mouse	Aluminum oxide	Yes, intratracheal instillation of 18 and 54 µg.	No change in *Saa3* mRNA expression in lung tissue.	(Gutierrez et al., 2023)
Mouse	Cube titanium dioxide	Yes, intratracheal instillation of 18, 54 and 162 µg.	No change in *Saa3* mRNA expression in lung tissue. No change in *Saa1* mRNA expression in liver tissue.	(Danielsen et al., 2020)

Quantitative Understanding of the Linkage

Response-response relationship

The interaction of insoluble nanomaterials with the lungs (Key event 1495) (measured in dosed surface area: dosed mass multiply by specific surface area) is correlated to the expression of *Saa3* mRNA levels in mice lung tissue (Key event 1438) and the responses show a linear regression, in female C57BL/6J mice 1 day after intratracheal instillation (Gutierrez et al., 2023) (Figure 1). The Pearson's correlation coefficient was 0.70 ($p < 0.001$) between log-transformed dosed surface area and log-transformed *Saa3* mRNA levels in mice lung tissue. The linear regression formula obtained was Log *Saa3*mRNA = 1.080*Log Dosed surface area + 0.9415 ($p<0.001$)(Gutierrez et al., 2023).

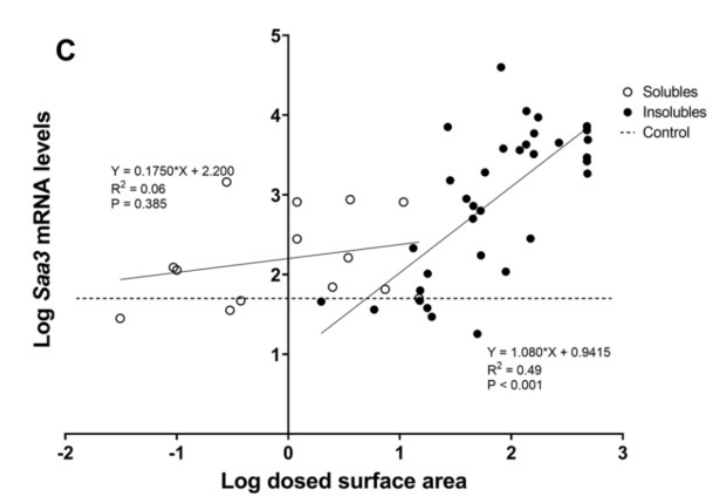

Figure 1. Correlations between dosed surface area and *Saa3* mRNA levels in lung tissue, 1 day after exposure to nanomaterials. Reproduced from Gutierrez et al. (2023).

Time-scale

After exposure to titanium dioxide nanoparticles in mice, expression of*Saa1* mRNA in the liver is short lasting, while expression of *Saa3* mRNA in lung tissue is longer lasting, as it has been observed 28 day after exposure (Wallin et al., 2017).

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Relationship: 2959: Interaction with the lung cell membrane leads to Systemic acute phase response

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with lung resident cell membrane components leading to atherosclerosis	non-adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Systemic acute phase response is measured as elevation of acute phase proteins in humans (mainly C-reactive protein and serum amyloid A), and serum amyloid A in mice has been shown after exposure to several stressors (see Empirical evidence).

Key Event Relationship Description

This KER presents the association between the interaction of stressors with the lung resident cell membrane components (Key event 1495) and the induction of systematic acute phase response (Key event 1439). The lungs consist of many different cell types. Some of these cell types are capable of detecting danger when in contact with stressors and transmit the signal to initiate the required inflammatory or immunological response (Franks et al., 2008; Hiemstra et al., 2015). Acute phase response is the systemic response to acute and chronic inflammatory states, where acute phase response genes are expressed in organs such as lung and liver, and subsequently secreted into systemic circulation (Gabay & Kushner, 1999). The two major acute phase proteins are C-reactive protein and serum

amyloid A (Gabay & Kushner, 1999). The evidence of the KER presented is based on animal studies (mice), controlled human studies and epidemiological studies

Evidence Supporting this KER

Biological Plausibility

The biological plausibility is high. Pulmonary inflammation occurs when stressors interact with the airways (Moldoveanu et al., 2009) and acute phase response is induced during inflammatory conditions (Gabay & Kushner, 1999). It has been shown (see table below) that exposure to different stressors produces an increase of acute phase proteins in blood [i.e. C-reactive protein (CRP) and serum amyloid A (SAA)] in humans and mice.

Empirical Evidence

Any substance that is inhaled will interact with a component of the respiratory system, including cells. Any study that shows that inhalation exposure leads to systemic acute phase response is considered evidence for this KER, even if the specific interaction between the substance and the respiratory system has not been investigated.

The table below presents the evidence for this KER. Exposure through the respiratory system (inhalation or intratracheal instillation) of stressors was considered as interaction with lung resident cell membrane components (Key event 1495), while systemic acute phase response is measured as the concentration of acute phase protein in blood plasma or serum (Key event 1439).

Species	Stressor	Substance interaction with lung residents cell membrane components	Systemic acute phase response	Reference
Mouse	Carbon black nanoparticles	Yes, intratracheal instillation of 162 µg.	Yes, significant increase of plasma serum amyloid A (SAA) at 1 and day 28 after exposure.	(Bourdon et al., 2012)
Mouse	Multiwalled carbon nanotubes	Yes, intratracheal instillation of 18, 54 and 128 µg.	Yes, increased levels of plasma SAA3 after 1 day, with 128 µg.	(Saber et al., 2013)
Mouse	Multiwalled carbon nanotubes (referred as CNT_{small})	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, increased plasma SAA3 1, 3 and 28 days after exposure to 162 µg, and 3 days after exposure to 18 and 54 µg.	(Poulsen, Saber, Mortensen, et al., 2015; Poulsen, Saber, Williams, et al., 2015)
Mouse	Multiwalled carbon nanotubes (referred as CNT_{large})	Yes, 18, 54 and 162 µg. intratracheal instillation of	Yes, increased plasma SAA3 1 and 3 days after exposure to 162 µg, and 3 days after exposure to 54 µg.	(Poulsen, Saber, Mortensen, et al., 2015; Poulsen, Saber, Williams, et al., 2015)
Mouse	Graphene oxide	Yes, intratracheal instillation of 18, 54 and 162 µg.	Yes, increased SAA3 plasma levels 3 days after exposure to 54 and 162 µg.	(Bengtson et al., 2017)
Mouse	Multiwalled carbon nanotubes	Yes, intratracheal instillation of 54 µg.	Yes, increased SAA1/2 and SAA3 plasma levels 1 day after exposure to. No change in SAA1/2 and SAA3 28 and 92 days after exposure.	(Poulsen et al., 2017)
Mouse	Multiwalled carbon nanotubes	Yes, intratracheal instillation of 6, 18 and 54 µg.	Yes, increased SAA1/2 plasma levels 1 day after exposure. No change in SAA1/2 28 and 92 days after exposure. Increased SAA3 plasma levels 1 days after exposure. Increased SAA3 plasma levels 28 and 92 days after exposure.	(Poulsen et al., 2017)
Mouse	Carbon black	Yes, intratracheal instillation of 162 µg.	Yes, increased SAA3 plasma levels 1 days after exposure. No change in SAA3 28 and 92 days after exposure. No change in SAA1/2 plasma levels.	(Poulsen et al., 2017)
Mouse	Particulate matter from non-commercial airfield	Yes, intratracheal instillation of 6, 18 and 54 µg.	Yes, increased plasma SAA3 levels after exposure to 54 µg.	(Bendtsen et al., 2019)

Species	Stressor	Substance interaction with lung residents cell membrane components	Systemic acute phase response	Reference
Mouse	Diesel exhaust particles	Yes, intratracheal instillation of 18, 54 and 54 µg.	Yes, increased plasma SAA3 levels after exposure to 54 µg.	(Bendtsen et al., 2019)
Mouse	Nanofibrilated celluloses (FINE NFC, BIOCID FINE NFC and AS)	Yes, intratracheal instillation of 6 and 18 µg.	FINE NFC increased plasma SAA3 1 day after exposure to 6 and 18 µg, while AS increased SAA3 after exposure to 18 µg. After 28 days, only 6 µg of FINE NFC increased plasma SAA3.	(Hadrup, Knudsen, et al., 2019)
Mouse	Copper oxide	Yes, intratracheal instillation of 2, 6 and 12 µg.	Yes, increased plasma SAA1/2 level after exposure to 6 µg.	(Gutierrez et al., 2023)
Mouse	Tin dioxide	Yes, intratracheal instillation of 54 and 162 µg.	Yes, increased plasma SAA3 after exposure to 162 µg.	(Gutierrez et al., 2023)
Mouse	Titanium dioxide	Yes, intratracheal instillation of 162 µg.	Yes, increased plasma SAA3 and SAA1/2 after exposure to 162 µg.	(Gutierrez et al., 2023)
Mouse	Carbon black	Yes, intratracheal instillation of 162 µg.	Yes, increased plasma SAA3 and SAA1/2 after exposure to 162 µg.	(Gutierrez et al., 2023)
Mouse	Singlewalled carbon nanotubes	Yes, pharyngeal aspiration of 40 µg.	Yes, increase serum CRP, haptoglobin and SAP 1 day after exposure.	(Erdely et al., 2011)
Mouse	Multiwalled carbon nanotubes	Yes, pharyngeal aspiration of 40 µg.	Yes, increase serum CRP, haptoglobin and SAP 1 day after exposure. No changes after 28 days.	(Erdely et al., 2011)
Mouse	Serum amyloid A	Yes, intratracheal instillation (2 µg) once a week for 10 weeks.	Yes, increased levels of endogenous serum SAA3.	(Christophersen et al., 2021)
Human	Welding fumes	Yes, median exposure to welders ($PM_{2.5}$) was 1.66 mg/m^3 and 0.04 mg/m^3 for controls, during 5.3 h.	No changes in serum C reactive protein (CRP) 6 hours after exposure, but significantly increased serum CRP levels 16 hours after welding.	(Kim et al., 2005)
Human	Wood smoke	Yes, 4h exposure to 240-280 µg/m^3.	Yes, significant increase in blood SAA after exposure, and 3 and 20 h after exposure, no change in CRP.	(Barregard et al., 2006)
Human	Brazing fumes	Yes, 6h exposure to 1.4, 2 and 2.5 mg/m^3.	Yes, increased blood CRP 24h after exposure to 2 and 2.5 mg/m^3.	(Brand et al., 2014)
Human	Fumes from welding aluminium	Yes, 6h exposure to 2.5 mg/m^3.	Yes, significantly increased blood CRP 24 after exposure. No change after exposure nor a week after exposure.	(Hartmann et al., 2014)
Human	Fumes from welding zinc coated materials	Yes, 6h exposure to 2.5 mg/m^3.	Yes, significantly increased blood CRP 24 after exposure. No change after exposure nor a week after exposure.	(Hartmann et al., 2014)
Human	Traffic related particulate matter	Yes, exposure during work hours.	Yes, serum CRP and SAA were significantly and positively associated with increases in exposure.	(Meier et al., 2014)
Human	Fumes from brazing galvanized steel, using aluminum bronze wire	Yes, 6h exposure to 2.5 mg/m^3.	Yes, significant increase in serum CRP and SAA 29 h after exposure. No change 6 nor 10 h after exposure.	(Baumann et al., 2016)

Species	Stressor	Substance interaction with lung residents cell membrane components	Systemic acute phase response	Reference
Human	Fumes from welding galvanized steel and aluminum, using zinc wire	Yes, 6h exposure to 2 mg/m^3.	Yes, significant increase in serum CRP and SAA 29 h after exposure. No change 6 nor 10 h after exposure.	(Baumann et al., 2016)
Human	Fumes from brazing galvanized steel using zinc wire	Yes, 6h exposure to 2 mg/m^3.	Yes, significant increase in serum CRP 29 h after exposure. No change 6 nor 10 h after exposure.	(Baumann et al., 2016)
Human	Dust from pulp and paper mill	Yes, exposure during working hours.	Yes, blood CRP, SAA and fibrinogen were significantly and positively associated with the exposure.	(Westberg et al., 2016)
Human	Zinc welding fumes	Yes, 6h exposure to 2.5 mg/m^3	Yes, significant plasma SAA increase at 24 h. No effect at 6h.	(Baumann et al., 2018)
Human	Copper welding fumes	Yes, 6h exposure to 2.5 mg/m^3	Yes, significant plasma SAA increase at 24 h. No effect at 6h.	(Baumann et al., 2018)
Human	Zinc and copper welding fumes	Yes, 6h exposure to 2.5 mg/m^3	Yes, significant plasma SAA increase at 24 h. No effect at 6h.	(Baumann et al., 2018)

Additional empirical evidence can be found in the following links:Additional evidence KER 2959_1 and Additional evidence KER 2959_2.

Uncertainties and Inconsistencies

In the case of nanomaterials, it has been shown that physicochemical characteristics as size, surface area, surface functionalization, shape, composition, among others, affect the magnitude and duration of acute phase response in mice (Bengtson et al., 2017; Gutierrez et al., 2023; Poulsen et al., 2017).

It has been observed that in most controlled human studies, an increase in CRP and/or SAA was observed after exposure to particulate matter (Baumann et al., 2018; Monse et al., 2018, 2021; Walker et al., 2022; Wyatt et al., 2020). However, in other human studies the exposure did not induce acute phase response(Andersen, Saber, Clausen, et al., 2018; Andersen, Saber, Pedersen, et al., 2018), maybe due to a low level of exposure(Andersen et al., 2019).

The table below presents inconsistencies for this KER, where substance interaction with lung resident cell membrane components has occurred, while systemic acute phase response was not observed. Exposure through the respiratory system (inhalation or intratracheal instillation) of stressors was considered as interaction with lung resident cell membrane components, while systemic acute phase response is measured as the concentration of acute phase protein in blood plasma or serum.

Species	Stressor	Substance interaction with lung residents cell membrane components	Systemic acute phase response	Reference
Mouse	Diesel exhaust particles	Yes, inhalation of 20 mg/m^3 for 90 min, in 4 consecutive days.	No effect.	(Saber et al., 2009, 2013)
Mouse	Carbon black	Yes, inhalation of 20 mg/m^3 for 90 min, in 4 consecutive days.	No effect.	(Saber et al., 2009, 2013)
Mouse	Reduced graphene oxide	Yes, intratracheal instillation of 18, 54 and 162 µg.	No, no change in SAA3 plasma concentration 3 days after exposure.	(Bengtson et al., 2017)
Mouse	Crocidolite	Yes, intratracheal instillation of 6 and 18 µg.	No change in SAA1/2 nor SAA3 plasma levels.	(Poulsen et al., 2017)
Mouse	Particulate matter from commercial airport	Yes, intratracheal instillation of 6, 18 and 54 µg.	No change in plasma SAA3.	(Bendtsen et al., 2019)
Mouse	Carbon black	Yes, intratracheal instillation of 54 µg.	No change in plasma SAA3.	(Bendtsen et al., 2019)
Mouse	Uncoated zinc oxide nanoparticles	Yes, intratracheal instillation of 0.2, 0.7 and 2 µg.	No effect on plasma SAA3.	(Hadrup, Rahmani, et al., 2019)
Mouse	Coated zinc oxide nanoparticles	Yes, intratracheal instillation of 0.2, 0.7 and 2 µg.	No effect on plasma SAA3.	(Hadrup, Rahmani, et al., 2019)
Mouse	Zinc oxide	Yes, intratracheal instillation of 0.7 and 2 µg.	No change in plasma SAA3 or SAA1/2 levels.	(Gutierrez et al., 2023)

Species	Stressor	Substance interaction with lung residents cell membrane components	Systemic acute phase response	Reference
Mouse	Aluminum oxide	Yes, intratracheal instillation of 18 and 54 µg.	No change in plasma SAA3 or SAA1/2 levels.	(Gutierrez et al., 2023)
Human	Particulate matter and gas from fire extinguishing exercise	Yes, exposure during training exercises.	No change was observed on blood CRP or SAA levels.	(Andersen, Saber, Clausen, et al., 2018)
Human	Gas and particulate matter from firefighting activities	Yes, exposure during firefighting.	No change was observed on blood CRP or SAA levels.	(Andersen, Saber, Pedersen, et al., 2018)
Human	Diesel exhaust	Yes, 6h per day for 3 days.	No change was observed on blood CRP or SAA levels.	(Andersen et al., 2019)

Quantitative Understanding of the Linkage

Response-response relationship

The interaction of insoluble nanomaterials with the lungs (Key event 1495) (measured in dosed surface area: dosed mass multiply by specific surface area) is correlated to serum amyloid A (SAA)3 and SAA1/2 plasma levels (Key event 1439) and the responses show a linear regression, in female C57BL/6J mice 1 day after intratracheal instillation (Gutierrez et al., 2023) (Figure 1 and Figure 2).

The Pearson's correlation coefficient was 0.92 ($p < 0.001$) between log-transformed dosed surface area (dosed mass multiply by specific surface area) and log-transformed SAA3 plasma levels (Figure 1). The linear regression formula obtained was Log SAA3 = 0.9459 *Log Dosed surface area – 2.854 ($p=0.01$). In the case SAA1/2, the correlation coefficient was 0.83 ($p<0.05$) between log-transformed dosed surface area and log-transformed SAA1/2 plasma levels was, and the linear regression formula was Log SAA1/2 = 0.6368 *Log Dosed surface area +0.09524 ($p=0.01$) (Figure 2) (Gutierrez et al., 2023).

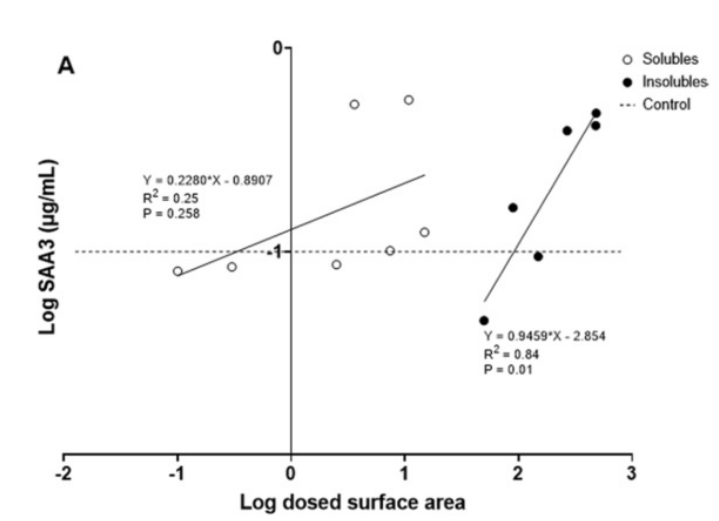

Figure 1. Correlations between pulmonary dosed surface area and SAA3 protein in plasma, 1 day after exposure to nanomaterials. Reproduced from Gutierrez et al. (2023).

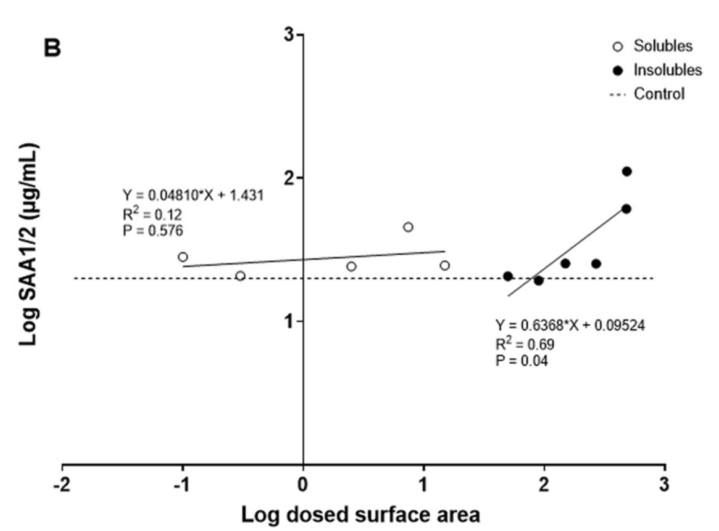

Figure 2. Correlations between pulmonary dosed surface area and SAA1/2 protein in plasma, 1 day after exposure to nanomaterials. Reproduced from Gutierrez et al. (2023).

Time-scale

In mice, increased serum amyloid A (SAA) protein levels are observed 1 and 3 days after most exposures, however increased SAA levels are not frequently observed 28 or 90 days after exposure (Bourdon et al., 2012; Hadrup et al., 2019; Poulsen et al., 2017; Poulsen, Saber, Mortensen, et al., 2015; Poulsen, Saber, Williams, et al., 2015).

In humans, increased SAA and C-reactive protein has been observed 22h and 2 days after exposure to zinc oxide, but not 3 days after exposure (Monse et al., 2021). After exposure to zinc oxide, copper oxide or a mix both, SAA levels were elevated 24h after exposure in humans, but not 6h after exposure (Baumann et al., 2018).

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Relationship: 3052: Increased proinflammatory mediators leads to Systemic acute phase response

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with lung resident cell membrane components leading to atherosclerosis	non-adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Acute phase response is conserved in vertebrate species (Cray, Zaias, & Altman, 2009).

Key Event Relationship Description

This KER presents the association between the secretion of pro-inflammatory mediators (Key event 1496) and the induction of systemic acute phase response (Key event 1439). Pro-inflammatory mediators are secondary messengers that initiate and regulate inflammatory reactions. They are secreted during inflammation in all species. Acute phase response is the systemic response to acute and chronic inflammatory states, that includes changes in plasma

concentration of proteins (Gabay & Kushner, 1999). The evidence of the KER presented is based on animal studies (mice), controlled human studies and epidemiological studies.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility is high. The production of acute phase proteins during acute phase response is induced by the release of pro-inflammatory markers as interleukin (IL)-6, IL-1β, and tumor necrosis factor α (TNF-α) at inflammatory sites (Gabay & Kushner, 1999; Mantovani & Garlanda, 2023).

In this KER, pulmonary inflammation has been considered as an indirect marker of the release of pro-inflammatory factors because the release of inflammatory mediators (i.e. cytokines and chemokines) recruits immune cells to inflammation sites (Janeway, Murphy, Travers, & Walport, 2008). In mice, pulmonary inflammation is commonly assessed as the number or fraction of neutrophils in the broncheoalveolar lavage fluid (BALF) (Van Hoecke, Job, Saelens, & Roose, 2017).

Empirical Evidence

The table below presents evidence for this KER. Secretion of pro-inflammatory mediators is measured as change in concentration of pro-inflammatory markers in blood or increase neutrophil numbers in blood or bronchoalveolar lavage fluid (BALF) (Key event 1496), while systemic acute phase response is measured as the concentration of acute phase proteins in blood plasma or serum (Key event 1439).

Species	Stressor	Secretion of pro-inflammatory mediators	Systemic acute phase response	Reference
Mouse	Carbon black nanoparticles	Yes, significant increase of neutrophil number 1, 3 and 28 days after exposure.	Yes, significant increase of plasma serum amyloid A (SAA) at 1 and day 28 after exposure.	(Bourdon, Halappanavar, et al., 2012; Bourdon, Saber, et al., 2012)
Mouse	Multiwalled carbon nanotubes (referred as CNT_{small})	Yes, increased neutrophil numbers in BALF 1, 3 and 28 days after exposure to 18, 54 and 162 µg.	Yes, increased plasma SAA3 1, 3 and 28 days after exposure to 162 µg, and 3 days after exposure to 18 and 54 µg.	(Poulsen, Saber, Mortensen, et al., 2015; Poulsen, Saber, Williams, et al., 2015)
Mouse	Multiwalled carbon nanotubes (referred as CNT_{large})	Yes, increased neutrophil numbers in BALF 1, 3 and 28 days after exposure to 18, 54 and 162 µg.	Yes, increased plasma SAA3 1 and 3 days after exposure to 162 µg, and 3 days after exposure to 54 µg.	(Poulsen, Saber, Mortensen, et al., 2015; Poulsen, Saber, Williams, et al., 2015)
Mouse	Graphene oxide	Yes, increased neutrophil numbers in BALF 1 and 3 days after exposure to 18, 54 and 162 µg.	Yes, increased SAA3 plasma levels 3 days after exposure to 54 and 162 µg.	(Bengtson et al., 2017)
Mouse	Multiwalled carbon nanotubes (MWCNT)	Yes, increased neutrophil numbers in BALF 1 day after exposure to 6, 18 and 54 µg. Increased neutrophil numbers in BALF 28 days after exposure to 6, 18 and 54 µg. Increased neutrophil numbers in BALF 92 days after exposure to 54 µg.	Yes, increased SAA1/2 and SAA3 plasma levels 1 day after exposure. No change in SAA1/2 and SAA3 plasma leves28 and 92 days after exposure.	(Poulsen et al., 2016, 2017)
Mouse	Carbon black	Yes, increased neutrophil numbers in BALF after 1, 28, 92 days after exposure.	Yes, increased SAA3 plasma levels 1 days after exposure. No change in SAA3 28 and 92 days after exposure. No change in SAA1/2 plasma levels.	(Poulsen et al., 2016, 2017)
Mouse	Particulate matter from non-commercial airfield	Yes, increased neutrophil numbers in BALF 1 day after exposure to 18 and 54 µg.	Yes, increased plasma SAA3 levels after exposure to 54 µg.	(Bendtsen et al., 2019)
Mouse	Diesel exhaust particles	Yes, increased neutrophil numbers in BALF 1 day after exposure to 54 and 162 µg, and 28 days after exposure to 162 µg.	Yes, increased plasma SAA3 levels after exposure to 54 µg.	(Bendtsen et al., 2019)

Species	Stressor	Secretion of pro-inflammatory mediators	Systemic acute phase response	Reference
Mouse	Nanofibrilated celluloses (FINE NFC, BIOCID FINE NFC and AS)	Yes, increased neutrophil numbers in BALF 1 day after exposure to 6 and 18 µg of FINE NFC, 18 µg of AS, and 18 µg of BIOCID FINE NFC. Increased neutrophil numbers in BALF 28 days after exposure to 6 and 18 µg of FINE NFC, and 18 µg of AS.	FINE NFC increased plasma SAA3 1 day after exposure to 6 and 18 µg, while AS increased SAA3 after exposure to 18 µg. After 28 days, only 6 µg of FINE NFC increased plasma SAA3.	(Hadrup, Knudsen, et al., 2019)
Mouse	Copper oxide	Yes, increased neutrophil numbers in BALF 1 day after exposure to 2, 6 and 12 µg.	Yes, increased plasma SAA1/2 level after exposure to 6 µg.	(Gutierrez et al., 2023)
Mouse	Tin dioxide	Yes, increased neutrophil numbers in BALF 1 and 28 days after exposure to 162 µg.	Yes, increased plasma SAA3 after exposure to 162 µg.	(Gutierrez et al., 2023)
Mouse	Titanium dioxide	Yes, increased neutrophil numbers in BALF 1 and 28 days after exposure.	Yes, increased plasma SAA3 and SAA1/2 after exposure to 162 µg.	(Gutierrez et al., 2023)
Mouse	Carbon black	Yes, increased neutrophil numbers in BALF 1 and 28 days after exposure.	Yes, increased plasma SAA3 and SAA1/2 after exposure to 162 µg.	(Gutierrez et al., 2023)
Mouse	Serum amyloid A	Yes, increased neutrophil numbers in BALF.	Yes, increased levels of endogenous plasma SAA3.	(Christophersen et al., 2021)
Human	Welding fumes	Yes, significant increase in blood neutrophil numbers 6 hours after exposure, but no change 16 hours after welding.	No changes in serum C reactive protein (CRP) 6 hours after exposure, but significantly increased serum CRP levels 16 hours after welding.	(Kim et al., 2005)
Human	Wood smoke	Yes, significant lower increase in serum interleukin (IL)-6 3 h after exposure than exposure to clean air. No change immediately after exposure and 20 h after exposure. No change in serum tumor necrosis factor α (TNF-α).	Yes, significant increase in blood SAA immediately after exposure, and 3 and 20 h after exposure, no change in CRP.	(Barregard et al., 2006)
Human	Fumes from brazing galvanized steel, using aluminum bronze wire	Yes, significant increase in serum IL-6 levels 10 h after exposure.	Yes, significant increase in serum CRP and SAA 29 h after exposure. No change 6 nor 10 h after exposure.	(Baumann et al., 2016)
Human	Fumes from welding galvanized steel and aluminum, using zinc wire	Yes, significant increase in serum IL-6 levels 10 h after exposure.	Yes, significant increase in serum CRP and SAA 29 h after exposure. No change 6 nor 10 h after exposure.	(Baumann et al., 2016)
Human	Fumes from brazing galvanized steel using zinc wire	Yes, significant increase in serum IL-6 levels 10 h after exposure.	Yes, significant increase in serum CRP 29 h after exposure. No change 6 nor 10 h after exposure.	(Baumann et al., 2016)
Human	Zinc oxide	Yes, dose-response relationship in blood neutrophils 24 h after exposure.	Yes, dose-response relationship in CRP and SAA blood levels 24 h after exposure.	(Monse et al., 2018)
Human	Fumes from small arms firing	Yes, increased blood neutrophils 24h after exposure.	Yes, increased blood CRP levels 24h after exposure	(Sikkeland et al., 2018)

Species	Stressor	Secretion of pro-inflammatory mediators	Systemic acute phase response	Reference
Human	Ambient particulate matter	Yes, significant decrease of blood neutrophils.	Yes, increased blood levels of SAA and CRP 1h and 20h after exposure.	(Wyatt et al., 2020)
Human	Micro-sized zinc oxide	Yes, increased neutrophil number in blood 22 h after exposure.	Yes, increased blood CRP 22h and 2 days after exposure. No changes in CRP or SAA 3 days after exposure.	(Monse et al., 2021)
Human	Nano-sized zinc oxide	Yes, increased neutrophil number in blood 22 h after exposure.	Yes, increased blood CRP and SAA 22h and 2 days after exposure. No changes in CRP or SAA 3 days after exposure.	(Monse et al., 2021)

Uncertainties and Inconsistencies

Wyatt et al. observed a decrease in blood neutrophil numbers in humans after exposure to ambient particulate matter although an increase in serum amyloid A (SAA) and C reactive protein (CRP) was observed. It was mentioned this might be due to the translocation of neutrophil from major vessels to smaller arteries (Wyatt et al., 2020).

In the study by Meier et al., the authors obtained a negative association between particulate matter with a diameter of less than 2.5 μm ($PM_{2.5}$) exposure and blood levels of tumor necrosis factor (TNF-α) and interleukin (IL)-6, while SAA and CRP were positive associated with the exposure. The authors mentioned these results might be due the time point where the samples were taken (Meier et al., 2014).

Barregard et al. also observed that IL-6 levels were lower after exposure to wood smoke than after exposure to clean air. They suggested that this response was due to a possible sequestering of cytokines in the pulmonary capillary bed (Barregard et al., 2006).

The table below presents inconsistencies for this KER, where secretion of pro-inflammatory mediators has been observed after exposure to a stressor, while systemic acute phase response was not observed, or viceversa. Secretion of pro-inflammatory mediators was measured as change in concentration of pro-inflammatory markers in blood or increase neutrophil numbers in blood or bronchoalveolar lavage fluid (BALF), while systemic acute phase response was measured as the concentration of acute phase in blood plasma or serum.

Species	Stressor	Secretion of pro-inflammatory mediators	Systemic acute phase response	Reference
Mouse	Diesel exhaust particles	Yes, significant increase of neutrophils in BALF.	No effect	(Saber et al., 2005, 2009, 2013)
Mouse	Reduced graphene oxide	Yes, increased neutrophil numbers 1 and 3 days after exposure to 162, and 90 days after exposure to 18, 54 and 162 μg.	No, no change in serum amyloid A (SAA)3 plasma concentration 3 days after exposure.	(Bengtson et al., 2017)
Mouse	Crocidolite	Yes, increased neutrophil numbers in BALF after 1 and 28 days after exposure to 6 and 18 μg, and 92 days after exposure to 18 μg.	No change in SAA1/2 nor SAA3 plasma levels.	(Poulsen et al., 2016, 2017)
Mouse	Particulate matter from commercial airport	Yes, increased neutrophil numbers in BALF 1 day after exposure to 18 and 54 μg.	No change in plasma SAA3.	(Bendtsen et al., 2019)
Mouse	Carbon black	Yes, increased neutrophil in BALF after 1, 28 and 90 days of exposure.	No change in plasma SAA3.	(Bendtsen et al., 2019)
Mouse	Coated zinc oxide nanoparticles	Yes, increased neutrophil numbers in BALF 1 and 3 days after exposure to 2 μg, and 28 days after exposure to 0.2 and 0.7 μg.	No effect on plasma SAA3.	(Hadrup, Rahmani, et al., 2019)
Mouse	Zinc oxide	Yes, increased neutrophil numbers in BALF 1 day after exposure to 0.7 μg.	No change in plasma SAA3 or SAA1/2 levels.	(Gutierrez et al., 2023)
Mouse	Aluminum oxide	Yes, increased neutrophil numbers in BALF 1 and 28 days after exposure to 54 μg.	No change in plasma SAA3 or SAA1/2 levels.	(Gutierrez et al., 2023)

Species	Stressor	Secretion of pro-inflammatory mediators	Systemic acute phase response	Reference
Human	Brazing fumes	No significant change in blood neutrophils.	Yes, increased blood C reactive protein (CRP) after exposure to 2 and 2.5 mg/m^3.	(Brand et al., 2014)
Human	Fumes from welding aluminium	No significant change in blood neutrophils 24 h nor 7 days after exposure.	Yes, significantly increased blood CRP 24 after exposure. No change after exposure nor a week after exposure.	(Hartmann et al., 2014)
Human	Fumes from welding zinc coated materials	No significant change in blood neutrophils 24 h nor 7 days after exposure.	Yes, significantly increased blood CRP 24 after exposure. No change after exposure nor a week after exposure.	(Hartmann et al., 2014)
Human	Traffic related particulate matter	No, IL-6 had no significant association with exposure, and TNF-α had a negative significant association with the exposure.	Yes, serum CRP and SAA were significantly and positively associated with increases in exposure.	(Meier et al., 2014)
Human	Emissions from iron foundries	No significant increase in blood levels of IL-6 and IL-8.	Yes, blood SAA levels increased with increasing particulate matter exposure. No significant effects were observed for CRP.	(Westberg et al., 2019)
Human	Emissions from pine wood stove (three stone fire stove)	No significant change in blood levels of IL-6, IL-8 and TNF-α.	Yes, increased CRP and SAA blood levels 24 h after exposure. No change 3h after exposure.	(Walker et al., 2022)

Quantitative Understanding of the Linkage

Response-response relationship

Neutrophil number in brochoalveolar lavage fluid (BALF) (indirect measure of the secretion of proinflammatory mediators – Key event 1496) correlates with plasma SAA3 levels (Key event 1439), in female C57BL/6J mice 1 day after intratracheal instillation of metal oxide nanomaterials (Figure 1). The Pearson's correlation coefficient was 0.79 ($p<0.001$) between log-transformed neutrophil number in BALF and log-transformed SAA3 plasma protein levels (Gutierrez et al., 2023).

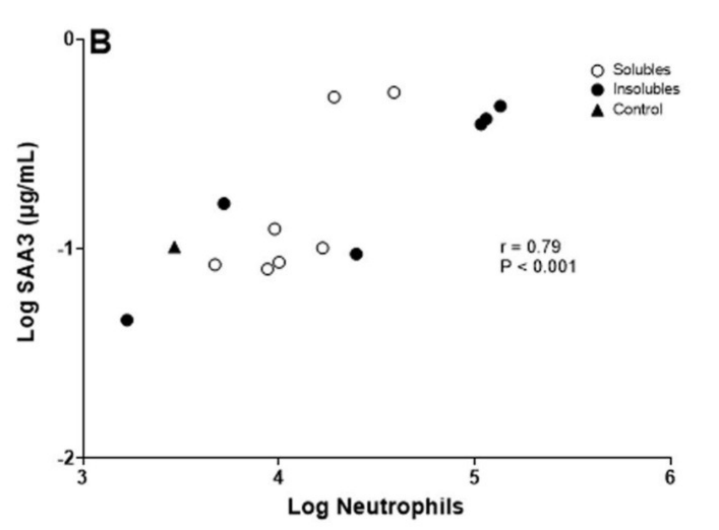

Figure 1. Correlation between neutrophil numbers and SAA3 plasma protein levels in mice 1 day after exposure to nanomaterials. Reproduced from Gutierrez et al. (2023).

A linear dose-response has also been found between neutrophil numbers in BALF and SAA3 plasma protein levels in

mice, 1 day after exposure to multiwalled carbon nanotubes (Figure 2) (Poulsen et al., 2017).

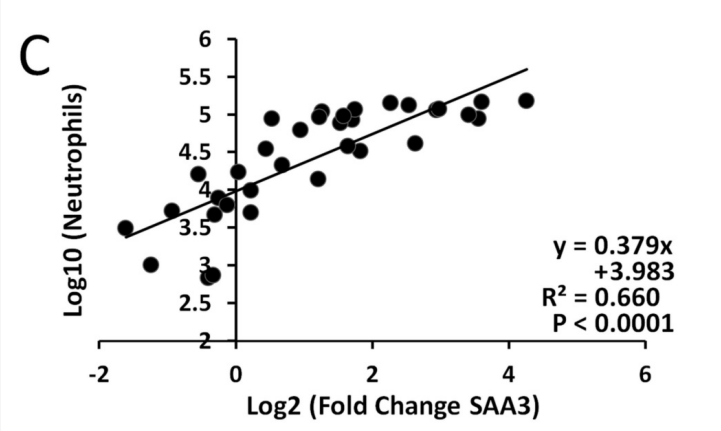

Figure 2. Transformed SAA3 protein vs. transformed neutrophil influx. Reproduced from Poulsen et al. (2017).

Time-scale

It has been shown that the concentration of pro-inflammatory mediators increases before acute phase proteins:

- In patients with atherosclerotic renal stenosis, blood interleukin (IL)-6 increased in the first hour after renal artery stenting and reached its highest concentration at 6h, while C-reactive protein (CRP) increased 6h after the treatment, peaking at 24h after treatment (Li et al., 2004).
- In human infants undergoing cardiopulmonary bypass, it has been observed that blood concentrations of IL-6 significantly increased after cessation of the procedure and remained elevated 24h later, while CRP started increased 6h after bypass and kept increasing at 12h and 24h after bypass (Allan et al., 2010).

Known Feedforward/Feedback loops influencing this KER

Interleukin (IL)-1, IL-6 and TNF- α can decrease acute phase response by decreasing their own production through the induction of corticosteroids (Uhlar & Whitehead, 1999).

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Relationship: 2960: Interaction with the lung cell membrane leads to Atherosclerosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with lung resident cell membrane components leading to atherosclerosis	non-adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adults	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Mouse models of human atherosclerosis has been shown to present atherosclerotic lesion progression after exposure to concentrated ambient particles, welding fumes and diesel exhaust particles (Chen & Nadziejko, 2005; Erdely et al., 2011; M. R. Miller et al., 2013).

In humans, epidemiological studies have shown that air pollution, as a stressor that interacts with the lungs, is a risk

factor for cardiovascular diseases (Vaduganathan, Mensah, Turco, Fuster, & Roth, 2022).

Key Event Relationship Description

This KER presents the association between the interaction of stressors with the lung resident cell membrane components (Key event 1495) and atherosclerosis (Key event 1443) as the adverse outcome. The evidence of the KER presented is based on mouse models of human atherosclerosis and human epidemiological studies.

Evidence Supporting this KER

The evidence for this KER was based on literature search on the search engine PubMed, epidemiological data and novel experimentation.

Biological Plausibility

The biological plausibility is moderate. Exposure to different stressors have been shown to induce the progression of atherosclerotic in mouse models of human atherosclerosis (see below). In humans, it has been hypothesized that air pollution, an example of stressor that interacts with the lungs, and cardiovascular diseases are linked by three pathways: i) translocation of inflammatory mediators from the lungs to the systemic circulation, ii) activation of alveolar receptors that results in the alteration of autonomic response and changes in cardiovascular function, and iii) translocation of particles (stressors) from the lungs to the systemic circulation (M. R. Miller & Newby, 2020; Van Eeden, Leipsic, Paul Man, & Sin, 2012).

Empirical Evidence

Any substance that is inhaled will interact with a component of the respiratory system, including cells. Any study that shows that inhalation exposure leads to atherosclerosis is considered evidence for this KER, even if the specific interaction between the substance and the respiratory system has not been investigated. For this KER, exposure through the respiratory system (inhalation, aspiration or intratracheal instillation) of stressors was considered as interaction with lung resident cell membrane components (Key event 1495), while atherosclerosis is measured as atherosclerotic lesions (i.e. plaques) size or area (Key event 1443).

- ApoE$^{-/-}$ and double knockout ApoE-/-LDLr$^{-/-}$ mice exposed to concentrated ambient particles (110 µg/m^3) for 6h/d, 5d/week for 5 months develop severe atherosclerosis. ApoE$^{-/-}$ mice exposed to concentrated ambient particles presented a 57% increase in aortic intima surface coverage by atherosclerotic lesion than mice exposed to air (Chen & Nadziejko, 2005).
- Intrapharyngeal aspiration of singlewalled carbon nanotubes into ApoE$^{-/-}$ mice (20 µg/mouse every 2 weeks for 8 weeks) induced a significant increase in plaque progression (Li et al., 2007).
- ApoE$^{-/-}$ mice on a Western diet showed an increase in atherosclerotic lesion area after exposure to fumes from gas metal arc-stainless steel welding (40 mg/m^3) for 3h/day for 10 days (Erdely et al., 2011).
- A modest increase in atherosclerotic plaque area was observed in ApoE$^{-/-}$ mice after intratracheal instillation of titanium dioxide nanoparticles (0.5 mg/kg) once a week for four weeks (Mikkelsen et al., 2011).
- ApoE$^{-/-}$ mice, fed a Western diet and exposed to diesel exhaust particles through oropharyngeal aspiration (35 µg) twice a week for four weeks, presented an increased atherosclerotic lesions area(M. R. Miller et al., 2013).
- Intratracheal instillation of human serum amyloid A once a week for 10 weeks in ApoE$^{-/-}$ mice (on Western-type diet) induced atherosclerotic plaque progression (Christophersen et al., 2021).

In addition, several epidemiological studies have shown that exposure to particulate matter from air pollution is associated to cardiovascular diseases (i.e. due to progression of atherosclerosis):

- A prospective study in six cities from USA showed that air pollution was associated with death from cardiopulmonary diseases (Dockery et al., 1993).
- In Dublin, there was a decrease in black smoke concentration in air, along with a significant decrease in the number of cardiovascular deaths, after the 1990 ban on coal sales (Clancy, Goodman, Sinclair, & Dockery, 2002).

Uncertainties and Inconsistencies

ApoE$^{-/-}$ mice seem to have a moderate plaque progression even when feed a normal diet, instead of high-fat diet, and exposed to the stressor for a short period (Mikkelsen et al., 2011).

Quantitative Understanding of the Linkage

Response-response relationship

- A decrease of 70% of black smoke (35 µg/m^3) was observed along with a 10.3% decrease ($p<0.0001$) in cardiovascular deaths following the ban of coal in Dublin (Clancy et al., 2002).
- An increase of 10 µg of $PM_{2.5}$ (particulate matter with a diameter of less than 2.5 µm) was associated with 24% increased risk of cardiovascular event and a 76% increased risk of death from a cardiovascular disease, in postmenopausal women from USA (K. A. Miller et al., 2007).

- A $PM_{2.5}$ increase of 5 µg/m^3 was associated with 21% increased risk of death from cerebrovascular disease, while an increase of 10 µg/m^3 of PM_{10} (particulate matter with a diameter of less than 10 µm) was associated with an 22% increased risk of death from cerebrovascular disease (Beelen et al., 2014). These results where analyzed from 22 European cohort studies on long-term exposure to air pollution and associations with cardiovascular diseases mortality (Beelen et al., 2014).
- Results from 11 cohort studies on long-term exposure to air pollution and incidence of acute coronary events showed a 13% increased risk of coronary events associated to 5 µg/m^3 increase of $PM_{2.5}$, and a 12% increased risk of coronary events associated to 10 µg/m^3 increase of PM_{10} (Cesaroni et al., 2014).

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