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Abstract: Hydrogen sulfide (H2S) represents one of the main pollutants originating from both geologic phenomena such as volcanoes, geysers, fumaroles and hot springs, and geothermal plants that produce heat and electricity. Many increasing data suggest that H2S retains a variety of biological properties, and modulates many pathways related to cardiovascular pathophysiology although its role as beneficial/adverse determinant on cardiovascular disease (CVD) is not clearly established. In this review, the current knowledge on the association between H2S exposure and risk of CVD in geothermal areas has been examined. The few epidemiological studies carried out in geothermal areas suggest, in some cases, a protective role of H2S towards CVD, while in others a positive association between exposure to H2S and increased incidence of CVD. Most of the studies have an ecological design that does not allow to produce evidence to support a causal relationship and also often lack for an adequate adjustment for individual CVD risk factors. The review has also considered the potential role of two other aspects not sufficiently explored in this relationship: the production of endogenous H2S that is a gasotransmitter producing beneficial effects on cardiovascular function at low concentration and the intake of H2S-releasing drugs for the treatment of patients affected by hypertension, inflammatory diseases, and CVD. Thus, a threshold effect of H2S and the shift of action as beneficial/adverse determinant given by the sum of exogenous exposure and endogenous production cannot be excluded. In this complex scenario, an effort is warranted in the future to include a more comprehensive evaluation of risk for CVD in relation to H2S emissions, especially in geothermal areas.

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Science of the Total Environment

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Cover Letter

Article submission:

Hydrogen sulfide and cardiovascular disease. doubts, clues, and pitfalls from studies in geothermal areas

The review we present to Science of the Total Environment deals with the assessment of the relationship between exposure to hydrogen sulfide (H_2S), which represents one of the main pollutants originating from both geologic phenomena such as volcanoes, geysers, fumaroles and hot springs, and geothermal plants that produce heat and electricity, and its effects on cardiovascular disease (CVD). Whereas the few epidemiological studies conducted in geothermal areas found inconsistent results, suggesting in some cases a protective role of H_2S towards CVD and in others a positive association between exposure to H_2S and increased incidence of CVD and ischemic heart disease, several aspects of this potential relationship were not sufficiently explored. To date, the studies conducted were prevalently by ecological design and the associations without adequate adjustment for environmental and lifestyle confounding factors. Furthermore, we have considered the role of two other topics: i) the production of endogenous H_2S that is a gasotransmitter producing beneficial effects on cardiovascular function at low concentration and ii) the possible intake of H_2S-releasing drugs for the treatment of patients affected by hypertension, inflammatory diseases, and CVD. Hence, in this complex scenario, a further effort should be performed in the future to include a more comprehensive evaluation of cardiovascular risk in relation to H_2S emissions, in particular in geothermal areas.

Given the growing exploitation of geothermal energy all over the world to move from a fossil-fuel centralised system towards a more distributed fossil-free system and its potential association with CVD, which remains the leading cause of death worldwide, we hope that this review could be of your interest in continuity with our review recently published on Science of the Total Environment: “Bustaffa E, Cori L, Manzella A, Nuvolone D, Minichilli F, Bianchi F, Gorini F. The health of communities living in proximity of geothermal plants generating heat and electricity: A review. Sci Total Environ. 2020 Mar 1;706:135998”, and with the original article “Nuvolone D, Petri D, Pepe P, Voller F. Health effects associated with chronic exposure to low-level hydrogen sulfide from geothermoelectric power plants. A residential cohort study in the geothermal area of Mt. Amiata in Tuscany. Sci Total Environ. 2019 Apr 1;659:973-982.”

Hoping that this review will be positively considered by the Journal Editors,

Yours Sincerely,

The authors,

Francesca Gorini, Elisa Bustaffa, Kyriazoula Chatzianagnostou, Fabrizio Bianchi, Cristina Vassalle

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*Title Page

Hydrogen sulfide and cardiovascular disease: doubts, clues, and pitfalls from studies in geothermal areas

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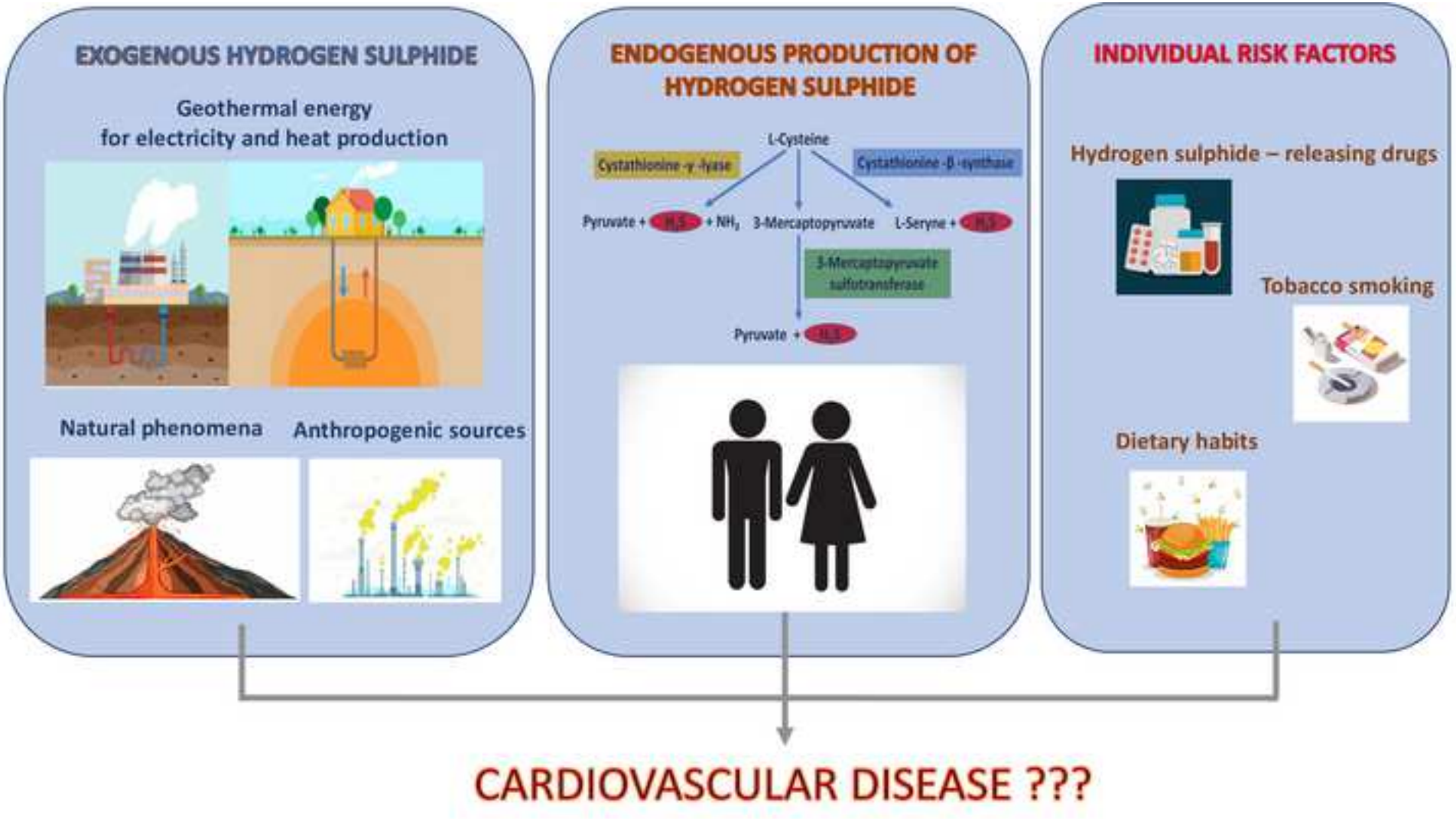
*Graphical Abstract



***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

- One the main air pollutants emitted in geothermal areas is hydrogen sulfide (H_2S).
- Weak signals suggest an association between H_2S and cardiovascular disease (CVD).
- Mortality and hospitalization results from epidemiological studies are conflicting.
- The contribution of endogenous H_2S production is crucial in this evaluation.
- A comprehensive analysis should consider all individual risk factors for CVD.

Abstract

Hydrogen sulfide (H_2S) represents one of the main pollutants originating from both geologic phenomena such as volcanoes, geysers, fumaroles and hot springs, and geothermal plants that produce heat and electricity. Many increasing data suggest that H_2S retains a variety of biological properties, and modulates many pathways related to cardiovascular pathophysiology although its role as beneficial/adverse determinant on cardiovascular disease (CVD) is not clearly established. In this review, the current knowledge on the association between H_2S exposure and risk of CVD in geothermal areas has been examined. The few epidemiological studies carried out in geothermal areas suggest, in some cases, a protective role of H_2S towards CVD, while in others a positive association between exposure to H_2S and increased incidence of CVD. Most of the studies have an ecological design that does not allow to produce evidence to support a causal relationship and also often lack for an adequate adjustment for individual CVD risk factors. The review has also considered the potential role of two other aspects not sufficiently explored in this relationship: the production of endogenous H_2S that is a gasotransmitter producing beneficial effects on cardiovascular function at low concentration and the intake of H_2S-releasing drugs for the treatment of patients affected by hypertension, inflammatory diseases, and CVD. Thus, a threshold effect of H_2S and the shift of action as beneficial/adverse determinant given by the sum of exogenous exposure and endogenous production cannot be excluded. In this complex scenario, an effort is warranted in the future to include a more comprehensive evaluation of risk for CVD in relation to H_2S emissions, especially in geothermal areas.

Keywords: hydrogen sulfide; geothermal; cardiovascular disease; cardiovascular risk; health; threshold effect

1. Introduction

Geothermal energy, which is the energy contained in rocks and fluids within the earth's crust and able to generate geological phenomena (e.g., volcanoes, geysers, fumaroles and hot springs), can be recovered to produce thermal and electric energy (Nuvolone et al., 2019; Bustaffa et al., 2020). In 2015, a total of 82 countries was reported to use geothermal energy for a direct utilization (i.e., thermal energy; Lund and Boyd, 2016), while worldwide electricity production from geothermal resources has been estimated to achieve up to 8.3% by 2050, serving 17% of world population (Bertani, 2016). Although geothermal energy is generally considered a more sustainable source of energy compared to carbon and fossil fuel plants and the third thermal renewable energy source in Italy, an important environmental issue related to geothermal industrial development is the emission of non-condensable gases to the atmosphere (Somma et al., 2017; Manzella et al., 2018). In addition to carbon dioxide, methane, volatile metals, silicates, carbonates, metal sulfides and sulfates and traces of mercury (Hg), arsenic, antimony, selenium and chromium, geothermal power plants also emit hydrogen sulfide (H_2S) in relative high amounts (Somma et al., 2017; Bustaffa et al, 2020). Hydrogen sulfide may originate from different environmental natural sources (e.g., volcanoes, sulfur springs, undersea vents, swamps, bogs, crude petroleum and natural gas, generally in the range of $\mu g/m^3$) as well as from human-derived activities (e.g., petroleum refineries, tanneries, natural gas, petrochemical, oven, and food processing plants, municipal sewers and sewage treatment plants, manure processes, and paper mills, in the range of mg/m^3), thus occupational H_2S exposure is generally greater compared to the one from natural environment (WHO, 2003). Hydrogen sulfide route of exposure is essentially inhalatory and, in this context, it is important to note that H_2S may persist in the atmosphere more days, depending on different factors (e.g., season, irradiation, ventilation) (Finzi and Brusasca, 1991).

In urban areas, concentrations of H_2S generally range between 1.0 and 3.0 $\mu g/m^3$ (Kurtidis et al., 2008) while in proximity of volcanic and geothermal areas H_2S can reach air levels up to 1,500 $\mu g/m^3$, as measured in Roturua, New Zealand, where the largest population center in the world is located over an active geothermal field directly used for bathing and wellness (Fisher, 1999; Bates et al., 2002; Horwell et al., 2005). In Italy, geothermal fields are used for electricity generation and all the 36 geothermal plants, which are placed in the

southern part of the Tuscany region, cover over 30% of the regional electricity needs (Razzano and Cei, 2015; Manzella et al., 2019). Most of the geothermal plants are currently equipped with filters for the abatement of H_2S, therefore H_2S emission levels amount to 2–12 $\mu g/m^3$ (period of average exposure up to 90 days) (ARPAT, 2018; Manzella et al., 2018), which are lower than those recommended by the World Health Organization (WHO) (an average of 20 $\mu g/m^3$ over a period of 90 days; WHO, 2003). Human health effects resulting from exposure to H_2S are well known, and while very low concentrations (0.011 mg/m^3) represent the odor threshold, increasing levels have been associated with bronchial constriction (2.8 mg/m^3), eye irritation (5.0-29.0 mg/m^3), olfactory paralysis (>140 mg/m^3), up to death (>700 mg/m^3) (WHO, 2003).

Cardiovascular disease (CVD) remains the globally leading cause of death, taking an estimated 17.9 million lives in 2016, which represent the 31% of all the deaths worldwide (WHO, 2017). Increases of predisposing diseases or comorbidities such as hypertension, diabetes, obesity, in addition to an ageing population, constitute the main cause of the rising number of subjects living with CVD (Savarese and Lund, 2017; von Lueder and Agewal, 2018). In particular, hypertension, defined as a systolic blood pressure > 140 mm Hg and a diastolic blood pressure < 90 mm Hg, is one of the major risk factors for atherosclerosis (Alexander. 1995; Špinar, 2012). The number of adults with hypertension increased to 1.13 billion people in 2015, most (two-thirds) living in low- and middle-income countries (WHO, 2019).

In addition to these acknowledged cardiovascular risk factors, the hypothesis that ambient air pollutants might contribute to the occurrence and development of CVD has been confirmed over the last years. Ambient air pollution is considered responsible for 4.2 million deaths worldwide, contributing to 7.6% of all deaths in 2016, and has been estimated to cause about 25% of all deaths and disease from ischemic heart disease (IHD) (WHO, 2018). Natural phenomena and anthropogenic sources emit complex mixtures of air pollutants, many of which are harmful to health (Lee et al., 2014). In particular, fine particulate matter <2.5 μm ($PM_{2.5}$) is the most important environmental risk factor correlated with global cardiovascular mortality and disability (Rajagopalan et al., 2018). On the other hand, H_2S retains a variety of biological properties, and modulates numerous pathways related to cardiovascular pathophysiology (e.g., through modulation of oxidative stress, apoptosis, angiogenesis, vasodilation and activity of endothelial nitrogen synthase),

87 although its role as beneficial/adverse determinant on CVD according to its localization and concentration is
88 not completely elucidated (Yang et al., 2020).
89 The few epidemiological studies carried out in geothermal areas evaluating the relationship between H_2S and
90 CVD are inconclusive, as some evidenced a protective role of H_2S in atherosclerosis onset and development,
91 while in others a positive association between exposure to H_2S and increased incidence of CVD and CV risk
92 factors has been observed. In this complex scenario, we also discuss further aspects, the production of
93 endogenous H_2S and the intake of commonly used H_2S-releasing drugs that may affect final H_2S "two-edged
94 sword" effects, beneficial at low concentration but potentially harmful at higher concentration, which merit
95 to be assessed in the CVD-H_2S relationship.
96
97

98 ## 2. Hydrogen sulfide and cardiovascular risk in geothermal areas

99 Although the growing concern of WHO towards the health effects potentially associated to the exposure to
100 emissions from the exploitation of geothermal energy, in the last three decades only a limited number of
101 epidemiological studies have explored the health status of populations residing in areas where geothermal
102 fluids are used to produce heat and electricity (Bustaffa et al., 2020) (Table 1). Moreover, the available
103 results from the evaluation of health hazards relating the cardiovascular system and chronic-level of H_2S
104 exposure are conflicting (Lewis and Copley, 2015). Using morbidity and mortality data, a significant
105 increase of incidence for diseases of circulatory system (standardized incidence rate (SIR), 95% Confidence
106 Interval (95%CI): =1.05; 95%CI=1.02–1.07) and of mortality for hypertensive disease (standardized
107 mortality rate (SMR), SMR=1.61; 95%CI=1.24–2.05), in particular among men of the Maori ethnic group,
108 was observed in the geothermal Rotorua area compared to the rest of New Zealand (Bates et al., 1998, 1997).
109 While in these studies the whole population was assumed to be exposed uniformly to the geothermal
110 emissions, in a subsequent investigation the authors examined hospital discharges classifying residential
111 census area units of patients in Rotorua by exposure level to H_2S (high, medium, low, corresponding to 1400,
112 700, 0-55 $\mu g/m^3$, respectively; Horwell et al., 2005), which was based on measurements from passive
113 samplers located around the city for specified periods of time (Bates et al., 2002). The previous findings

were only partially corroborated, and a significantly elevated incidence was shown for diseases of the circulatory system (SIR=1.39; 95%CI=1.29–1.50) and IHD (SIR=1.53; 95%CI=1.35–1.73) in relation to H_2S high-level, with a little evidence of exposure-related trends, but not for hypertensive disease (Bates et al., 2002).

More recently, an Italian residential cohort study evaluated the health effects of chronic exposure to H_2S in six municipalities of the geothermal district of Mt. Amiata, which currently has five active geothermal power plants (Nuvolone et al., 2019) (Table 1). Exposure to chronic levels of H_2S was assessed using dispersion modelling, and estimates were validated using data recorded from fixed monitoring sites, both for meteorological parameters and H_2S metrics. Each cohort member residing in the area was georeferenced and assigned H_2S exposure metrics estimated and based on residential address. The authors reported significantly lower rates of mortality than those expected for IHD (SMR=0.85; 95%CI=0.76–0.95) and acute myocardial infarction (AMI) (SMR=0.75; 95%CI=0.63–0.93) using H_2S metric as a continuous variable. Conversely, a positive significant association was detected between linear H_2S exposure and hospitalization for diseases of the circulatory system (SHR=1.04; 95%CI=1.01–1.07) and heart failure (HF) (SHR=1.14; 95%CI=1.04–1.17), whereas no association was observed for hypertensive disease, IHD, and AMI (Nuvolone et al., 2019). These results are consistent with those of two previous ecological investigations conducted in the whole Tuscany geothermal areas, which found a reduction of mortality than that expected for diseases of the circulatory system and IHD though levels of exposure to H_2S in these studies were not available (Minichilli et al., 2012; Bustaffa et al., 2017).

Recently, a case crossover study explored the short-term health effects associated with the daily exposure to H_2S emissions from all Tuscan geothermal power plants using individual data of mortality, hospitalization and emergency department visits (Nuvolone et al., 2020) (Table 1). Each georeferenced case was linked to the H_2S daily level derived from 18 monitoring sites, comparing the pattern of exposure to H_2S in the event day with the pattern in the same weekday during the three weeks before the event. An increased mortality for cardiovascular causes (odds ratio (OR), 90% Confidence Interval (90%CI): OR=1.22, 90%CI=1.03–1.44) was associated with an increase of 10 $\mu g/m^3$ of H_2S daily levels exclusively among men, whereas no

140 associations were observed between H_2S daily levels and urgent hospital admissions and emergency
141 departments visits for CVD (Nuvolone et al., 2020)

Table 1. Summary of the principal characteristics of epidemiological studies investigating the relationship between H_2S exposure and cardiovascular disease

Reference	Country	Study design – study period	Exposure assessment – H_2S concentration	Health outcome assessment	Confounders	Limits
Bates et al. 1997	New Zealand - Rotorua	Ecological 1981-1990	Air monitoring sites Median concentration 35 μg/m^3 (measure performed in 1978)	Mortality data in conjunction with census data.	Age, gender, ethnicity, calendar year.	Possibility of ecological fallacy; assumption of uniform exposure; possibility of ethnic misclassification; lack of updated measurements of H_2S and other air pollutant levels
Bates et al. 1998	New Zealand - Rotorua	Ecological 1981-1990	Air monitoring sites Median concentration 35 μg/m^3 (measure performed in 1978)	Cancer registry and hospital discharge data in conjunction with census data.	Age, gender, ethnicity, calendar year.	Possibility of ecological fallacy; assumption of uniform exposure; lack of updated measurements of H_2S levels and other pollutant levels; possibility of systematic biases in recording data; lack of information about individual risk factors.
Bates et al. 2002	New Zealand - Rotorua	Ecological 1993-1996	Passive samplers mapping H_2S variations both in summer and in winter. Exposure level classified in high (1400 μg/m^3), medium (700 μg/m^3), low (0-55 μg/m^3) and linked to residential census area units.	Hospital discharge data in conjunction with residential census area units.	Age, gender, ethnicity.	Possibility of ecological fallacy; categorization of exposure based on the residential location at the time of diagnosis; lack of information of residential history; selection bias related to the access to the public hospitals; missing adjustment for other confounders such as life habits, socioeconomic status and other environmental exposures, seasonal variation.
Minichilli et al. 2012	Italy: Traditional and Mount Amiata areas	Ecological Sixteen municipalities ~ 43,000 inhabitants 2000-2006	Three air monitoring sites Mean concentration in the years 1997-2008 Traditional area: 0.6-19.1 μg/m^3 Mt. Amiata area: 8.5-16.5 μg/m^3	Mortality data and hospital discharges records linked to census data.	Deprivation index.	Use of the residence at municipal level as a proxy of exposure to both environmental and socioeconomic factors; possibility of ecological fallacy; lack of adjustment for other confounding factors; inclusion of only primary diagnoses in hospital discharge analyses
Bustaffa et al. 2012	Italy: Traditional and Mount Amiata areas.	Ecological Sixteen municipalities ~ 40,000 inhabitants 2003-2012	Three monitoring sites to derive daily H_2S time series and assign exposure to cases at individual level. Daily mean concentration Traditional Area: 4.6-17.9 μg/m^3 Mt. Amiata area: 5.6-6.2 μg/m^3	Mortality data linked to census data	Deprivation index.	Use of the residence at municipal level as a proxy of exposure to both environmental and socioeconomic factors; possibility of ecological fallacy.
Nuvolone et al. 2019	Italy: Mount Amiata area	Residential cohort Six municipalities 33,804 subjects 1998-2016	Six monitoring sites and CALPUFF – CALMET – WRF modelling to evaluate spatial variability of exposure at individual level. H_2S max 90 days: 0.5-33.5 μg/m^3	Mortality and first hospital discharges data linked to the medical records of the georeferenced cohort members	Gender, socioeconomic status (available at census block level), calendar period.	Ecological fallacy as regards socioeconomic status; lack of information for other confounders such as diet, life habits and other pollutants; H_2S exposure estimated only at residence; inclusion of only primary diagnoses in hospital discharge analyses.
Nuvolone et al. 2020	Italy: Traditional and Mount Amiata areas.	Case-crossover ~ 37,000 inhabitants Sixteen municipalities 2000-2017	Eighteen fixed monitoring sites to derive daily H^2S time series and assign exposure to cases at individual level. Nine sites to collect meteorological data. Daily mean concentration Traditional Area: 4.6-17.9 μg/m^3 Mt. Amiata Area: 5.6-6.2 μg/m^3	Individual data of mortality, urgent hospital admissions and emergency department visits. All residence addresses of cases were georeferenced	Age group, gender, geothermal area, period, seasonability.	Small population and cases under study; possibility of information bias for exposure (time spent outdoors, occupational exposure); no adjustment for other pollutants.

3. Hydrogen sulfide endogenous production

It is known that exposures to very high H_2S concentration levels (i.e., >250 ppm /~ 350 mg/m^3) may provoke a sudden death, loss of consciousness, and pulmonary edema (ACGIH, 2010; ATSDR 2016). One of the main mechanisms involved at molecular level is related to oxygen/adenosine triphosphate (O_2/ATP) modulation. In fact, if at low concentration H_2S increases O_2 uptake and ATP, these same reactions are inhibited at higher H_2S doses (20-40 μM) through the inhibition of the enzyme cytochrome c-oxidase (Olson and Straub, 2016). Increased H_2S blood concentration resulted associated with higher inflammation (myeloperoxidase activity, tumor necrosis factor α-TNFα blood levels, and histological inflammatory findings in experimental models), and elevated H_2S levels were observed in endotoxic shock (Li et al., 2005). Moreover, H_2S at micromolar concentration can provoke DNA oxidative damage through its capacity to generate superoxide, hydrogen peroxide, and hydroxyl radical (Hoffman et al., 2012). It has been also observed that H_2S genotoxic damage induced in Chinese hamster ovary cells at these doses, may be effectively quenched by co-treatment with butylhydroxyanisole, a radical scavenger, which further attests for the oxidative nature of this injury (Attene-Ramos et al., 2007).

Less clear is the scientific evidence of the long-term effects of chronic low-dose H_2S exposures, which do not yet allow to define a major health risk (e.g., respiratory, neurological, cardiovascular, reproductive and developmental systems, and cancer), giving the observed controversial results also as a consequence of the many significant limitations presented by several studies (Lewis and Copley, 20015).

Previously recognized as a toxic gas, more recently H_2S has been associated to other gasotransmitters, such as nitric oxide (NO) and carbon monoxide, produced endogenously in small quantities, with various beneficial effects on the cardiovascular system, as well as on nervous and gastrointestinal systems due to cytoprotective, antioxidative, and anti-inflammatory properties (Sun et al., 2020). Endogenously, H_2S is synthetized by enzymatic or non-enzymatic pathways. Enzymatic generation occurs via the activities of three enzymes: cystathionine β-synthase, cystathionine γ-lyase, and 3-mercaptopyruvate sulfotransferase, together with cysteine amino transferase (Yang et al., 2019). Anti-inflammatory, antioxidant, anti-apoptotic, anti-hypertrophic effects are also ascribed to H_2S (Donnarumma et al., 2017). In the cardiovascular setting, the major effects include cardioprotection, heart rate decrease, inotropic and proangiogenic effects, decrease

blood pressure and vasodilation (Tomasova et al., 2016). Accordingly, reduced levels of H_2S have been found in patients with acute or stable coronary artery disease (Gao et al., 2015), diabetes (Yang e al., 2020), hypertension (van Goor et al., 2016), and HF (Kovačić et al., 2012). In particular, the link between H_2S and NO appears strong in cardioprotection, as H_2S is able to activate endothelial NO synthase and increase NO generation (Chatzianastasiou et al., 2016). Furthermore, there is evidence of an emerging role of H_2S in myocardial fibrosis, which is a key determinant of heart dysfunction and HF (Zhang et al., 2015). H_2S resulted to prevent transforming growth factor (TGF)-β1-stimulated differentiation of fibroblasts to myofibroblasts through inhibition of the TGF-β1/Smad3 signaling pathway, and this antifibrotic effect may be of particular interest for the therapeutic possibilities in the treatment of HF (Yang et al., 2019).

H_2S also retains epigenetic effects by modulation of DNA methylation, histone deacetylase, and microRNA expression (MicroRNA 21, Micro RNA 129) (Weber et al., 2017, 2016; Corsello et al., 2018).

Nonetheless, as many other molecules (e.g., antioxidants), H_2S may likely exert beneficial effects at low physiological concentration on different pathways, with the main involvement of oxidative stress and NO, whereas it may be detrimental when available in excess (Gaggini et al., 2020). In this context, it is important to remind one limitation to assess local H_2S concentration, due to the lack of a unique "gold standard" method, therefore reported physiological H_2S concentration greatly vary (low μM – ten to hundreds μM), also due to the variability of detection methods and different tissues in which the measurement is performed (Nagy et al., 2014).

Mouth and gastrointestinal tract bacteria may also produce H_2S through metabolism of sulfhydryl proteins such as in cysteine degradation (Chun-Mei et al., 2017). This is of particular interest, given the number of recent experimental and clinical data evidencing that alterations in the composition, function, and metabolites of microbiota can induce dysbiosis, which in turn affects the onset and development of CVD (Tang et al., 2017). It has been suggested that H_2S produced in the colon may target the cardiovascular system and contribute to the control of arterial blood pressure (Tomasova et al., 2016). Furthermore, the action of H_2S at intestinal level appears to be dual and opposite depending on concentration, with beneficial effects at nanomolar to low micromolar levels with the maintenance of the mucus layer integrity, inflammation decrease, but able to induce deleterious effects at higher values (high micromolar to

millimolar), which lead to increased inflammatory processes, and promotion of cancer onset and progression (Blachier et al., 2019).

4. Hydrogen sulfide-releasing drugs

The involvement of H_2S in many pathophysiological pathways, and the wide number of patients (e.g., hypertension, inflammatory diseases, cardiovascular disease, cancer) potentially interested for this therapeutic option, renders its use in the drug design rationale (Verma et al., 2017). In addition to the cases in which the reduction of circulating levels of H_2S is necessary, most of the efforts are directed towards the identification of reliable donors releasing appropriate amounts of H_2S, for an adequate time period. Two main different approaches to supply H_2S can be adopted in order to generate a H_2S precursor or, alternatively, to stimulate the endogenous system. For it concerns precursor, some molecules have been identified and tested as H_2S donors, such as the most tested sodium hydrosulfide-NaHS, and sodium sulfide-Na_2S. In particular, NaHS administration in experimental models has been proven to lower blood pressure, to improve left ventricular hypertrophy and fibrosis and decrease neointima formation, to reduce smooth muscle cell proliferation, and to prevent impairment of balloon-induced vascular relaxation in a rat model of balloon- induced neointima hyperplasia (Meng et al., 2007; Yang et al., 2014; Ni et al., 2018).

Due to the mutual NO/H_2S interactions, Na_2S/S-nitrosoglutathione (Na_2S/GSNO: 10 μmol L^{-1}/1 μmol L^{-1}) administration has been proved as more effective in terms of vasorelaxation compared to each compound administered alone, suggesting the efficacy of H_2S donors in combined treatment in the intrarenal arteries of patients with arterial hypertension (Cacanyiova et al., 2017).

Nevertheless, these molecules present a short half-life, which may be a complication giving a low effectiveness in treating chronic diseases (Hsieh et al., 2019). As a consequence, GYY4137 Dichloromethane complex and polysulfides, novel molecules characterized by a more gradual release of H_2S, appear promising (Cao et al., 2019). This is of particular significance in view of the results obtained by comparing two H_2S donors, namely the fast-releasing-NaHS and GYY4137, which point out that H_2S effects are influenced by the level and by the generation rate of H_2S, as well (Whiteman et al., 2010). These compounds were evaluated according to the generation of pro- and anti-inflammatory biomarkers in lipopolysaccharide

(LPS)-treated murine RAW264.7 macrophages. GYY4137 was reported to significantly and concentration-dependently reduce pro-inflammatory biomarker release (interleukins such as IL-1β, IL-6, TNF-α, and prostaglandin E2) and increase instead the anti-inflammatory IL-10 cytokine. By contrast, NaHS revealed a biphasic opposite response, that is decreased or increased proinflammatory biomarker levels at low and high concentrations, respectively. Therefore, a different H_2S behavior should be carefully evaluated when testing potential H_2S donors according to H_2S production rate.

Thiosulfate, already in clinical use to treat calcific uremic arteriopathy in dialysis patients, is generated during H_2S metabolism, acting as H_2S donor. Treatment of hypertensive rats with thiosulfate is beneficial in lowering blood pressure and reducing organ damage (van Goor et al., 2016). Interestingly, intravenous sodium thiosulfate has been found to prevent and delay the vascular calcification progression in hemodialysis (HD) patients (Saengpanit et al., 2018), indicating that it may be considered a promising therapeutic strategy to delay and treat structural and functional vascular wall abnormalities in the CVD patient setting. In addition, very recent data suggested that patients who received sodium thiosulphate treatment, showed a reduction of iliac artery and heart valves calcification as well as several other cardiovascular functional parameters (reduced pulse wave velocity, lower carotid intima-media thickness, better echocardiographic parameters of left ventricular hypertrophy) (Djuric et al., 2020). However, one limitation to this treatment remains its intravenously administration, thus the development of gastric acid resistant capsules is expected in this field.

A further application for H_2S is the treatment of myocardial fibrosis (Kang et al., 2020). Experimental data suggested that many pathways can be involved in these effects from different types of exogenous H_2S, such as canonical Wnt and TGF-β1/Smad3, PKC-ERK1/2MAPK, PI3K/AKT and JAK⁄STAT signaling pathways (Liu et al., 2018a, 2018b; Long et al., 2019; Yang et al., 2019).

Of interest, nanoparticles formed by the self-assembly of phospholipid molecules, and used as carriers for the delivery of S-propargyl-cysteine - a novel H_2S donor - have been tested in rats, resulting efficacious to inhibit myocardial fibrosis via the inhibition of the TGF-β1/Smad signaling pathway (Tran et al., 2019). Since they were reported in the ‘80s, site-specific drug delivery nanocarriers have been developed in parallel to advance in nanotechnology and biotechnology, especially in the cancer scenario (Tomlinson et al., 1987).

However, although the development of a drug with specific on-site delivery may offer several advantages over common drug administration regimens (e.g. targeted drug transport to specific target sites, prolonged drug half-life, time-controlled release, and decreased drug immunogenicity and cytotoxicity), its application in the clinical practice is still challenging (Tomlinson et a., 1987, Vivek et al., 2014; Datz et al., 2016).

Dietary intake may represent another determinant influencing H_2S levels, and an interesting possibility to improve H_2S deficiency that may occur in many diseases (Łowicka and Bełtowski, 2007). In this regard, garlic and garlic-derived organic polysulfides, such as diallyl trisulfide and diallyl disulfide, act as H_2S donors through the reaction of thiols (e.g. glutathione), and are characterized by a different production rate (i.e., the first one as fast H_2S donor, the second as slow one) (Liang et al., 2015). Moreover, diallyl trisulfide acts as a stimulator of the endogenous system upregulating the expression and enzymatic activity of cystathionine gamma-lyase, one of H_2S-producing enzymes, responsible for endogenous H_2S production (Xu et al., 2020). Dietary formulations, such as SG-1002 that is an oral H_2S donor, have been tested in experimental model, resulting beneficial for the cardiovascular system through the modulation of different cellular pathways (e.g., NO synthase-NO- cyclic guanosine monophosphate) (Kondo et al., 2013; Barr et al., 2015; Shimizu et al., 2018; Rushing et al., 2019).

Furthermore, many commonly used cardiovascular drugs may increase H_2S levels, such as angiotensin-converting enzyme inhibitors (e.g., ramipril; Wiliński et al., 2010), statins (Xu et al., 2014), digoxin (Wiliński et al., 2011), metformin (Ma et al., 2020) and nonsteroidal anti-inflammatory drugs (Zhang et al., 2019).

Thus, if by one side H_2S can be a promising pharmacological option, to the other there is the possibility of reaching local adverse toxic level as the sum of different exogenous/endogenous sources, which surely require further evaluation (Figure 1). Notably, it should be considered that H_2S can also be stored as bound sulfane sulfur, which in turns may release H_2S in response to physiological stimuli (Kimura, 2011). Free blood sulfide is generally at the sub-to low micromolar concentration, but ten to hundred micromolar sulfide reserves are available, acting as a buffer to maintain free sulfide constantly below toxic levels but supplying additive sulfide pool when necessary under different conditions (Nagy et al., 2014).

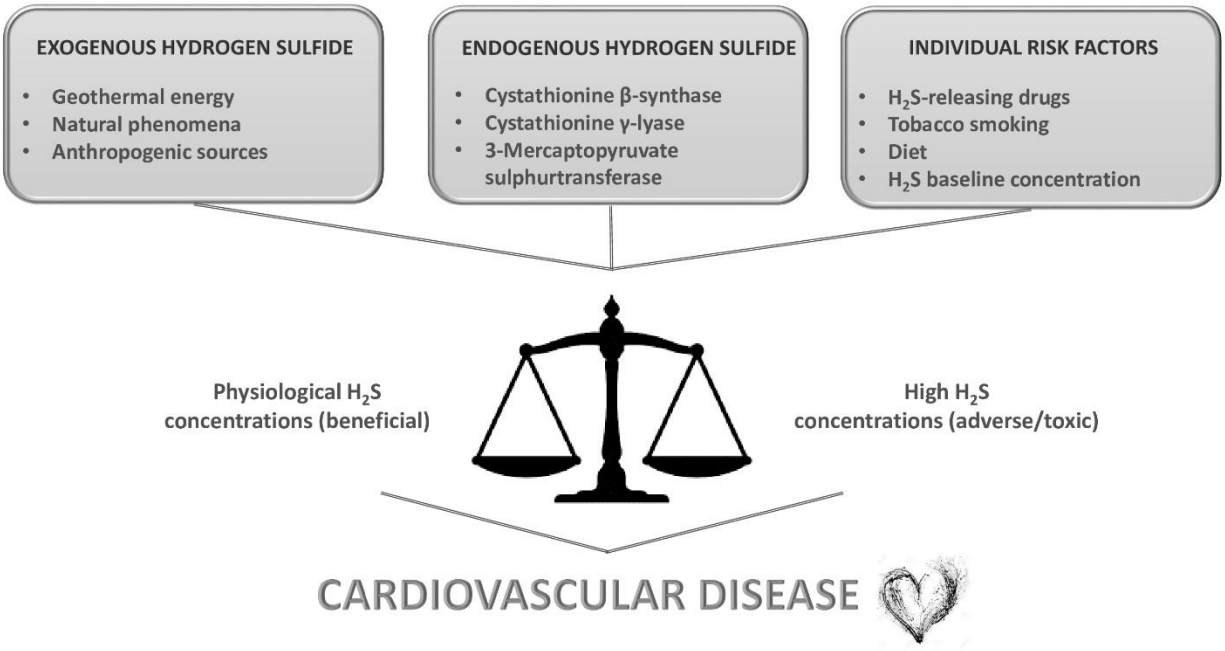


Figure 1. Factors influencing levels of hydrogen sulfide in humans and their association with cardiovascular disease.

Abbreviations: H_2S: hydrogen sulfide

5. Discussion

Overall, the findings of the studies examined do not allow to draw definitive conclusions. In fact, while Rotorua inhabitants exhibited excesses of CVD incidence and of mortality for hypertensive disease, in Italian geothermal areas, a significant decreased mortality for CVD compared to neighbor areas was observed, except for men with short-term exposure to high H_2S levels. This is an important point, because when considering sex/gender differences, effectively there are many emerging dissimilarities in the CVD regarding coronary biology and anatomy, cardiovascular risk factor susceptibility, epigenetics modification, symptoms according to which the disease occurs, and cytochrome metabolism, which may explain different health consequences and disease manifestations in males and females (Al-Husein et al., 2018; Haider et al., 2020). At the moment, these gender- and sex-specific determinants, which may also contribute to different response in the H_2S-CVD relationship remain largely unknown, and surely merit further investigation.

In addition, a positive significant relationship was also found at increasing exposure to H_2S and hospitalization for CVD as a group and for HF (Nuvolone et al., 2019).

To date, the health effects of low-level exposure to H_2S related to the emissions from natural or anthropogenic sources have not been sufficiently explored. In particular, a relatively low number of studies have evaluated the cardiovascular risk associated with the chronic exposure to H_2S in geothermal areas, and most of them have an ecological design that does not allow to produce evidence to support a causal relationship.

In addition to being affected by the well-known ecological bias due to the non-use of individual data (Lewis and Copley, 2015), the studies carried out are characterized by a missing or weak adjustment for confounding factors such as individual information on residential and medical history, diet, tobacco smoking, alcohol abuse, occupational exposure, as well as data about other environmental co-exposures.

An important point to consider is the extent of the environmental exposure. For example, in order to compare the consequences on cardiovascular health of exogenous H_2S respect to that produced endogenously, it is important to point out that, although not updated, the median concentration of H_2S in Rotorua district is 35 $\mu g/m^3$, even though it can reach up to 1400 $\mu g/m^3$, while in Italian geothermal areas the equipment of most plants with filters has led to a significant reduction of H_2S emissions, which are almost always below the limits recommended by WHO. According to a recent research, the baseline level of

endogenous H_2S in the plasma of healthy volunteers lies in the range of 70 - 125 μM (Karunya et al, 2019) that is 10^3 –10^5 times the environmental concentration of H_2S in geothermal areas. Hence, in addition to a partial inadequateness of epidemiological studies in exploring further risk factors that at least in part explain the excess of CVD as a whole and of other heart disease subtypes, necessarily other contributors exert a crucial action in this process.

It should be noted that the classification of CVD is often referred to specific groups or large groups of diseases, which cannot be interpreted univocally, i.e., circulatory system disease, ischemic heart disease and myocardial infarction, since partly overlapping. In addition, in the studies conducted in New Zealand, a diversity of disease classification between Rotorua and the rest of the country cannot be ruled out.

The studies carried out in Rotorua did not always report updated H_2S concentration measurements and did not evaluate Hg exposure, which may have had been elevated (Bates et al, 1998, 1997). In the study of Nuvolone et al. (2019), adopting an advanced design based on an accurate exposure reconstruction of a resident cohort and a high-quality dispersion modelling, information on individual risk factors and other environmental pollutants were missing. The Mt. Amiata area was the third largest Hg mining district worldwide, in operation until the 1980s but not yet fully reclaimed (Bustaffa et al, 2020; Lattanzi et al, 2019). Both the recent investigations performed in Italian geothermal areas (Nuvolone et al, 2020; 2019) did not include Hg, nitrogen oxides, sulfur dioxide, $PM_{2.5}$, and coarse particles with the diameter from 2.5 to 10 μm (PM_{10}), in the exposure assessment.

Approximately 40.5% of the population is predicted to have some form of CVD by 2030 (Heidenreich et al, 2011) and beyond the role of ageing population, a complex interplay between genetic predisposition and environmental factors, including pollution, has been acknowledged to exert an important influence on CVD risk, progression, and severity (Bhatnagar, 2017). Of all ambient air pollutants, $PM_{2.5}$ poses the greatest health risks for a wide variety of acute and chronic illnesses and premature deaths, but predominantly from cardiovascular and respiratory outcomes (Ren et al, 2017). A systematic review and meta-analysis reported the association between short-term exposure to $PM_{2.5}$, along with nitrogen dioxide (NO_2) and carbon monoxide, and a higher risk of AMI (Mustafic et al, 2012). In addition to short-term effects, long-term exposure to $PM_{2.5}$ and PM_{10} was associated to an increased risk for mortality of CVD and IHD (Pinault e al, 2017; Weichenthal et al, 2014; Lipsett et al, 2011); exposure to nitrogen oxides was also related to death for

CVD (Lipsett et al, 2011). Moreover, short-term exposure to $PM_{2.5}$, PM_{10} and sulfur dioxide and long-term exposure to PM_{10} and NO_2 were significantly associated with hypertension in each 10 $\mu g/m^3$ increment (Cai et al, 2016). Even exposure at low $PM_{2.5}$ levels (< 10 $\mu g/m^3$ annual mean according to WHO guidelines; WHO, 2005) is considered a risk to health, and in particular to cardiovascular events (Di et al., 2017; Pinault et al., 2017; Crouse et al, 2012). Therefore, although in the Italian geothermal districts concentrations of PM_{10} and NO_2 measured by using mobile instruments were below the thresholds recommended by WHO (Nuvolone et al, 2020, 2019), an effect of other air pollutants besides H_2S on cardiovascular risk cannot be ruled out.

Hg, often detected in the Tuscan geothermal areas, can precipitate from the atmosphere into the soil where a few insoluble mercury sulfide precipitates are methylated by bacteria to form methylmercury. This organic form can accumulate in the food chain and represents the most harmful Hg species to which humans can be exposed (Clarkson et al, 2003). Epidemiological studies reported that dietary intake of Hg is positively associated with risk for AMI and IHD (Salonen et al, 2000, 1995), mortality for CVD (Salonen et al, 1995), as well as with a negative impact on blood pressure (Choi et al, 2009; Valera et al, 2009), carotid intima-media thickness (Choi et al, 2009) and heart rate variability (Valera et al, 2011; Yaginuma-Sakurai et al, 2010). Notably, a Korean recent study performed around abandoned metal mines showed a significant correlation between residents with higher blood Hg levels and hypertension (Kim et al, 2019).

Thus, in the relationship between H_2S and CVD, it would be essential evaluate the presence and levels of other pollutants, which may can play a significant role in the onset and development of the atherosclerotic disease. Moreover, the possibility of synergistic/antagonist interactions, currently still unclear and assessed in specific contexts according to the presence of different types of environmental pollution, should be the focus of further research.

Another important point to consider is related to smoking habits, a recognized risk factor for atherosclerosis, responsible of approximately one of every four deaths from CVD, according to the 2014 Surgeon General's Report on smoking and health (CDC, 2014). Current tobacco smokers have at least a double risk of developing most significant types of CVD including AMI and HF compared to individuals who never smoked (Bancks et al, 2019). Recent data suggests the interactive role between cigarette smoking and some environmental pollutions in increasing cardiovascular risk, which deserves to be considered also in the

relationship between H_2S and CVD (Zhang et al., 2020). Nonetheless, with the exception of the study by Nuvolone et al. (2020), which investigated acute health outcomes related to short-term exposure to H_2S controlling time-invariant personal factors (age, gender, smoking, habits, body mass index), no other available study performed in geothermal areas reported the confounding effect of smoking on CVD mortality and morbidity.

Diet, whose effects on CVD risk and progression are inconsistent due to the health characteristics and genetic backgrounds of populations, instead influences all the main CVD risk factors such as hypertension, obesity, and cholesterol levels (Bhatnagar, 2017). Therefore, the lack of control for individual information on dietary intake can result as over or under estimate of measure of association. In particular, the diet is not only a confounder but also a modifier as well, and, moreover, the effect of smoking in CVD can vary depending on diet status (Hajian Tilaki, 2012). It should be further considered that high protein and fat content in the diet seem to increase H_2S production, whereas high carbohydrate consumption has an opposite effect (Teigen et al., 2019). Therefore, dietary sulfur intake along with the abundance of sulfate reducing bacteria in the gastrointestinal tract were considered the primary determinants of H_2S production, at concentration able to affect the cardiovascular system (Teigen et al., 2019). As reported in the previous sections, many common drugs can release H_2S at concentration able to affect circulating concentration of this molecule and, consequently, physiopathological pathways.

Hence, this burden of biological, metabolic as well environmental and methodological determinants would be considered in the assessment of the effects of H_2S on health and diseases. In this context, how these factors affect the balance between the low and high H_2S bioavailability that may be alternatively related to the beneficial/adverse effects on health, and for which the exogenous contribution and endogenous production together add up for the final outcome, should be evaluated in an integrated environment-health surveillance system on the health status of communities living in geothermal areas (Table 2).

Table 2. Determinants modulating hydrogen sulfide levels to be evaluated in the relationship between hydrogen sulfide, health status and cardiovascular disease

Sex differences
Source type (environmental *vs* endogenous productions)
Extent of environmental exposure (industrial *vs* geothermal)
Presence/level of other environmental pollutant classes
Interference/synergism between pollutants
Disagreement between methods/assays
Smoking habit interference and other cardiovascular risk factors
Dietary contribution
Gastrointestinal tract bacterial activity
Endogenous releasable reserves
Hydrogen sulfide - releasing drugs
Balance between low concentration (beneficial) effects *vs* high (adverse) consequences levels
Meteorological factors (e.g., wind speed and direction, cloud cover, precipitation and geographical distribution)
A more specific evaluation of disease categories (e.g., circulatory system disease, ischemic heart disease, myocardial infarction)

6. Conclusion

The evaluation of exposure to H_2S, especially in geothermal areas, is of relevant interest for its potential effects to cardiovascular risk. The few epidemiological studies conducted suggest only weak and even conflicting indications, in particular as regards the relationship between chronic H_2S exposure and mortality for CVD. This assessment, if on the one hand is limited by the study design and the lack of adequate adjustment for confounders known to be associated with an increased risk of CVD, on the other it cannot disregard the possibility of a threshold effect. Therefore, at the endogenous levels of H_2S normally produced, which have a protective effect, the exogeneous H_2S, as the sum of ambient exposure, intake of drugs and dietary habits, must to be added, giving rise to possible detrimental consequences (Figure 1). In order to perform a correct evaluation, future epidemiological studies should consider all the known individual risk factors for CVD and have reliable dispersion models for estimating individual environmental exposures both to H_2S and other atmospheric pollutants. A comprehensive analysis on the association between exposure to H_2S, which remains a widespread pollutant not only in geothermal districts, and CVD occurrence (also

distinguishing among specific diseases), is warranted to provide further elements in the complex relationship environment and health, as well as novel insights into the etiopathogenesis of CVD, which is currently the leading cause of premature death in the world.

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Tables 1-2

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Table 1. Summary of the principal characteristics of epidemiological studies investigating the relationship between H2S exposure and cardiovascular disease

Reference	Country	Study design – study period	Exposure assessment – H_2S concentration	Health outcome assessment	Confounders	Limits
Bates et al. 1997	New Zealand - Rotorua	Ecological 1981-1990	Air monitoring sites Median concentration 35 µg/m^3 (measure performed in 1978)	Mortality data in conjunction with census data.	Age, gender, ethnicity, calendar year.	Possibility of ecological fallacy; assumption of uniform exposure; possibility of ethnic misclassification; lack of updated measurements of H_2S and other air pollutant levels
Bates et al. 1998	New Zealand - Rotorua	Ecological 1981-1990	Air monitoring sites Median concentration 35 µg/m^3 (measure performed in 1978)	Cancer registry and hospital discharge data in conjunction with census data.	Age, gender, ethnicity, calendar year.	Possibility of ecological fallacy; assumption of uniform exposure; lack of updated measurements of H_2S levels and other pollutant levels; possibility of systematic biases in recording data; lack of information about individual risk factors.
Bates et al. 2002	New Zealand - Rotorua	Ecological 1993-1996	Passive samplers mapping H_2S variations both in summer and in winter. Exposure level classified in high (1400 µg/m^3), medium (700 µg/m^3), low (0-55 µg/m^3) and linked to residential census area units.	Hospital discharge data in conjunction with residential census area units.	Age, gender, ethnicity.	Possibility of ecological fallacy; categorization of exposure based on the residential location at the time of diagnosis; lack of information of residential history; selection bias related to the access to the public hospitals; missing adjustment for other confounders such as life habits, socioeconomic status and other environmental exposures, seasonal variation.
Minichilli et al. 2012	Italy: Traditional and Mount Amiata areas	Ecological Sixteen municipalities ~ 43,000 inhabitants 2000-2006	Three air monitoring sites Mean concentration in the years 1997-2008 Traditional area: 0.6-19.1 µg/m^3 Mt. Amiata area: 8.5-16.5 µg/m^3	Mortality data and hospital discharges records linked to census data.	Deprivation index.	Use of the residence at municipal level as a proxy of exposure to both environmental and socioeconomic factors; possibility of ecological fallacy; lack of adjustment for other confounding factors; inclusion of only primary diagnoses in hospital discharge analyses
Bustaffa et al. 2012	Italy: Traditional and Mount Amiata areas.	Ecological Sixteen municipalities ~ 40,000 inhabitants 2003-2012	Three monitoring sites to derive daily H_2S time series and assign exposure to cases at individual level. Daily mean concentration Traditional Area: 4.6-17.9 µg/m^3 Mt. Amiata area: 5.6-6.2 µg/m^3	Mortality data linked to census data	Deprivation index.	Use of the residence at municipal level as a proxy of exposure to both environmental and socioeconomic factors; possibility of ecological fallacy.
Nuvolone et al. 2019	Italy: Mount Amiata area	Residential cohort Six municipalities 33,804 subjects 1998-2016	Six monitoring sites and CALPUFF – CALMET – WRF modelling to evaluate spatial variability of exposure at individual level. H_2S max 90 days: 0.5-33.5 µg/m^3	Mortality and first hospital discharges data linked to the medical records of the georeferenced cohort members	Gender, socioeconomic status (available at census block level), calendar period.	Ecological fallacy as regards socioeconomic status; lack of information for other confounders such as diet, life habits and other pollutants; H_2S exposure estimated only at residence; inclusion of only primary diagnoses in hospital discharge analyses.
Nuvolone et al. 2020	Italy: Traditional and Mount Amiata areas.	Case-crossover ~ 37,000 inhabitants Sixteen municipalities 2000-2017	Eighteen fixed monitoring sites to derive daily H^2S time series and assign exposure to cases at individual level. Nine sites to collect meteorological data. Daily mean concentration Traditional Area: 4.6-17.9 µg/m^3 Mt. Amiata Area: 5.6-6.2 µg/m^3	Individual data of mortality, urgent hospital admissions and emergency department visits. All residence addresses of cases were georeferenced	Age group, gender, geothermal area, period, seasonability.	Small population and cases under study; possibility of information bias for exposure (time spent outdoors, occupational exposure); no adjustment for other pollutants.

Table 2. Determinants modulating hydrogen sulfide levels to be evaluated in the relationship between hydrogen sulfide, health status and cardiovascular disease

Sex differences
Source type (environmental *vs* endogenous productions)
Extent of environmental exposure (industrial *vs* geothermal)
Presence/level of other environmental pollutant classes
Interference/synergism between pollutants
Disagreement between methods/assays
Smoking habit interference and other cardiovascular risk factors
Dietary contribution
Gastrointestinal tract bacterial activity
Endogenous releasable reserves
Hydrogen sulfide - releasing drugs
Balance between low concentration (beneficial) effects *vs* high (adverse) consequences levels
Meteorological factors (e.g., wind speed and direction, cloud cover, precipitation and geographical distribution)
A more specific evaluation of disease categories (e.g., circulatory system disease, ischemic heart disease, myocardial infarction)

Figure 1

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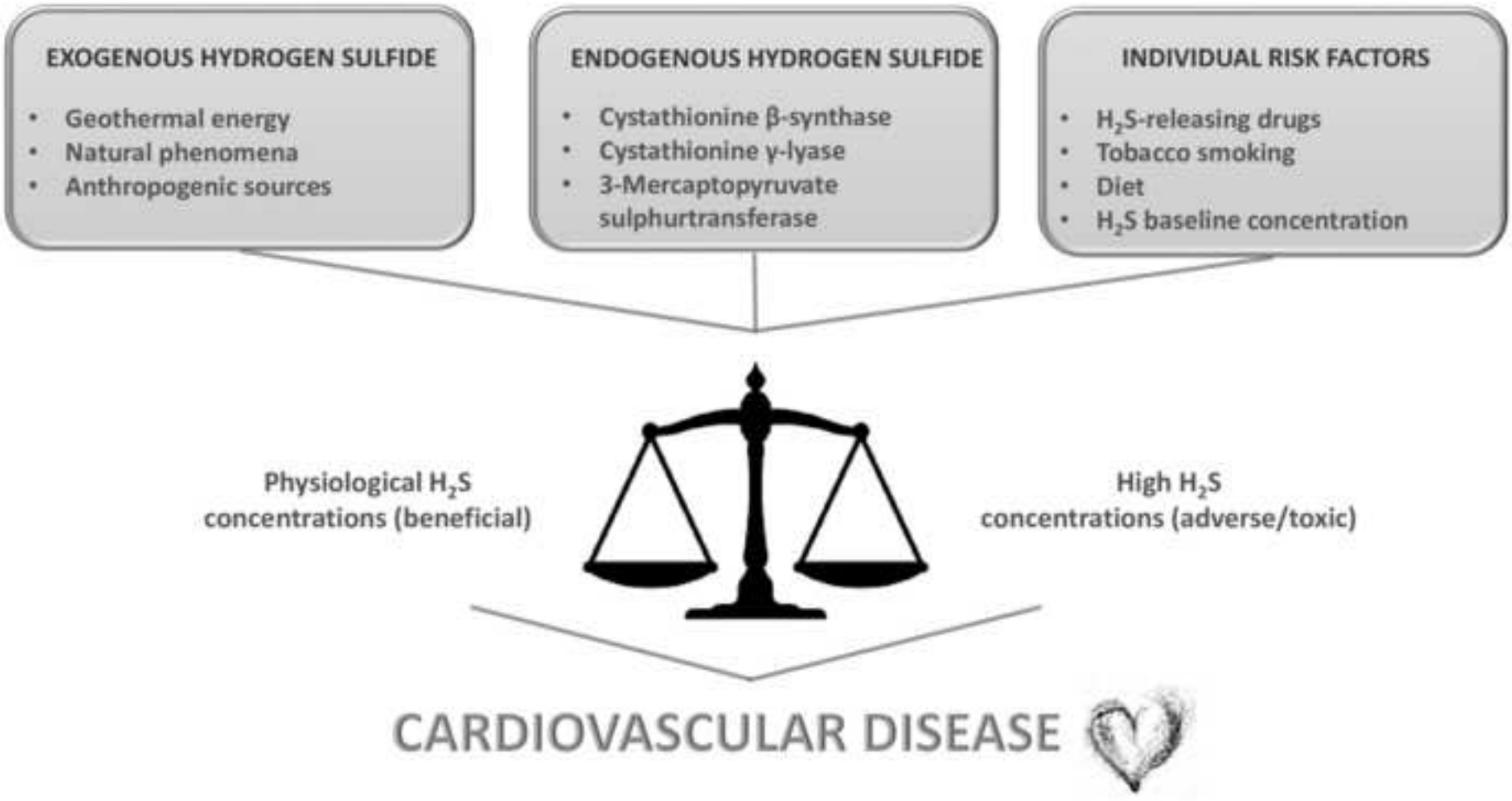

***conflict of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: