Predictive role of vital parameters for the outcomes of systemic inflammation and the role of TRP channels in pharmacological modulation of body temperature

Doctoral (PhD) thesis

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List of abbreviations

A1 ankyrin-1

AP acute pancreatitis

APACHE acute physiology and chronic health evaluation

CI confidence interval

ES estimated logit mortality rate

i.p. intraperitoneal(ly)

i.v. intravenous(ly)

KO knockout

LOS length of hospital stay

MA metabolic acidosis

NH_4Cl ammonium chloride

s.c. subcutaneous

SDM(s) standardized difference(s) in means

SE standard error

SIRS systemic inflammatory response syndrome

T_b body temperature

TRP transient receptor potential

V1 vanilloid-1

WT wild type

I. Introduction

I/1. Impact of body temperature and blood pH on outcomes of systemic inflammation

I/1.1. Sepsis and acute pancreatitis: two distinct manifestations of systemic inflammation

Systemic inflammation is a complex, generalized pathophysiological process, which can be clinically manifested in numerous forms, including systemic inflammatory response syndrome, sepsis, severe sepsis, septic shock, and multiple-organ dysfunction and failure (Bone, et al., 1992). This response describes an inflammatory process independent of its cause, and can result from infections, as well as from noninfectious causes (e.g., trauma, burns, acute pancreatitis).

When the underlying origin of the systemic inflammatory response is infection, it is termed sepsis. The definition and clinical criteria of sepsis has changed over the past three decades (Bone, et al., 1992; Levy, et al., 2003; Singer, et al., 2016), and based on the latest consensus (Singer, et al., 2016), it is defined as evidence of infection plus life-threatening organ dysfunction. Sepsis constitutes a global burden for medical care with an estimated 31 million cases per year worldwide (Fleischmann, et al., 2016). The incidence of sepsis has remained considerable (Esteban, et al., 2007; Vincent, et al., 2014; Walkey, Lagu, & Lindenauer, 2015) and it is associated even nowadays with high mortality rates (Vincent, et al., 2014). Moreover, it also increases the risk of mortality for 5–8 years after the septic event (Cuthbertson, et al., 2013).

Acute pancreatitis (AP) is initially a sterile inflammation of the pancreas most commonly triggered by noninfectious causes such as bile stones or excessive use of alcohol. The incidence of AP increases worldwide, and it is one of the most common gastrointestinal causes for hospitalization with significant morbidity and mortality in the US (Lee & Papachristou, 2019). Although the mortality rate in mild and moderate AP is low, this value is still unacceptably high (30%) in its severe form (Parniczky, et al., 2016).

I/1.2. Association between body temperature and mortality in sepsis

Sepsis is often associated with changes of deep body temperature (T_b), which can be manifested as fever or hypothermia in experimental animals (Romanovsky, et al., 2005; Romanovsky & Szekely, 1998; Saper, Romanovsky, & Scammell, 2012), as well as in human patients (Cunha, 2012; Kushimoto, et al., 2014; Romanovsky & Szekely, 1998). Due to its frequent occurrence in systemic inflammation, deep T_b is regularly measured as one of the vital signs in the clinical praxis. In fact, many diagnostic and prognostic scoring systems of sepsis e.g., acute physiology and chronic health evaluation II (APACHE II), systemic inflammatory response syndrome (SIRS), include an abnormal deviation of T_b from the normal range (Bone, et al., 1992; Knaus, Draper, Wagner, & Zimmerman, 1985; Le Gall, Lemeshow, & Saulnier, 1993; Opal, 2005; Singer, et al., 2016). Usually T_bs below 36.0°C or above 38.0°C are considered equally pathological (Beverly, Walter, & Carraretto, 2016), which values are in accordance with the criteria of the systemic inflammatory response syndrome (Proud, 1953; Singer, et al., 2016). Based on experimental data from animal

studies, Romanovksy and colleagues (Romanovsky, et al., 2005; Romanovsky & Szekely, 1998) proposed that hypothermia and fever can both develop as two distinct adaptive mechanisms in sickness syndrome. The latter characteristically occurs at the beginning of an infection, representing an active fight against the pathogen, while the former is usually associated with progressed stage or severity of the disease and it aims to secure the vital systems of the host (Romanovsky, et al., 2005; Romanovsky & Szekely, 1998). The two adaptive strategies can develop sequentially (e.g., early phase fever followed by late phase hypothermia) as the severity of the disease progresses (Romanovsky & Szekely, 1998), but hypothermia can be also one of the initial developing events in animal models of endotoxin shock (Fonseca, et al., 2016), moreover, septic patients admitted to intensive care unit develop hypothermia more frequently in the early than in the late stages of their stay (Fonseca, et al., 2016). Despite the distinct pathological background of fever and hypothermia in systemic inflammation, both the increase and the decrease of T_b are evaluated commonly as equally severe signs in the clinical praxis (Beverly, et al., 2016). This can be, at least in part, due to the standpoint that fever and hypothermia both represent an adaptive (though different) biological response to infection (Romanovsky & Szekely, 1998). Accordingly, beneficial effects have been shown for elevated T_b on the clinical outcome of sepsis in clinical trials (Bryant, Hood, Hood, & Koenig, 1971; Megged, Yinnon, Raveh, Rudensky, & Schlesinger, 2006), although no association between fever and disease severity was also reported (Kushimoto, et al., 2013). Therapeutic (i.e., induced) hypothermia has also been shown to improve the outcome of sepsis in human studies (Blair, Henning, Hornick, & Cowley, 1964; Villar & Slutsky, 1993), but in case of spontaneously occurring

hypothermia usually a positive association with mortality rate was found (Clemmer, et al., 1992; Drewry, Fuller, Skrupky, & Hotchkiss, 2015; Kushimoto, et al., 2013). The definite association of T_b and mortality rate in a large study population has remained unknown.

I/1.3. Blood pH and the outcomes of acute pancreatitis

The inflammatory response is accompanied by changes in vital parameters, including T_b, heart rate, and respiratory rate. The changes in blood pH are also common in systemic inflammation (White, et al., 2018). Changes in acid-base balance significantly alter the release of inflammatory mediators which can affect the outcome of systemic inflammation. The bicarbonate production is one of the most important functions of the pancreas, which is required to maintain its constant "milieu intérieur," thereby to prevent premature activation of pancreatic proteases (Hegyi & Petersen, 2013; Pallagi, Hegyi, & Rakonczay, 2015; Pallagi, et al., 2011). When pancreatic bicarbonate production is challenged by local or systemic acid load (i.e., metabolic acidosis, MA), the resulting lower pH can facilitate pancreatic enzyme activation and deteriorate cell damage (Reed, et al., 2011). Furthermore, animal experiments in rats showed that injection of acidic contrast solution either into the pancreatic duct or into the vein significantly increased the severity of acute AP (Bhoomagoud, et al., 2009; Noble, Romac, Vigna, & Liddle, 2008). Beside an external acid load, the pancreatic pH balance can be also compromised by tissue injury such as AP, which can lead to acidification of local tissues, thus deteriorate cell damage (Behrendorff, Floetenmeyer, Schwiening, & Thorn, 2010). The luminal pH of the main pancreatic duct

was also lower in human patients with AP compared to controls (Takacs, Rosztoczy, Maleth, Rakonczay, & Hegyi, 2013), suggesting that the development of AP is accompanied by a decrease of local pH. Multiple mechanisms have been implicated in AP which can lead to MA, including direct mechanisms such as the loss of bicarbonate-rich pancreatic juice via pancreatic fistula or drainage (Rice, Ismail, & Pillow, 2014), as well as indirect ones through lactic acidosis which can sequentially occur in AP due to shock, sepsis, cardiovascular failure, or upper gastrointestinal bleeding (Zhan, Guo, Yang, Li, & Li, 2015). However, the interaction between AP and systemic pH is still not fully clarified.

Acidosis is often considered as a marker of disease severity, viz., a by-product of systemic dysregulation, and as such it is a proven prognostic factor in the assessment of critically ill patients (Vincent & Moreno, 2010). Despite the fact that scoring systems, which are used to help the diagnosis and the assessment of the progression of AP, include the changes in systemic pH balance of the patients (e.g., APACHE II and Ranson scores), clinical trials aiming to reveal a correlation between the acid-base status and the outcome of AP are scarce. To our knowledge, the sole published human study, which aimed to directly answer this question showed that changes in the parameters of systemic acid-base status can predict mortality in AP (Sharma, et al., 2014). On the contrary, the necessity of arterial blood gas sampling was questioned in patients with AP in another human study (Ward, Gilbert, Mulchandani, & Garrett, 2008). With regards to the results obtained in experimental animals, a detailed analysis of the correlation between systemic pH and the outcome of AP would be of utmost importance, because it could establish blood pH as a predictor of the severity and the outcome of the disease and, arguably, identify acidosis as a therapeutic target in AP.

I/2. Transient receptor potential (TRP) channels in pharmacological modulation of body temperature

I/2.1. The thermal effects of TRP vanilloid-1 (V1) antagonists

TRP channels are multimodal ion channels that act as sensors of chemically toxic and physical stimuli (Chen, Mu, Zhu, Mukherjee, & Zhang, 2020). Total of 28 different TRP channel proteins are known in mammals, which can be divided into seven subfamilies based on amino acid sequence homology: TRPA (Ankyrin), TRPC (Canonical), TRPM (Melastatin), TRPML (Mucolipin), TRPN (NO-mechano-potential, NOMP), TRPP (Polycystin), TRPV (Vanilloid). The TRPV1 channels are expressed throughout the thermal shell and core including neural and non-neural tissues (Caterina, et al., 1997; Miller, Jones, Chauhan, & Anderson, 1990; Schicho, Florian, Liebmann, Holzer, & Lippe, 2004; Starowicz, Cristino, & Di Marzo, 2008; Szallasi, et al., 1995; Tominaga, et al., 1998), and their thermoregulatory role was proposed long years (Jancso-Gabor, Szolcsanyi, & Jancso, 1970). In the 1990s, number of pharmaceutical companies have started developing TRPV1 antagonists (Holzer, 2008; Y. Lee, et al., 2015). During the in-vivo testing of TRPV1 antagonists, an unexpected adverse effect on T_b, hyperthermia, were repeatedly observed in animal studies and human clinical trials alike. Usually, when studying the TRPV1-antagonizing property of compounds, pharmaceutical companies use capsaicin, low extracellular pH, and heat (> 42°C) stimuli to activate this channel. It is now known that TRPV1 antagonists can affect these three modes differentially (for review, see Blumberg, Pearce, & Lee, 2011; Romanovsky, et al., 2009). Upon systemic administration many

TRPV1 antagonists cause hyperthermia in a variety of laboratory animal species (Garami, et al., 2020). However, not all TRPV1 antagonists are equal as far as their ability to cause hyperthermia (Watabiki, et al., 2011). Some TRPV1 antagonists have been shown to be hyperthermic in several mammalian species, whereas others cause hyperthermia only in particular species. Intriguingly, some TRPV1 antagonists (e.g., A-1165901, A-425619, AMG7905, and AMG8562) cause hypothermia instead of hyperthermia (Garami, Pakai, et al., 2018; Lehto, et al., 2008; Mills, et al., 2008). And yet other compounds appear to affect T_b regulation in a species-specific fashion.

I/2.2. Ammonium chloride-induced hypothermia

Ammonium chloride (NH_4Cl) is a systemic and urinary acidifying agent that can be used in the treatment of metabolic alkalosis (Doxiadis, Goldfinch, & Holt, 1953; Martin & Matzke, 1982; Sellers & Kast, 1945). As an expectorant, it is also a common ingredient of many cough mixtures, which explains why the excessive consumption of such over-the-counter medications can lead to MA (MacRury, Neilson, & Goodwin, 1987; Wong, et al., 2001) that was also observed in subjects receiving NH_4Cl in other types of medicines (Relman, Shelburne, & Talman, 1961; Sleisenger & Freedberg, 1951; Wood, 1955). Extending its application areas, more recently, the use of NH_4Cl was implicated in COVID-19 (Baker, Williams, Tropsha, & Ekins, 2020; Siami, et al., 2021).

The oral and/or parenteral administration of NH_4Cl is often used to induce systemic (extracellular) acidosis in animal models (Celotto, et al., 2016; Galicek, Seow, & Lingard,

1981; Nowik, Kampik, Mihailova, Eladari, & Wagner, 2010; Rothe & Schimek, 1984; Rumbus, et al., 2018) and in human experiments (Edge, et al., 2015; Kleger, et al., 2001; Reaich, Channon, Scrimgeour, & Goodship, 1992; Tizianello, De Ferrari, Gurreri, & Acquarone, 1977). Although interspecies differences, for example, between rats and mice, were demonstrated in the response to the same NH_4Cl-loading protocol (Nowik, et al., 2010; Stauber, et al., 2005), NH_4Cl administration has been widely used to study the effect of MA associated with different conditions, such as physical exercise (Bento, Fagian, Vercesi, & Gontijo, 2007; Edge, et al., 2015; Jara, Felsenfeld, Bover, & Kleeman, 2000; Lane & Adams, 1993) and iron metabolism (Coe, et al., 1975; Daher, et al., 2022).

In 1988, Gordon showed that the systemic administration of NH_4Cl leads to hypothermia in mice (Gordon, 1988). This finding can explain why its overconsumption was also associated with hypothermia in case of a human patient (MacRury, et al., 1987). However, the molecular mediators of the NH_4Cl-induced hypothermia have remained largely unknown. Our literature search did not reveal any studies that aimed to clarify the underlying mechanisms of this thermoregulatory phenomenon.

TRPV1 and TRPA1 channels are temperature-sensitive members of the TRP channel family, for the reason that in vitro they can be activated by noxious heat and cold, respectively (Romanovsky, et al., 2009; H. Wang & Siemens, 2015). In addition to thermal signals, they can be both activated by ligand agonists and by changes in pH, hence they function as polymodal receptors (Bamps, Vriens, de Hoon, & Voets, 2021; Holzer, 2011), especially on primary afferent neurons where they are often co-expressed (Huang, Li, Dhaka, Story, & Cao, 2012; Kobayashi, et al., 2005). Their thermosensor function has been well

established in pain sensation (Bamps, et al., 2021; Julius, 2013; Kashio & Tominaga, 2022), however, neither of them was found to serve as a thermosensor for the thermoregulation system in rodents (de Oliveira, et al., 2014; Steiner, et al., 2007). It was proposed that their activation by agonists other than temperature (by protons for TRPV1 and by sulfides for TRPA1) contributes to the regulation of deep T_b (Garami, Pakai, et al., 2018; Olah, et al., 2021). Interestingly, both channels can be activated by NH_4Cl (Dhaka, et al., 2009; Fujita, et al., 2008) and also by low pH (de la Roche, et al., 2013; Jordt, Tominaga, & Julius, 2000; Tominaga, et al., 1998; Wang, Chang, Allgood, Silver, & Liman, 2011). However, to our best knowledge, the contribution of the TRPV1 or TRPA1 channel to the thermal response to NH_4Cl has not been investigated yet.

In the present work, we studied whether NH_4Cl induces hypothermia also in rats in addition to mice to exclude the possibility of a mouse-specific effect, and whether the blockade of TRPV1 and TRPA1 channels influences the NH_4Cl-induced hypothermia by using genetic deletion of the channels. To avoid chronic compensatory mechanisms, which may develop for the function of a knocked out gene, we also used pharmacological inhibition of TRPV1 and TRPA1 in genetically unaltered animals.

II. Aims

The goal of the current work is to investigate the predictive role of vital parameters for the outcomes of systemic inflammation in human patients as well as to identify a potential pharmacological target for the modulation of T_b in animal experiments and human patients. The following main topics will be discussed in this thesis:

1. We studied the role of changes in T_b and blood pH as vital signs in the prediction of outcomes in two different manifestations of systemic inflammation (in sepsis and in AP) with meta-analysis. Regarding fever, different clinical trials came to controversial results in sepsis, while clinical trials aiming to reveal a correlation between the acid-base status and the outcomes of AP are scarce. We hypothesized that the increase and the decrease of T_b or blood pH from the normal range predicts the clinical outcomes in systemic inflammation differently.
2. Based on animal studies, nonthermal activation by protons is involved in thermoregulatory responses to TRPV1 antagonists. Therefore, we also aimed to examine the thermal effect of TRPV1 antagonists in humans using meta-analysis of clinical trials.
3. Previously, it has been shown that NH_4Cl causes hypothermia in mice. In order to exclude the possibility that the NH_4Cl-induced hypothermic response is specific only to mice, we aimed to detect NH_4Cl-induced hypothermia in rats as well. Although TRPV1 and TRPA1 channels can be activated by NH_4Cl, the contribution of these channels in the thermal response to NH_4Cl has not been investigated yet. Thus, we

studied the role of TRPV1 and TRPA1 channels in NH_4Cl-induced hypothermia using genetic deletion and pharmacological blockade of either channel in rodents.

III. Materials and Methods

III/1. Predictive role of body temperature and blood pH for the outcomes of systemic inflammation in humans

III/1.1. Body temperature and mortality in sepsis

III/1.1.1. Search strategy

A search of the PubMed, EMBASE, and Cochrane Controlled Trials Registry databases was performed with using the following Medical Subject Headings and search terms (from inception to February 2016): sepsis OR bacteremia OR "septic syndrome" AND ("body temperature" OR fever OR hypothermia OR normothermia OR hyperthermia) AND (mortality OR survival). We restricted our search to original human studies published in English without time period limitations. Publications reporting immunosuppressive conditions (e.g., cancer, transplantation, HIV infection) were not included in the analysis. As a specific example, in the EMBASE database, which identified the highest number of articles, the term “sepsis OR bacteremia OR "septic syndrome" AND ("body temperature" OR fever OR hypothermia OR normothermia OR hyperthermia) AND (mortality OR survival) NOT (cancer OR immunosuppressive OR aids OR hiv OR transplantation)” was entered, and then the following filters were selected: humans, English, article, article in press, conference abstract, conference paper, major clinical study, case control study, clinical trial, cohort analysis, comparative study, controlled clinical trial, controlled study, cross-sectional study, double blind procedure, medical record review, multicenter study,

observational study, outcomes research, phase 3 clinical trial, prospective study, randomized controlled trial, retrospective study. Our meta-analysis was conducted in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (Shamseer, et al., 2015). The analysis was based on the Participants, Intervention (prognostic factor), Comparison, Outcome model: in septic population, we aimed to assess the predictive role of T_b deviations on the mortality ratio. No review protocol has been registered for the current meta-analysis.

III/1.1.2. Study selection and data extraction

The titles and abstracts of the publications from the literature search were screened and the full text of potentially eligible articles was obtained. We included studies in which both the T_b values and the mortality ratios were reported for the same group(s) of patients with systemic inflammation accompanied by suspected or confirmed blood infection. From all included articles we extracted the sample size, the reported mean T_b value of the patients with its standard error (SE), and the mortality ratio within the group during 28–30 days in most cases. To analyze the influence of fever, normothermia, and hypothermia on the mortality ratio in sepsis we separated the collected data into three study groups based on the mean T_b of the patients.

III/1.1.3. Statistical analysis

We have used event rates (mortality rates) as effect size data. Studies were grouped by T_b as low (up to 36.0°C; n = 890), medium (36.1 to 38.0°C; n = 3,904) and high (above 38.0°C; n = 6,040) and forest plots in the three groups were used to describe mortality. Selection of the T_b groups was based on the SIRS criteria (Bone, et al., 1992; Singer, et al., 2016). Another grouping was conducted by mortalities, these were split into quartiles and the means of T_bs were compared by investigating the presence or absence of overlaps in the 95% confidence intervals (CI), just like in case of the grouping by T_b.

Between-study heterogeneity was tested with Q homogeneity test (P values of less than 0.05 were considered as indicators of significant heterogeneity) and with I^2 statistical test, where I^2 is the proportion of total variation attributable to between-study variability (an I^2 value of more than 50 was considered as indicating considerable heterogeneity). These two values were used to model selection purposes as well (fixed vs random). The tests revealed considerable heterogeneity in the overall study population ($Q = 809.509$; $I^2 = 89.25$) and also in all three T_b groups, in particular $Q = 270.447$; $I^2 = 85.58$ in the high, $Q = 373.357$; $I^2 = 90.63$ in the medium, and $Q = 36.843$; $I^2 = 70.14$ in the low T_b group. Consequently, we applied the random effect model in our forest plot and meta-regression analyses.

Publication bias was tested by inspecting the funnel plot. Meta-regression was performed to assess the overall effect of T_b to mortality. Except for the Pearson correlation analysis for which Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) was used, all analyses were performed with the Comprehensive Meta-Analysis software (Biostat, Inc., Engelwood, MJ, USA).

III/1.2. Blood pH and outcomes of acute pancreatitis

III/1.2.1. Search strategy

Our meta-analysis was conducted in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (Moher, Liberati, Tetzlaff, Altman, & Group, 2009; Olah, et al., 2018). The analysis was based on the Patients, Intervention (or indicator), Comparison, Outcome model: in patients with AP, we aimed to assess the predictive role of the change in pH status (as assessed by blood pH, bicarbonate concentration, base excess, or base deficit) on disease severity (indicated by clinical scores), length of hospital stay (LOS), and mortality ratio. This meta-analysis has been registered with PROSPERO (CRD42017055396). A search in the PubMed, EMBASE, and Cochrane Controlled Trials Registry databases was performed from inception to January 2017 using the following terms: "pancreatitis AND (mortality OR survival OR severity) AND ("arterial pH" OR "blood pH" OR "systemic pH" OR "base deficit" OR "base excess" OR bicarbonate OR HCO3- OR "anion gap" OR acidosis OR alkalosis OR acid-base)." We restricted our search to original human studies published in English without time period limitations. A manual search of the reference lists of relevant full-text articles was conducted to identify further potentially eligible articles.

III/1.2.2. Study selection and data extraction

The titles and abstracts of the publications from the literature search were screened and the full text of potentially eligible articles was obtained. We included studies in which blood pH or a related parameter (e.g., base excess, base deficit, or bicarbonate) and severity scores or LOS or mortality ratios were reported for the same group(s) of patients with AP. From all included articles we extracted the sample size, the reported mean pH or its related parameter for the studied patient groups with the corresponding SE or deviation, as well as the severity score, LOS, and mortality ratio within the group. To analyze the influence of the change in acid-base status on the severity and the outcome of AP, in each study we assigned the patient groups as a lower pH group and as a higher pH group, irrespective from the original basis for grouping used by the authors of the study. We used mortality ratio of the AP patients groups as the primary outcome. Regarding secondary outcomes, we used two commonly applied severity indices (i.e., APACHE II and Ranson scores) and the LOS. The APACHE II score estimates intensive care unit mortality based on laboratory values and vital signs, while Ranson score estimates mortality of patients with pancreatitis, based on initial and 48-hour lab values.

III/1.2.3. Statistical analysis

We used logit transformation of event rates for mortality ratios and standardized mean difference (SMD) for LOS and severity scores as the effect size data. The secondary

outcomes were compared between the lower and higher pH groups (see above) within each study, and then the estimated pooled mean values were calculated. The relevant studies were compared with standard meta-analysis tools (e.g., forest plot) in case of each outcome.

Between-study heterogeneity was assessed by I^2 statistical test, where I^2 is the proportion of total variation attributable to between-study variability (an I^2 value of more than 50 was considered as indication of considerable statistical heterogeneity). The selection of patients, study design, and the used methods showed variability among the studies included in our analyses, which also resulted in statistical heterogeneity. Since the lack of statistical significant results on these heterogeneity tests could be also due to the lack of power because of the small number of studies eligible for the analyses, we used the random effect model in case of each forest plot, similarly to our earlier meta-analysis (Rumbus, et al., 2017). Publication bias was assessed by funnel-plot analysis, Egger's test and Duval and Tweedie trim and fill method. Publication bias plots were used to assess whether studies with small sizes could have been missed in our analyses, however, due to the design of these tests they do not allow to firmly rule out the possibility that some papers missed the inclusion criteria of our search.

As a different statistical approach to reveal a correlation between systemic pH and mortality in moderate and severe forms of AP, we performed meta-regression analysis of those studies in which both blood pH and mortality rate were reported within the same patient group. The meta-analyses were performed with Comprehensive Meta-Analysis (version 3.3; Biostat, Inc., Engelwood, MJ, USA) and Stata (version 11.1; StataCorp, College Station, TX, USA) software.

III/2. The role of TRP channels in pharmacological modulation of body temperature

III/2.1. Relationship between body temperature and pH: TRPV1 antagonists induced thermal changes

III/2.1.1. Search strategy

We used standard meta-analysis tools, in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (Moher, et al., 2009). This analysis was registered with PROSPERO (CRD 42018095220).

III/2.1.2. Study selection and data extraction

We included the studies that reported deep T_b values in both TRPV1 antagonist-treated and placebo groups at least at two time points: (i) at shortly before the drug (or placebo) administration and (ii) at 3 h after the time of administration. Studies for which all the necessary information could not be obtained were excluded from the analysis. In certain cases, we had to make limited assumptions or simplifications, as explained in (Garami, et al., 2020). For each included study, we calculated the change in deep T_b as a difference between the T_b values at 3 h after the drug (placebo) administration vs. at the time of administration (0 h). We then calculated the difference between the T_b changes induced by

a drug and those induced by placebo (difference in means); we considered the latter difference to represent the thermal effect of the drug in our meta-analysis.

III/2.1.3. Statistical analysis

For all doses, the differences in means were standardized (based on variances) to obtain standardized differences in means (SDMs). The SDMs with 95% confidence intervals (CIs) were used as primary measures of effect size and are presented as a "forest plot". We considered each TRPV1 antagonist to be either mode-nonselective (ABT-102, AZD1386, and V116517) or mode-selective (NEO6860). To characterize the nonselective group, SDMs for the corresponding antagonists were weighted based on sample size and inversed variance.

III/2.2. The roles of TRPV1 and TRPA1 in NH_4Cl-induced hypothermia in rodents

III/2.2.1. Animals

Experiments were conducted in adult male rats and mice under protocols approved by the Institutional Animal Use and Care Committee of the University of Pecs (registration no.: BA02/2000-13/2021) and in accordance with the directives of the National Ethical Council for Animal Research and those of the European Communities Council (86/609/EEC). One hundred and ten Wistar rats and thirty-seven C57BL/6 mice were obtained from the Laboratory Animal Centre of the University of Pecs. In addition, mice

with ($^{-/-}$ aka knockout, KO) or without ($^{+/+}$ aka WT) a homozygous targeted mutation in the *Trpv1* gene (KO: n = 21; WT: n = 14) or in the *Trpa1* gene (KO: n = 24; WT: n = 16) were also obtained from the Laboratory Animal Centre of the University of Pecs, where they were bred and kept as described in details elsewhere (Bolcskei, et al., 2005; de Oliveira, et al., 2014). Mice from these strains were also used in previous studies aiming to investigate the thermoregulatory function of TRPA1 (de Oliveira, et al., 2014; Olah, et al., 2021) and TRPV1 (Garami, et al., 2017; Garami, Pakai, et al., 2018). The animals were housed in polycarbonate cages kept in temperature-controlled rooms on a 12 h light/dark cycle (lights on at 5:00 A.M.). The ambient temperature was maintained at 24-25°C and humidity at 30–40%. Standard rodent chow and tap water were available ad libitum. Animals were extensively habituated to the experimental setup, as described elsewhere (Garami, et al., 2011; Romanovsky, Ivanov, & Shimansky, 2002). At the time of the experiments, the mice and rats weighed 25 ± 1 and 328 ± 3 g, respectively.

III/2.2.2. Surgeries

Animals were anesthetized with intraperitoneal (i.p.) administration of a ketamine–xylazine cocktail (81.7 and 9.3 mg/kg for mice, 55.6 and 5.5 mg/kg for rats, respectively), and they received antibiotic protection intramuscularly (gentamicin, 6 mg/kg). To prevent intra- and postoperative hypothermia, during the surgery mice were kept on a heating pad (PECO Services Ltd., Brough, United Kingdom), and then they were allowed to recover from anesthesia in a temperature-controlled chamber (model MIDI F230S; PL Maschine

Ltd., Tarnok, Hungary) set to 31°C. Each rat and mouse was implanted with an i.p. catheter, additionally rats assigned to experiments with pharmacological antagonists were also implanted with an intravenous (i.v.) catheter during the same surgery. The i.p. and i.v. catheter implantations have been widely used in thermoregulation experiments, and the animals tolerated these interventions well (Garami, et al., 2017; Garami, Pakai, et al., 2018; Keringer, et al., 2022; Olah, et al., 2021). These preimplanted catheters were used for the non-stressful administration of substances to conscious animals in our experiments. In case of a bolus injection (i.p. or i.v.), the handling of the animal and the puncture of the abdominal wall with the needle would have resulted in pain and stress-induced hyperthermia (for review, see Romanovsky, et al., 2005), which could interfere with our results. All experiments were performed 3-5 days after the surgery.

For the non-stressful i.p. administration of the substances during the experiment, a polyethylene (PE)-50 catheter filled with pyrogen-free saline was implanted into the peritoneal cavity of each mouse and rat, similarly as in previous studies (Garami, et al., 2017; Garami, Pakai, et al., 2018). In brief, through a small midline incision on the abdomen, the internal end of the catheter was fixed to the left side of the abdominal wall with a suture, while the external end of the catheter was tunneled under the skin to the nape, where it was exteriorized and heat-sealed. The surgical wound was sutured in layers. The catheter was flushed with 0.1 ml of saline on the day after the surgery and every other day thereafter.

I.v. catheter implantation was performed in rats assigned to treatment with a TRPV1 or a TRPA1 antagonist. As described before (Garami, et al., 2017), the i.v. catheter was implanted during the same surgery as the i.p. catheter. A small longitudinal incision was

made on the ventral surface of the neck, left of the trachea. The left jugular vein was exposed, freed from its surrounding connective tissue, and ligated. A silicone catheter (with inner and outer diameter of 0.5 and 0.9 mm, respectively) was filled with heparinized (10 U/ml) saline, then it was inserted into the left jugular vein, passed into the superior vena cava, and secured in place with ligatures. The free end of the catheter was knotted and exteriorized at the nape. The skin wound was sutured. The catheter was flushed with heparinized saline on the day following the surgery and every other day thereafter.

III/2.2.3. Thermocouple thermometry

The mice and the rats were placed in cylindrical confiners and equipped with copper-constantan thermocouples (Omega Engineering, Stamford, CT, USA) to measure colonic temperature (a form of deep T_b). The colonic thermocouple was inserted beyond the anal sphincter (10 and 3 cm deep for rats and mice, respectively); fixed to the base of the tail with adhesive tape; and plugged into a data logger device (Cole-Palmer, Vernon Hills, IL, USA) connected to a computer. Animals in their confiners were then placed into a biochemistry incubator (model BJPX-Newark; Biobase; Jinan, China). As the expected T_b change was hypothermia, the ambient temperature was set to 25°C, which is slightly subneutral for rats and mice in this setup. The preimplanted i.p. and i.v. catheter (when present) was connected to a PE-50 extension, which was prefilled with the substance of interest and connected to a syringe placed in an infusion pump (model 975; Harvard Apparatus Inc., Holliston, MA, USA).

III/2.2.4. Drugs and drug administration

NH_4Cl was purchased from VWR Chemicals (Leuven, Belgium). On the day of an experiment, NH_4Cl was freshly dissolved in sterile water to achieve final concentrations of 32.1, 220 or 280 mg/ml. For the i.p. administration of NH_4Cl to mice, the working solution (32.1 mg/ml) was infused (10 ml/kg) over 16 minutes to deliver NH_4Cl at 321 mg/kg (~6 mmol/kg). In rats, the working solutions (220 and 280 mg/ml) were infused (1 ml/kg) over 5 minutes to deliver NH_4Cl at 220 and 280 mg/kg (ca. 4 and 5 mmol/kg), respectively. The decreased dose of NH_4Cl in the rats compared to mice was necessary, because rats were more sensitive to the effect of the same NH_4Cl-loading protocol on protein expression than mice (Nowik, et al., 2010). Control animals were infused with sterile water. All infusion rates and volumes were selected with the goal to minimize the stress and discomfort of the animals that may originate from the substance administration procedure itself. Similar i.p. infusion rates as in the present experiments were successfully applied in our previous studies for non-stressful administration of substances to mice (Garami, et al., 2017) and rats (Pakai, et al., 2018).

AMG 517, a highly potent TRPV1 antagonist (Garami, et al., 2010), and A967079, a highly potent TRPA1 antagonist (Chen, et al., 2011), were purchased from Tocris (Bristol, UK). A stock solution of AMG 517 (1 mg/ml) was prepared with 10% dimethyl sulfoxide (DMSO) and 10% Tween-80 in saline, aliquoted, and stored at -80°C. On the day of the experiment, the stock was diluted to give a working solution of AMG 517 at 210 μg/ml in 10% DMSO and 10% Tween-80 in saline. A stock solution of A967079 (10 mg/ml) was prepared with polyethylene glycol 400 (PEG 400), aliquoted, and stored at -80°C. On the

day of the experiment, the stock was diluted with PEG 400 and saline to give a working solution of A967079 at 5 mg/ml in 80% PEG 400 in saline. For the i.v. administration, the working solution of AMG 517 or A967079 (210 μg/ml or 5 mg/ml, respectively) was infused (1 ml/kg) over 10 minutes to deliver AMG 517 and A967079 at 210 μg/kg and 5 mg/kg, respectively. Both antagonists were infused i.v. to the rats 20 minutes before the i.p. infusion of NH_4Cl. Control rats were infused with the vehicle of the antagonist of interest. The efficacies of AMG 517 and A967079 administered i.v. at similar doses and rates as in the present experiments were shown in previous studies (Garami, et al., 2010; McGaraughty, et al., 2010).

To study the effect of AMG 517 on NH_4Cl-induced hypothermia in mice, we used a drug dose and administration model that was successfully applied earlier (Wanner, et al., 2012). The working solution (or its vehicle) was injected subcutaneous (s.c.) as a bolus to deliver AMG 517 at a dose of 210 μg/kg just before setting up the mice for the experiment (i.e., ~120 minutes before the administration of NH_4Cl). Then, the mice were allowed to accommodate to the experimental conditions for ~2 hours before they received the non-stressful, i.p. infusion of NH_4Cl (321 mg/kg) through a preimplanted catheter. The 2-hour latency until the NH_4Cl infusion was needed to reduce the stress-induced hyperthermia resulting from the needle prick associated with the s.c. injection of AMG 517 (or its vehicle). Because of the long (31 h) half-life of AMG 517 in rodents (Doherty, et al., 2007), the effect of the drug could be reasonably expected at the time of NH_4Cl infusion even with this latency.

The drug administration procedures in the different experiments are summarized in Table 1.

Table 1 Summary of drug administration procedures in the experiments (Rumbus, et al., 2024)

Species (strain)	Pretreatment			NH_4Cl	Figure number
	Drug	Dose (mg/kg) and route	Time prior NH_4Cl (min)	I.p. dose (mg/kg)	
Rat (Wistar)	N/A			220; 280	12
Mouse (*Trpv1*$^{-/-}$ and $^{+/+}$)	N/A			321	13
Mouse (C57BL/6)	AMG 517	0.21 s.c.	-120	321	14
Rat (Wistar)	AMG 517	0.21 i.v.	-20	280	15
Mouse (*Trpa1*$^{-/-}$ and $^{+/+}$)	N/A			321	16
Rat (Wistar)	A967079	5 i.v.	-20	280	17

N/A, not applicable

III/2.2.5. Blood pH measurements

One hour after the i.p. administration of NH_4Cl, the animals were anesthetized with ketamine-xylazine cocktail, and then blood samples were collected by cardiac puncture with a heparinized syringe. The pH of the blood samples was measured by a pH meter (model OP-211/2; Radelkis Ltd., Budapest, Hungary) within 1 minute after collection. After the blood withdrawal, the animals were euthanized with sodium pentobarbital (100 mg/kg, i.p.).

III/2.2.6. Data processing and analysis

Data on deep T_b and on blood pH were compared by ANOVA, as appropriate. ANOVA was followed by the Student-Newman-Keuls post hoc test as in our earlier study (Keringer, et al., 2022). Sigmaplot 11.0 (Systat Software, San Jose, CA, USA) software was

used for statistical analyses. Differences were considered significant when $p < 0.05$. Data are presented as mean ± SE.

IV. Results

IV/1. Predictive role of body temperature and blood pH for the outcomes of systemic inflammation in humans

IV/1.1. Body temperature and mortality in sepsis

IV/1.1.1. Study selection

The flow chart of the study selection is presented in Figure 1. Until February 29, 2016 the electronic literature search identified altogether 6,083 studies from the PubMed, EMBASE, and Cochrane databases. After enabling filters for human studies and English language, 762 articles remained, which were screened on title and abstract for inclusion criteria. In 720 studies T_b or mortality rate was not suitably reported in the septic patients, these were also excluded, as a result 42 full-text publications were found eligible for statistical analysis which included data from a total of 10,834 septic patients.

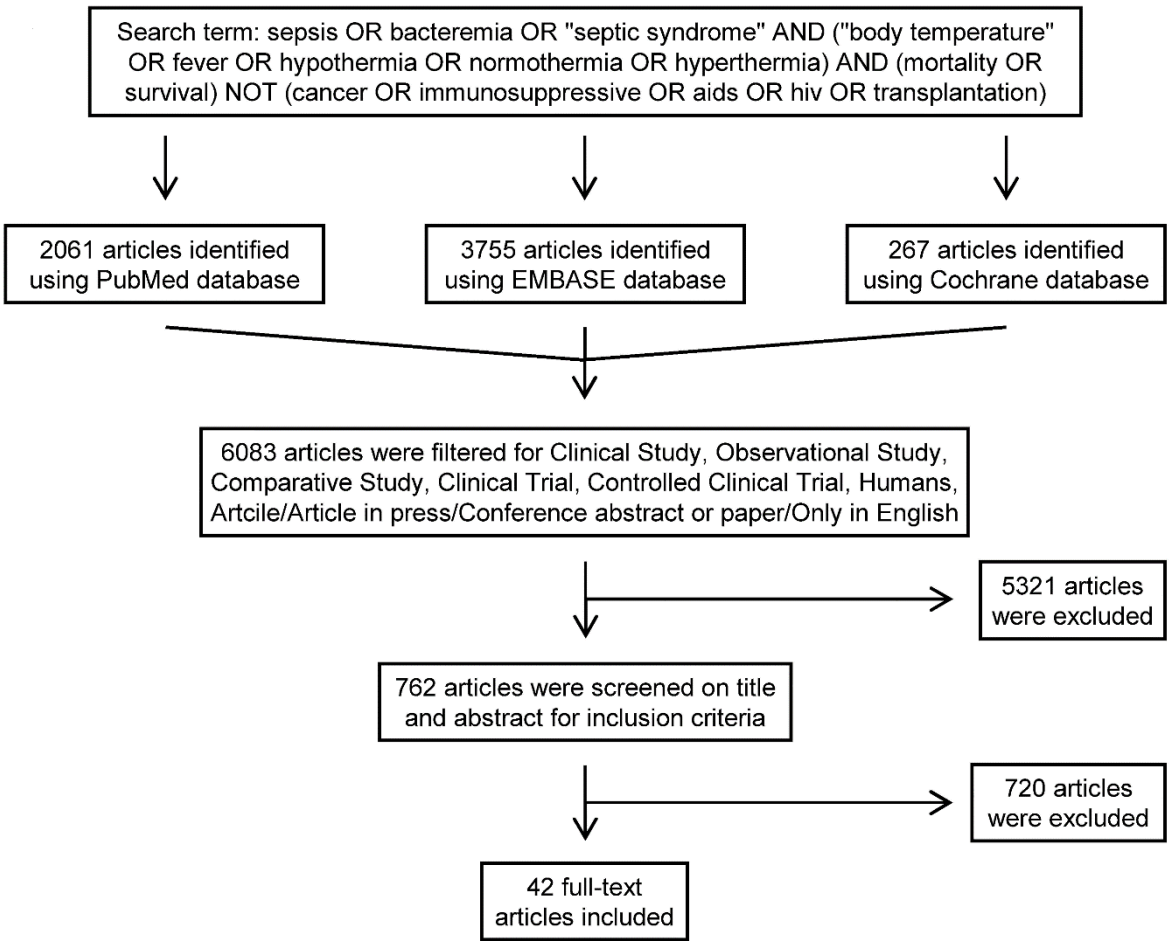

Figure 1. Flow chart of study selection and inclusion in our meta-analysis (Rumbus, et al., 2017).

IV/1.1.2. Incidence of mortality in septic patients with fever, normothermia, and hypothermia

As a rude approach, first we performed a common (Pearson) correlation analysis between T_b and mortality rate of all septic patients. A weak negative linear correlation was found ($y = -0.0909x + 3.6902$; $R2 = 0.2794$), which suggests an association between T_b and mortality in sepsis. This method, however, did not allow us to weight the collected data

according to the size of the studied populations, thus a detailed meta-analysis was needed. We divided the studies into three groups based on the T_b: fevbrile (defined as $T_b > 38.0°C$), normothermic ($T_b = 36.0\text{-}38.0°C$), and hypothermic ($T_b < 36.0°C$).

First, we investigated the incidence of mortality in fever associated with sepsis. We found 29 studies (Aceng, Byarugaba, & Tumwine, 2005; Arons, et al., 1999; Asiimwe, Abdallah, & Ssekitoleko, 2015; Bryant, et al., 1971; Clemmer, et al., 1992; Diekema, et al., 2003; Gozel MG, 2012; Harkness & Braun, 1989; Heffner, Horton, Marchick, & Jones, 2010; Hodgin & Sanford, 1965; Hoeboer, et al., 2012; Hung, et al., 2005; Kang, et al., 2012; Kirov, et al., 2001; Kushimoto, et al., 2013; Lee, et al., 2012; Marfin, Sporrer, Moore, & Siefkin, 1995; Megged, et al., 2006; Molina, et al., 2013; Neuberger, et al., 2015; Pittet, et al., 1996; Rhodes, et al., 1999; Schortgen, et al., 2012; Seguin, et al., 2012; Song, et al., 2012; Su, et al., 2012; Swenson, Hedrick, Popovsky, Pruett, & Sawyer, 2007; Taylor, Wills, Courval, & Molyneux, 1998; Weinstein, Murphy, Reller, & Lichtenstein, 1983), in which the authors reported fever (defined as $T_b > 38.0°C$) in sepsis. From these studies, 40 groups of septic patients could be separated and included in the analysis with the random effect model. The meta-analysis of the mortality rates in the septic patients with fever revealed an average event rate of 22.2% (95% CI, 19.2-25.5%; Z = -13.4331) (Figure 2). This percentage was significantly (P = 0.000) lower than the 50% chance of mortality, which could be regarded as a random outcome.

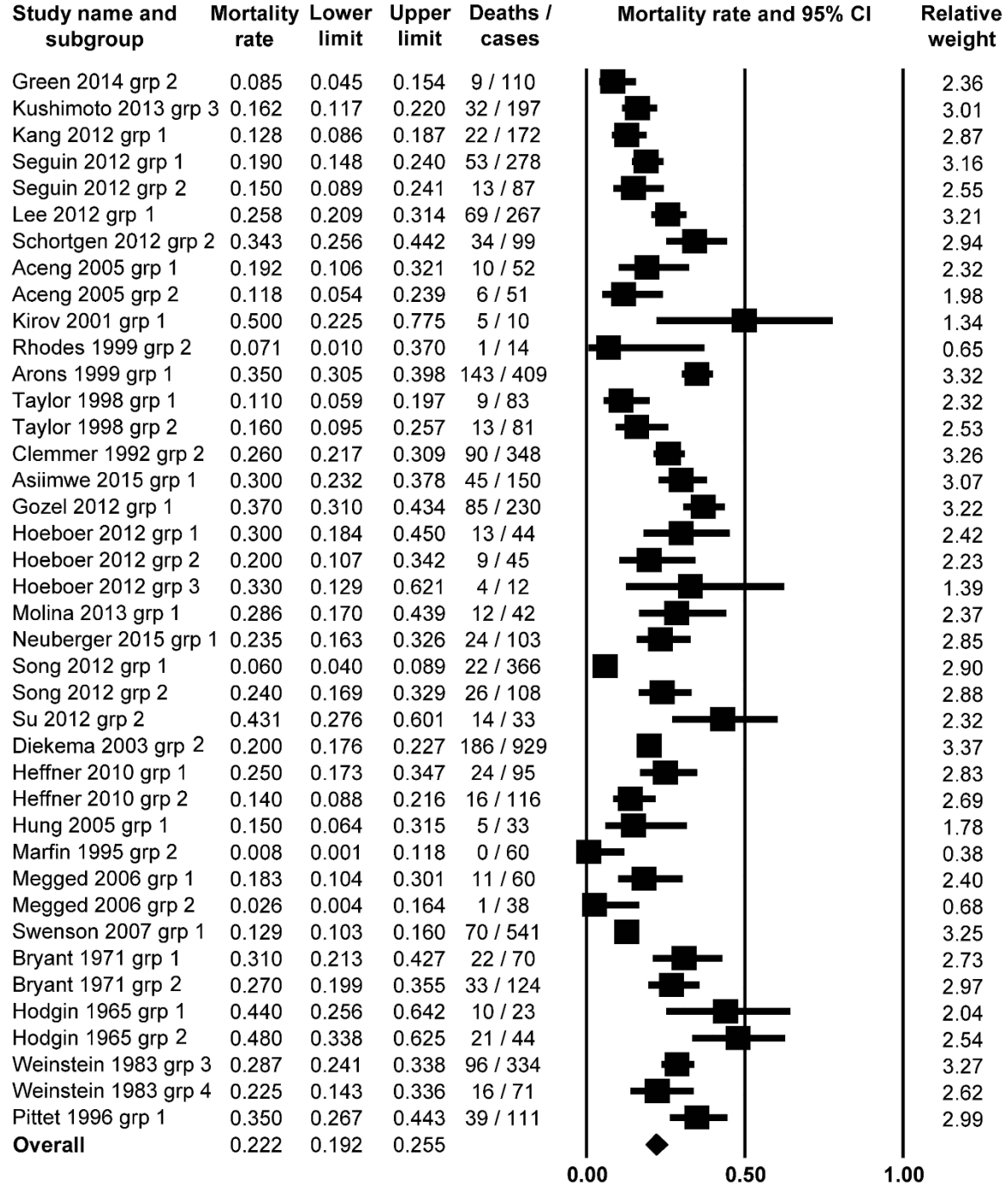

Figure 2. Forest plot analysis of mortality rate using random-effects model in septic patients with fever (body temperature above 38.0°C; n = 6,040) (Rumbus, et al., 2017).

Next, we analyzed the mortality ratios of patients who developed neither fever nor hypothermia in association with sepsis, therefore this population could be regarded as normothermic (T_b = 36.0-38.0°C). From the 25 studies, in which normal T_b was reported in the septic patients (Asiimwe, et al., 2015; Bernard, et al., 1997; Carl, Grossman, Behnke, Sessler, & Gehr, 2010; Drewry, Fuller, Bailey, & Hotchkiss, 2013; Drewry, et al., 2015; DuPont HL, 1969; Green, Ariathianto, Wong, Aboltins, & Lim, 2014; Hung, et al., 2005; Kang, et al., 2012; Kirov, et al., 2001; Kushimoto, et al., 2013; Marfin, et al., 1995; Memis, Karamanlioglu, Turan, Koyuncu, & Pamukcu, 2004; Molina, et al., 2013; Neuberger, et al., 2015; Pestana, et al., 2007; Rhodes, et al., 1999; Sarmin, Ahmed, Bardhan, & Chisti, 2014; Sawyer, et al., 2015; Schortgen, et al., 2012; Su, et al., 2012; Villar & Slutsky, 1993; Weinstein, et al., 1983; Young, et al., 2015), 36 subgroups of patients were separated, which were then analyzed with the random effect model. We found that the average mortality ratio was 31.2% (95% CI, 25.7-37.3%; Z = -5.7089) (Figure 3), which was higher than in the fever group. The mortality rate was significantly ($P < 0.001$) lower than 50% in this study population.

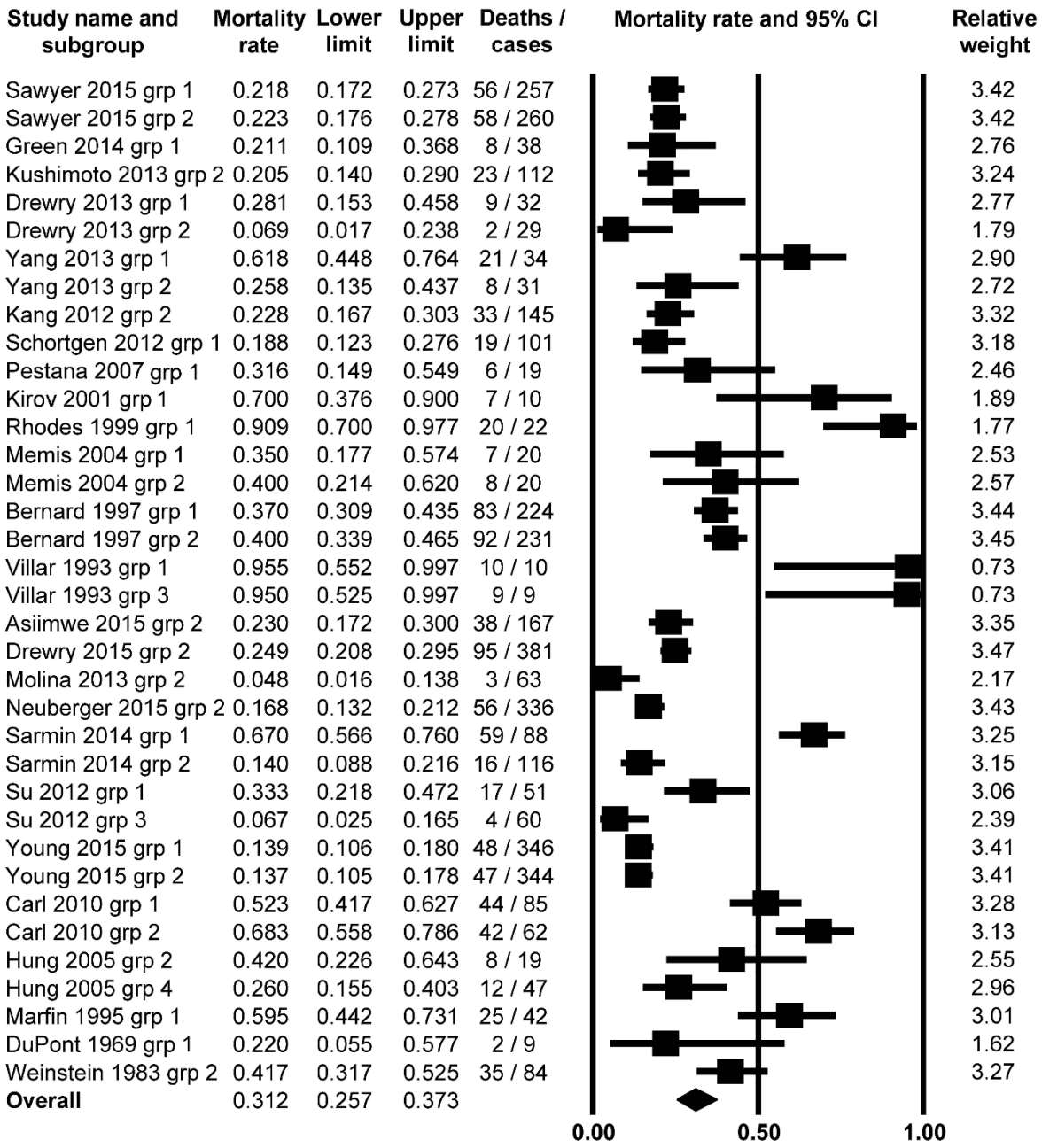

Figure 3. Forest plot analysis of mortality rate using random-effects model in septic patients with normothermia (body temperature between 36.1 and 38.0°C; n = 3,904) (Rumbus, et al., 2017).

Then, we examined the incidence of mortality in hypothermic (T_b < 36.0°C) septic patients. We identified 11 studies (Arons, et al., 1999; Bro-Jeppesen, et al., 2015; Clemmer,

et al., 1992; Diekema, et al., 2003; Drewry, et al., 2015; DuPont HL, 1969; Gozel MG, 2012; Hung, et al., 2005; Kushimoto, et al., 2013; Villar & Slutsky, 1993; Weinstein, et al., 1983), which included data on both T_b and mortality in septic patients. From these, the patients could be divided in 12 subgroups, which served as the basis of the meta-analysis. The random effect model revealed that the average mortality rate was the highest, 47.3% (95% CI, 38.9-55.7; Z = 0.520491) in the hypothermic patients (Figure 4), which did not significantly differ from the 50% random chance (P = 0.603).

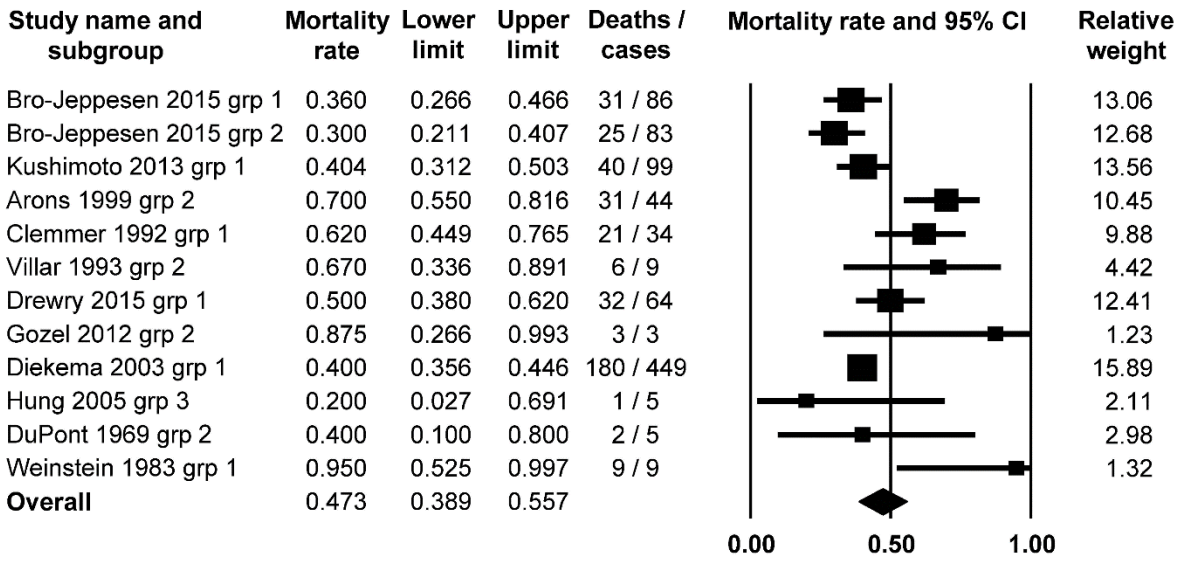

Figure 4. Forest plot analysis of mortality rate using random-effects model in septic patients with hypothermia (body temperature up to 36.0°C; n = 890) (Rumbus, et al., 2017).

As a further statistical approach, we also performed a meta-regression analysis on the collected data. We found a significant (P < 0.001) negative linear correlation between T_b and mortality rate (regression coefficient: -0.4318; 95% CI, -0.6699 - -0.1938) based on 51 studies included in the analysis (Figure 5).

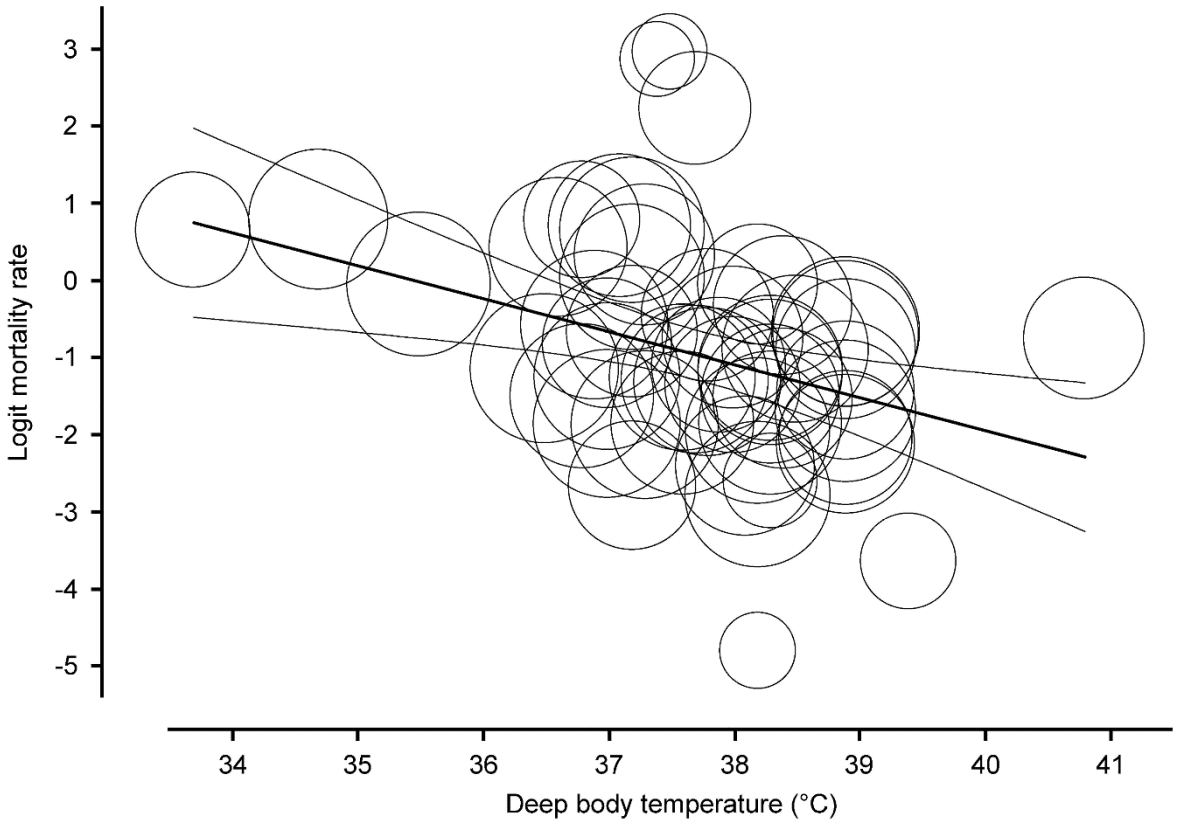

Figure 5. Meta-regression analysis of the association between body temperature and mortality ratio in septic patients (n = 10, 834) (Rumbus, et al., 2017).

Last, we divided the patients into quartiles (Q1-Q4) of mortality ratios (Q1: 0-25, Q2: 26-50, Q3: 51-75, and Q4: 76-100%) and calculated the average T_b for each mortality quartile. The weighted average T_bs were 38.1 (95% CI, 37.9-38.4°C), 37.8 (95% CI, 37.5-38.25°C), 37.6 (95% CI, 36.5-38.7°C), and 37.1°C (95% CI, 36.7-37.4°C) in the Q1, Q2, Q3, and Q4 groups, respectively. These results also indicate that in sepsis a higher T_b is associated with better outcome, while a lower T_b is related with higher risk of mortality. Of

note, the T_bs in Q1 and Q4 (i.e., in the groups with lowest and highest mortality, respectively) are clearly distinct from each other, as the 95% CIs do not overlap.

IV/1.2. Blood pH and outcomes in acute pancreatitis

IV/1.2.1. Study selection

The flow chart of the study selection is presented in Figure 6. Until January 2017 the electronic literature search identified altogether 1,076 studies from the PubMed, EMBASE, and Cochrane databases. After enabling filters for human studies and English language and removal of duplicates, 793 articles remained, which were screened on title and abstract for inclusion criteria. Full texts of the remaining 122 articles were reviewed in detail. In 109 studies pH parameters or outcomes were not suitably reported in the patients with AP, therefore these were also excluded. As a result, 13 full-text publications were found eligible for statistical analysis which included data from a total of 2,311 patients (De Campos, et al., 2008; Eachempati, Hydo, & Barie, 2002; Kaya, Dervisoglu, & Polat, 2007; Keskinen, et al., 2007; Lei, et al., 2013; Nair, Yadav, & Pitchumoni, 2000; Pupelis, Plaudis, Grigane, Zeiza, & Purmalis, 2007; Ranson, Rifkind, & Turner, 1976; Sharma, et al., 2014; Shen, et al., 2016; Shinzeki, et al., 2008; Zhan, et al., 2015; Zhu, Shi, & Sun, 2003).

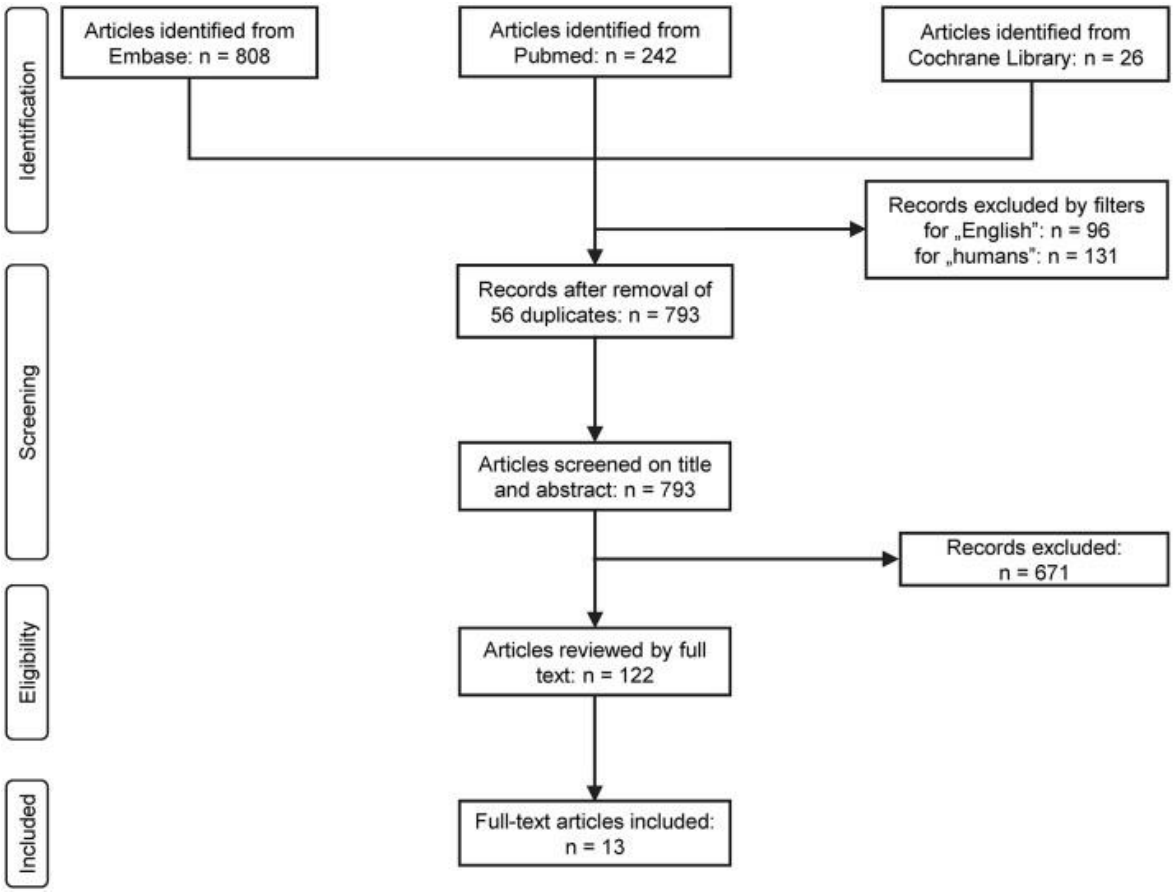

Figure 6. Flow chart of study selection and inclusion (Rumbus, et al., 2018).

IV/1.2.2. Reduction of blood pH is associated with higher mortality rate in AP

First, we investigated the association between systemic (blood) pH status and our strongest endpoint, namely the mortality. Our meta-analysis revealed a logit event rate of -0.09 (95% CI, -0.79, 0.61), corresponding to an average mortality rate of 51.0% (95% CI, 31.5, 70.1) in the more acidotic patient groups, while in the patient groups with higher pH or bicarbonate level the logit event rate was -3.68 (95% CI, -4.81, -2.55), which corresponds

to an average mortality rate of 3.0% (95% CI, 1.2, 7.1) (Figure 7). The mortality ratios were significantly different between the two groups ($P < 0.001$).

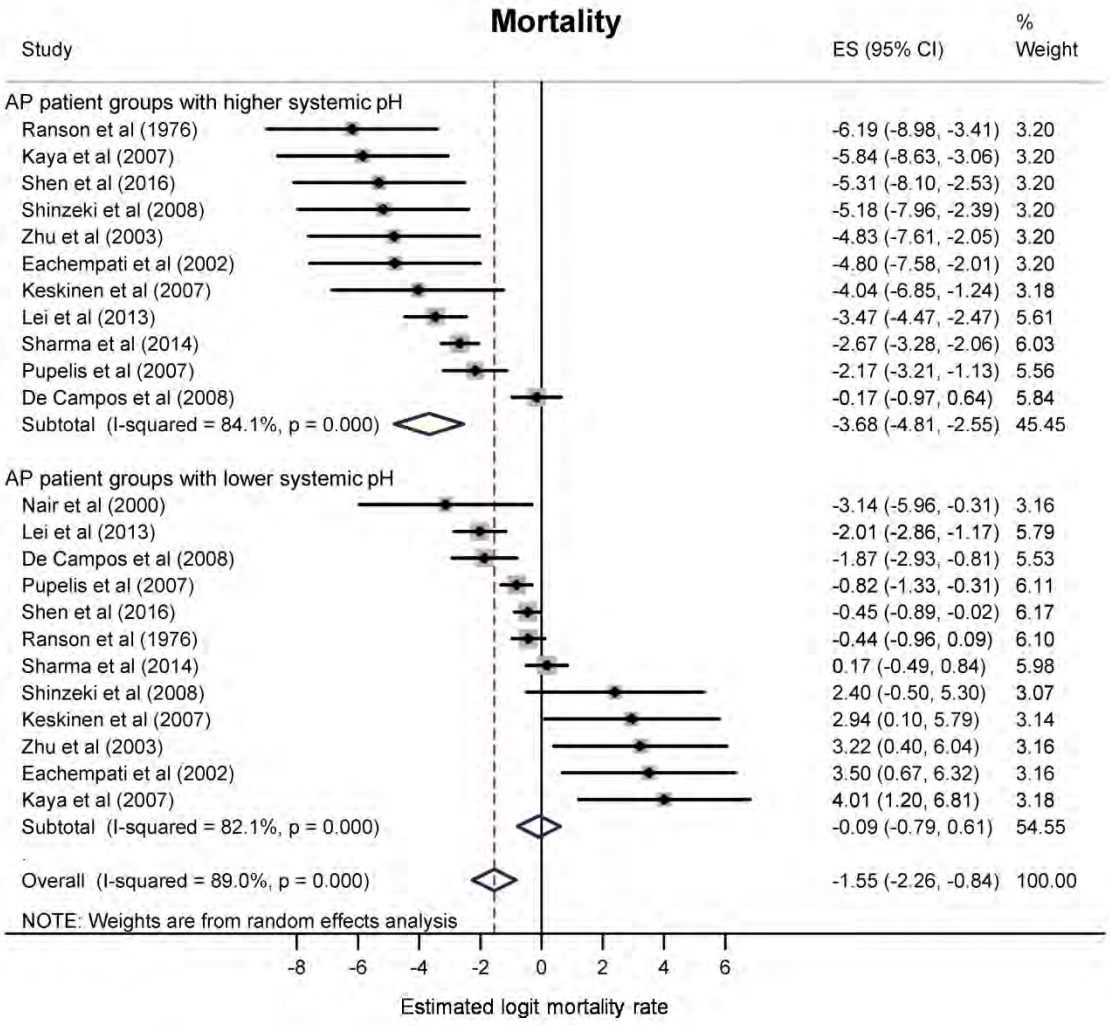

Figure 7. Forest plot of mortality rate using random-effects model in different systemic pH groups of patients with acute pancreatitis (AP). For each patient group, black circles and horizontal lines represent the estimated logit mortality rate (ES) and the corresponding confidence interval (CI), respectively. Lower ES corresponds with lower mortality rate and vice versa. Gray squares indicate the relative statistical weight of a given patient group. Open diamonds show the average ES and CI of patient groups with higher systemic pH (top), lower systemic pH (middle), and all patient groups (bottom) (Rumbus, et al., 2018).

IV/1.2.3. Lower pH or bicarbonate concentration worsen the severity of AP

We wanted to know whether the change in acid-base status can also predict the severity of AP as assessed by clinical scores. Thus, we studied the association between blood pH and clinical severity scores. We found two scores, the Ranson and the APACHE II scores, which were reported in sufficient number of studies for statistical analysis (De Campos, et al., 2008; Eachempati, et al., 2002; Kaya, et al., 2007; Keskinen, et al., 2007; Lei, et al., 2013; Nair, et al., 2000; Pupelis, et al., 2007; Ranson, et al., 1976; Sharma, et al., 2014; Shen, et al., 2016; Shinzeki, et al., 2008; Zhan, et al., 2015; Zhu, et al., 2003). Meta-analysis revealed that the estimated SMDs of the Ranson score (0.92, 95% CI, 0.58, 1.26) and the APACHE II score (1.38, 95% CI, 0.95, 1.81) were significant between the patient groups with lower pH or bicarbonate levels compared with less acidotic groups of patients ($P < 0.001$) (Figure 8A and B). These standardized values correspond to 1.60 (95% CI, 0.77, 2.42) higher Ranson score and 7.40 (95% CI, 5.05, 9.75) higher APACHE II score in the more acidotic patients with AP. The correlation found between lower blood pH and higher clinical scores could be expected as these scores also include blood pH (Vincent & Moreno, 2010), nevertheless, these results confirm the feasibility of our meta-analysis approach to reveal an interaction between systemic pH and the outcome of AP.

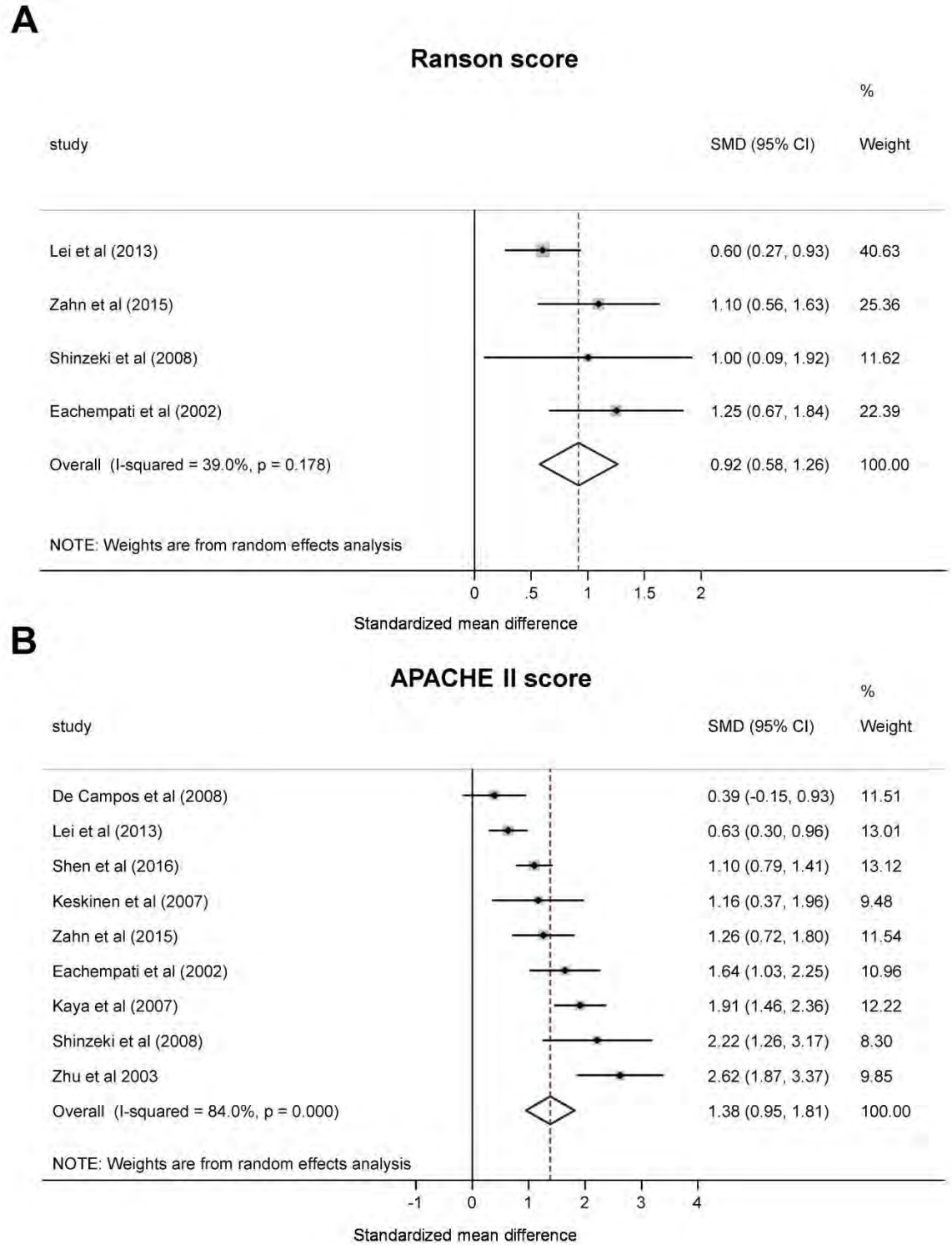

Figure 8. Forest plot of (A) Ranson scores and (B) Acute Physiology and Chronic Health Evaluation (APACHE II) scores using random-effects model in different systemic pH groups of patients with acute pancreatitis. Here and in Figure 9, in each study the standardized mean difference (SMD) of

the outcome was calculated between the patient group with lower and higher pH. Black circles represent the SMD for each study, while the left and right horizontal arms of the circles indicate the corresponding 95% confidence intervals (CI) for the SMD for each study. The size of the gray box is proportional to the sample size of the study; bigger box represents larger sample size, thus bigger relative weight of the study, and vice versa. Circles close to zero represent smaller SMD between the lower and higher pH groups in the given study. A positive SMD means higher score (Figure 8) or longer hospital stay (Figure 9) in the patient group with lower pH compared to the patient group with higher pH. The diamond on the bottom represents the averaged SMD calculated from the SMDs of all the individual studies. The vertical dashed line is determined by the two vertical points of the diamond and indicates the value of the averaged SMD of all studies. The horizontal points of the diamond represent the 95% CI of the averaged SMD (Rumbus, et al., 2018).

IV/1.2.4. Acidosis is associated with longer hospitalization in AP

Next, we analyzed the LOS in patients with AP by using the same grouping of acid-base status as we did for mortality and severity scores. For the meta-analysis, LOS was expressed as SMD between the patient groups. We found that this difference was significant between the more acidotic patient groups and the groups with higher pH or bicarbonate concentrations (0.89, 95% CI, 0.733, 1.043; $P < 0.001$) (Figure 9), which difference corresponds to 15.05 days (95% CI, 10.84, 19.19) longer LOS in the more acidotic AP patient group.

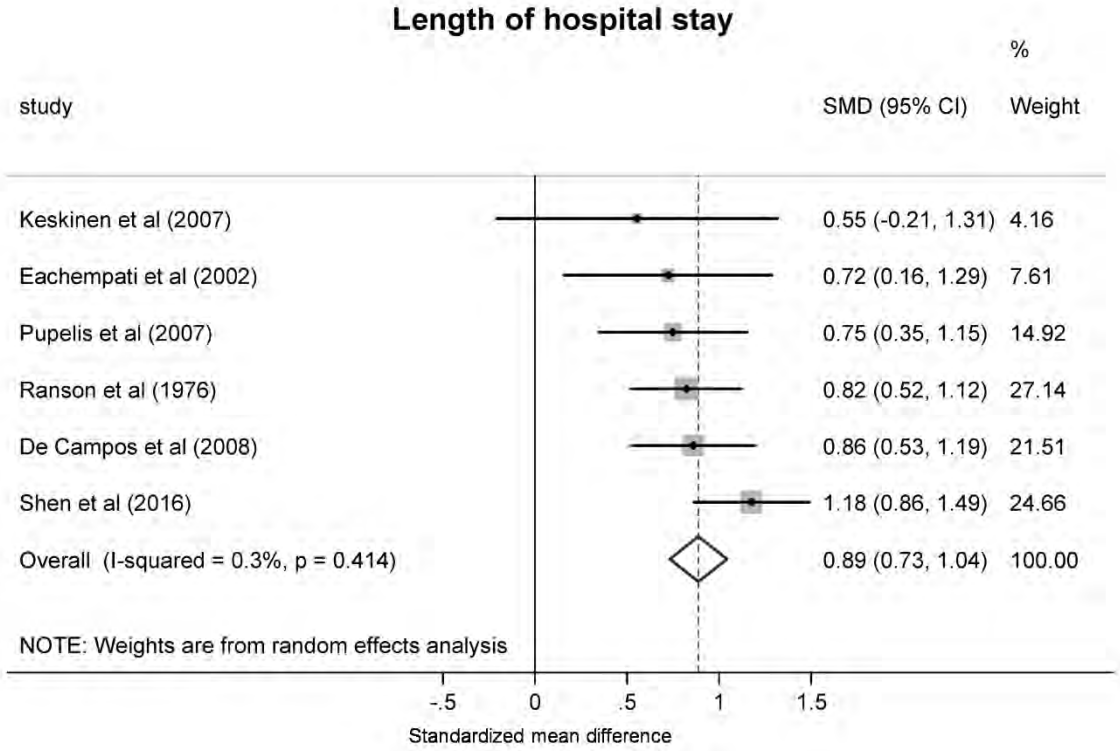

Figure 9. Forest plot analysis of the length of hospital stay using random-effects model in different systemic pH groups of patients with acute pancreatitis. For explanation, see the legend of Figure 8. SMD, standardized mean difference; CI, confidence interval (Rumbus, et al., 2018).

IV/1.2.5. Meta-regression analysis

As a further statistical approach to determine a correlation between blood pH and mortality in the more progressed forms of AP, we also performed a meta-regression analysis on the collected data. For that, we used those study groups, in which pH and mortality rate was reported in moderately severe or severe manifestations of AP for the same patient groups (Lei, et al., 2013; Shen, et al., 2016; Zhu, et al., 2003). We found a significant correlation between pH and mortality rate with a regression slope of -55.4 (95% CI, -97.9, -12.9; P =

0.011) (Figure 10). The potential reason for statistical heterogeneity, as revealed in the forest plots, could not be evaluated in the meta-regression analysis because of the small number of eligible studies.

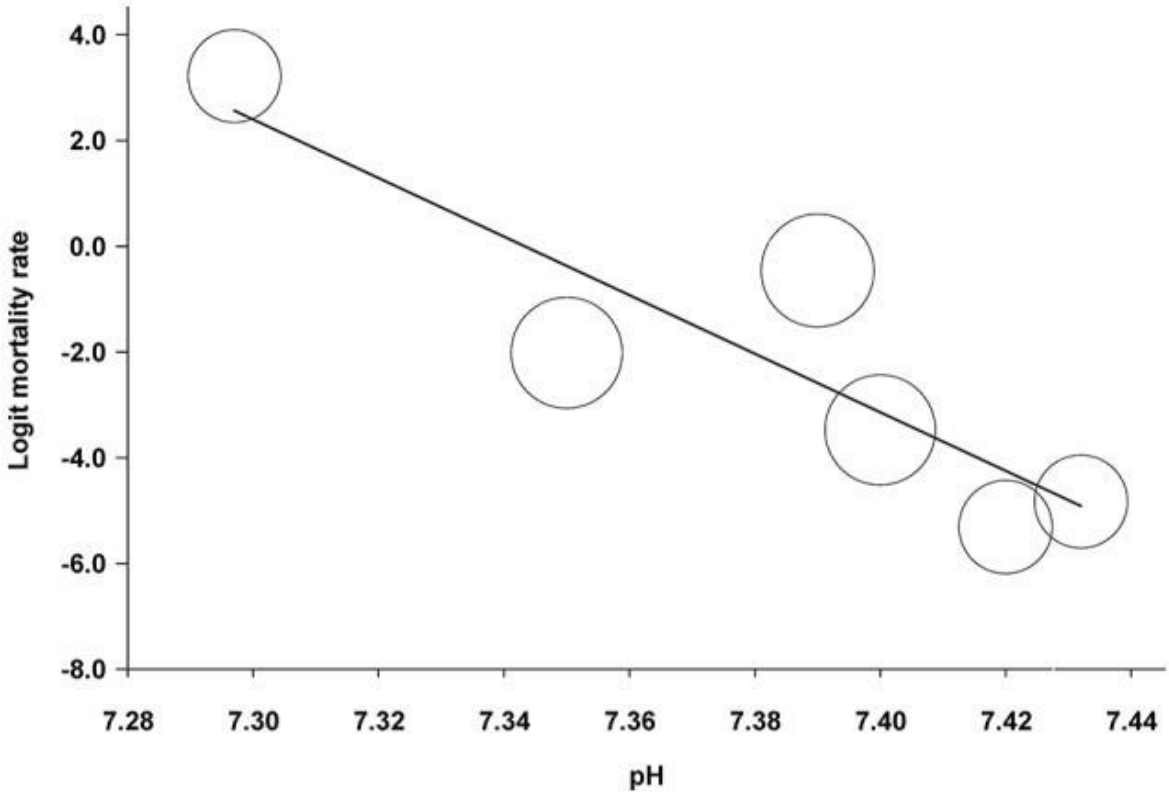

Figure 10. Meta-regression analysis of the association between blood pH and mortality rate in patients with moderately severe and severe forms of acute pancreatitis. The circles indicate estimated logit mortality rate calculated for each patient group. A lower calculated value corresponds with lower mortality rate and vice versa. The circle size is proportional to the precision of the estimated logit mortality rate. The solid black line represents the weighted regression line based on variance-weighted least squares (Rumbus, et al., 2018).

IV/2. The role of TRP channels in pharmacological modulation of body temperature

IV/2.1. Relationship between body temperature and pH: TRPV1 antagonists induced thermal changes. Meta-analysis of human clinical trials

All three antagonists in the mode-nonselective group caused hyperthermia, which was dose-dependent for those compounds that were administered at multiple doses, viz., ABT-102 and V116517 (Figure 11). The highest hyperthermic effect (SDM: 2.5; CI: 1.4-3.5) occurred in the group treated with the 86 μmol dose of ABT-102. The mean thermal effect of all doses of all mode-nonselective TRPV1 antagonists in the analyzed clinical trials was an SDM of 1.2 (CI: 0.9-1.6; $P < 0.001$). NEO6860, the only mode-selective TRPV1 antagonist that we were able to include in our analysis, did not cause hyperthermia at the dose used (1.2 mmol) but, instead, decreased the deep T_b (SDM: -0.7; CI: -0.3 to -1.1; $P < 0.001$).

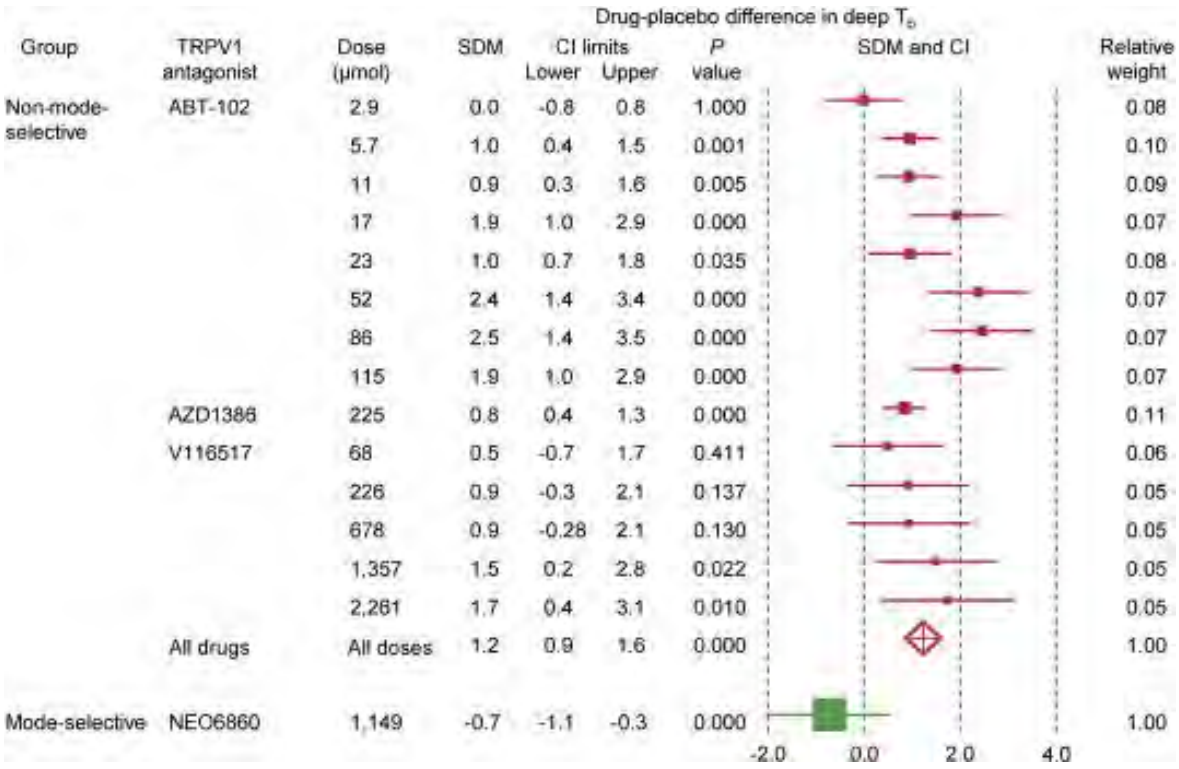

Figure 11. Forest plot of the thermal effects of TRPV1 antagonists. For each antagonist dose, an SDM (a measure of the T_b response) and CI were calculated as described in the text and are shown in the figure with a square and a horizontal bar, respectively. The area of the square is proportional to the sample size and inversed variance. The rhombus refers to the weighted mean SDM for the mode-nonselective group; the vertical diagonal of the rhombus points at the SDM value, whereas the horizontal diagonal represents the CI. Red symbols refer to mode-nonselective TRPV1 antagonists; the green square and bar refer to the selective antagonist NEO6860 (Garami, et al., 2020).

IV/2.2. NH_4Cl-induced hypothermia is attenuated by transient receptor potential channel vanilloid-1, but augmented by ankyrin-1 in rodents

IV/2.2.1. Systemic administration of NH_4Cl causes hypothermia in rats

First, we studied the thermal effect of NH_4Cl administered systemically (i.p.) to rats in order to exclude the possibility that the NH_4Cl-induced hypothermic response is specific only for mice. We found that the i.p. injection of NH_4Cl to the rats caused hypothermia, which was more pronounced at the higher dose (Figure 12). The colonic temperature of the rats started to drop promptly, already at 10 min after the injection of NH_4Cl at both doses. Compared to the baseline, the NH_4Cl-induced maximal mean (± SE) decrease in deep T_b was -0.4 ± 0.1°C at 20 min in case of 220 mg/kg and -0.8 ± 0.2°C at 30 min in case of 280 mg/kg. From that timepoint, the T_b of the NH_4Cl-treated rats gradually increased and reached the T_b level of the control (sterile water-treated) group by the end of the experiment. The T_b of the sterile water-treated rats tended to increase, and then to decrease during the experiment after the i.p. infusion, but it did not differ significantly from the baseline at any timepoints. The initial increase in the T_b could be caused by an unwanted stress response to sterile water infusion despite all of our efforts to minimize the discomfort associated with substance administration, while the gradual fall might have reflected ultradian T_b rhythms in rats maintained at an ambient temperature of ~25°C, which is below the thermoneutral zone. Statistically, both doses of NH_4Cl had a significant effect as compared to controls ($P < 0.001$ for both), and a statistical difference was also present between the lower and the higher dose groups ($P < 0.001$). At 220 mg/kg, the NH_4Cl-induced decrease in deep T_b was significant

compared to the control group between 20-50 min, while at 280 mg/kg, the T_b was significantly lower than in controls between 20-90 min. The development of hypothermia in response to NH_4Cl in rats is a novel finding of our study, especially considering that the effect occurred already at an i.p. dose of 220 mg/kg, which is almost 50% smaller than the threshold dose predicted earlier in mice (Gordon, 1988).

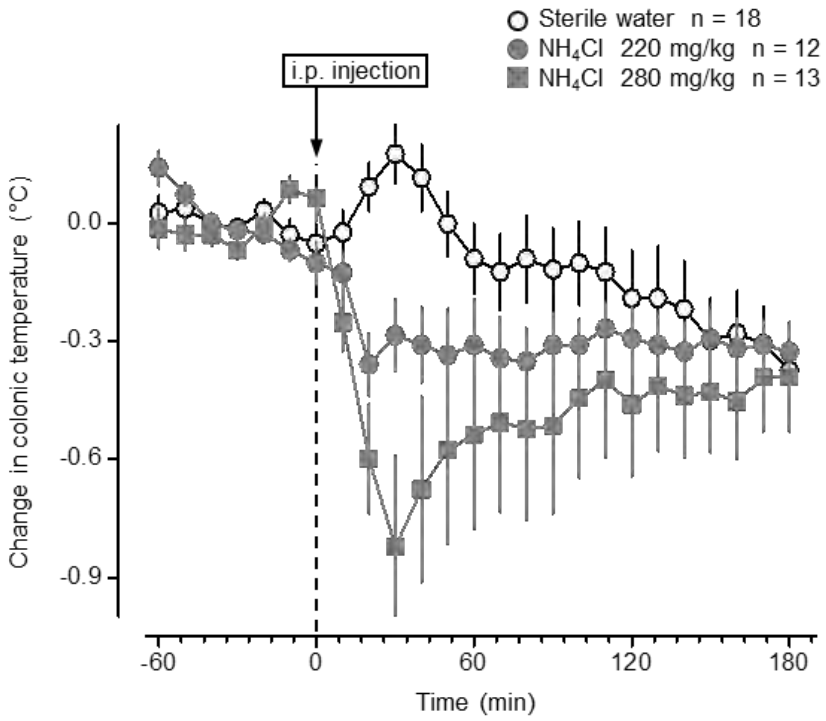

Figure 12. Dose-response curves. Changes in colonic body temperature in response to intraperitoneal (i.p.) administration of sterile water or ammonium chloride (doses indicated) in rats. Here and in Figures 13–17, n is the number of animals in each experimental group (Rumbus, et al., 2024).

IV/2.2.2. NH_4Cl-induced hypothermia is augmented in mice genetically lacking the TRPV1 channel

After we showed that the hypothermic response to NH_4Cl is not specific to mice only, we wanted to know if two of the most studied thermo-TRP channels, TRPV1 or TRPA1, are involved in this thermoregulatory response. In our first approach, we compared the hypothermic response to NH_4Cl between *Trpv1*$^{-/-}$ and *Trpv1*$^{+/+}$ mice. Since the threshold i.p. dose of NH_4Cl to trigger hypothermia in mice was above 300 mg/kg according to Gordon (Gordon, 1988), in our experiments we infused the mice i.p. with 321 mg/kg of NH_4Cl. As expected, at this dose NH_4Cl caused a sudden drop in the colonic temperature of *Trpv1*$^{+/+}$ mice, which reached the biggest mean decrease of -2.1 ± 0.5°C at 30 min post-injection (Figure 13). Compared to the vehicle (sterile water), the deep T_b of NH_4Cl-treated *Trpv1*$^{+/+}$ mice was markedly lower between 20-50 min post-injection ($P < 0.05$). In Trpv1$^{-/-}$ mice, NH_4Cl also caused hypothermia compared to sterile water, which was significant between 20-70 min ($P < 0.05$) with a maximal mean decrease of -4.0 ± 0.4°C ($P < 0.001$). Interestingly, however, the hypothermic response to NH_4Cl was much more pronounced in the *Trpv1*$^{-/-}$ mice than in their *Trpv1*$^{+/+}$ littermates (Figure 13). The intergenotype difference was significant ($P < 0.05$) between 20-60 min post-NH_4Cl administration with a maximum of ~2.0°C difference between the groups at 40 min ($P < 0.001$).

Nevertheless, it should be noted that the NH_4Cl-induced hypothermia developed in both genotypes of the mice (though to a different extent), which suggests that it also involves TRPV1-independent mechanisms.

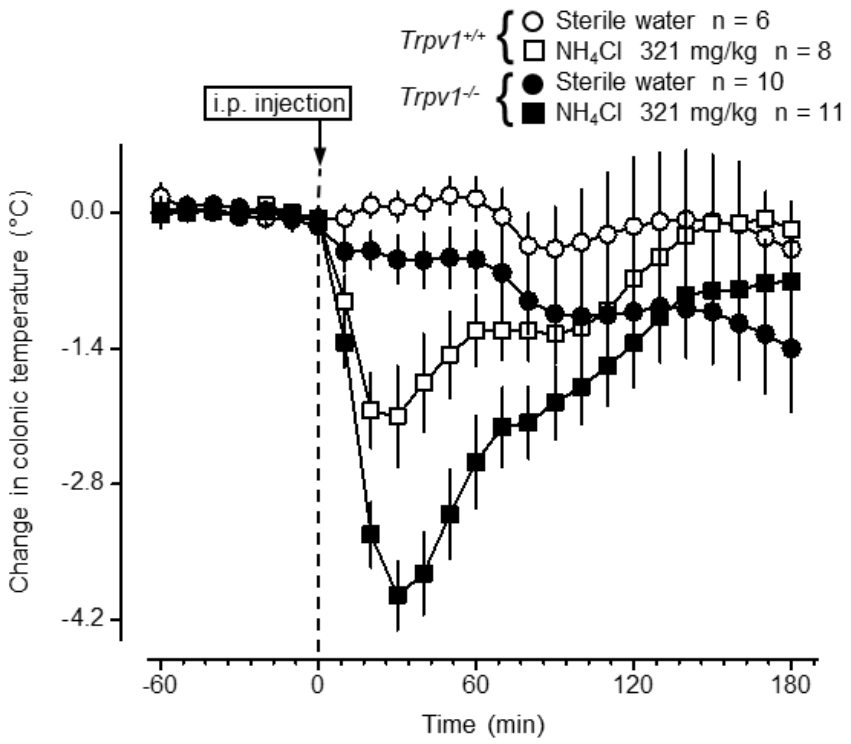

Figure 13. Colonic temperature responses of *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$ mice to i.p. administration of NH_4Cl (dose indicated) or sterile water (Rumbus, et al., 2024).

IV/2.2.3. *Pharmacological blockade of the TRPV1 channel exaggerates NH_4Cl-induced hypothermia in mice and rats*

Our findings in the genetically modified mouse model clearly indicated a bigger hypothermic response in *Trpv1*$^{-/-}$ mice. However, it could not be excluded that the *Trpv1*$^{-/-}$ mice had developed chronic compensatory mechanisms for the absence of TRPV1, which could have influenced the results. To avoid the potential development of chronic compensation, we decided to use genetically unaltered animals (viz., C57BL/6 mice and

Wistar rats) and block their TRPV1 channels acutely by a pharmacological antagonist, AMG 517.

AMG 517 is a highly potent and selective, in itself hyperthermia-inducing TRPV1 antagonist (Gavva, et al., 2008), which has been tested in hypothermic conditions associated with severe systemic inflammation and with general anesthesia (Garami, et al., 2017; Wanner, et al., 2012). Similarly as in our earlier study (Wanner, et al., 2012), in the present experiments the efficacy of AMG 517 could be confirmed by the higher deep T_b of the AMG 517-treated mice compared to vehicle-treated controls in the present experiments before NH_4Cl administration (Figure 14a). As it could be expected based on our results obtained in *Trpv1*$^{+/+}$ mice, in the vehicle-pretreated C57BL/6 mice the hypothermic response to NH_4Cl developed rapidly and it reached the biggest mean decrease of -2.4 ± 0.4°C at 30 min ($P < 0.001$ compared to sterile water) (Figure 14b). In the vehicle-pretreated mice, the NH_4Cl-induced decrease in deep T_b was significant ($P < 0.05$) compared to the sterile water-treated group between 20 and 90 min post-injection. Pretreatment with AMG 517 exaggerated the hypothermic effect of NH_4Cl to a maximal mean T_b decrease of -3.5 ± 0.6°C at 40 min ($P < 0.001$) compared to vehicle pretreatment (Figure 14b). The biggest difference between the mean T_b of the pretreatment groups was 1.6°C at 50 min post-NH_4Cl injection ($P < 0.001$). In response to NH_4Cl, the deep T_b of the AMG 517-pretreated mice was significantly lower than that of the vehicle-pretreated mice between 30 and 70 min ($P < 0.05$).

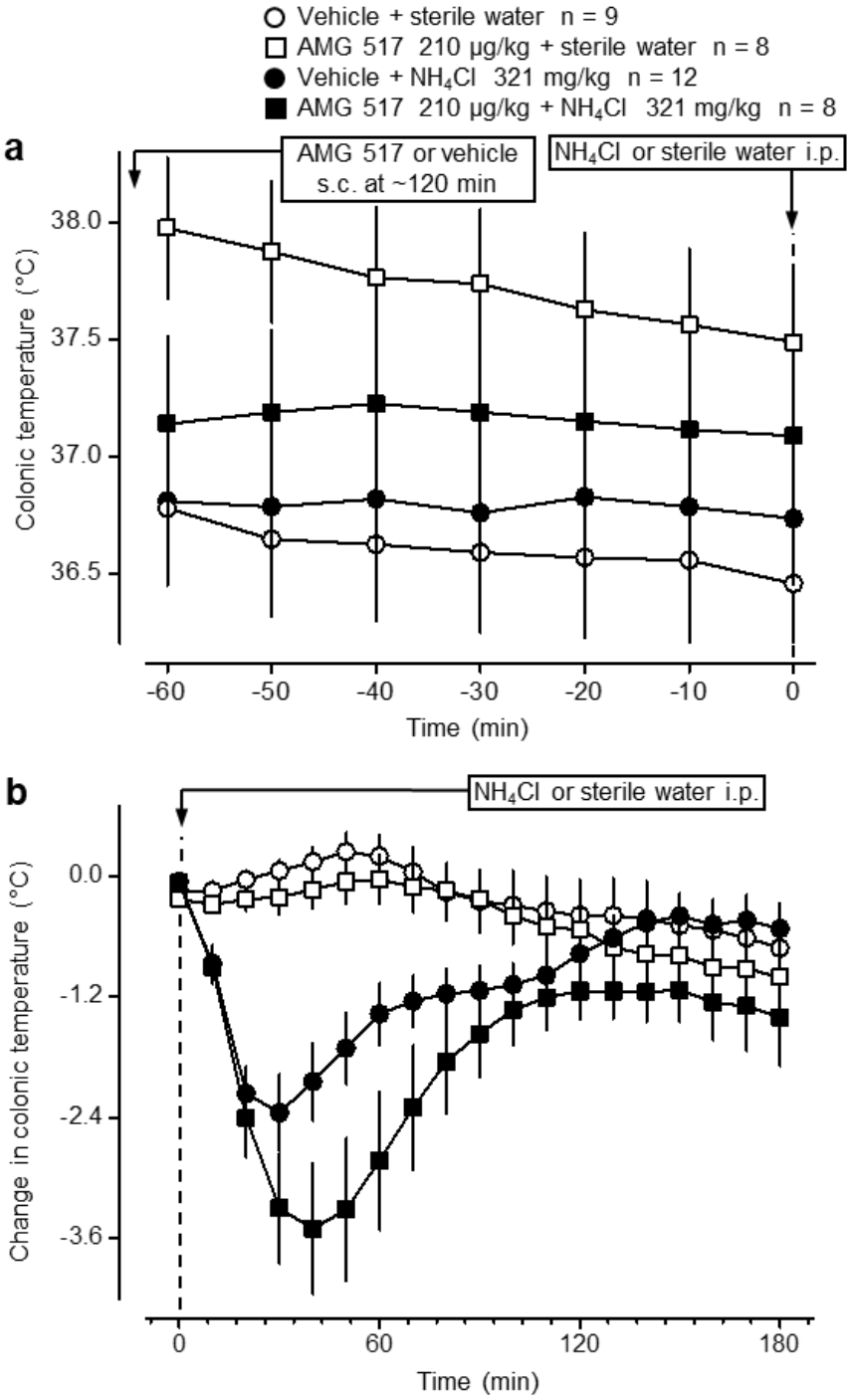

Figure 14. a Colonic temperature in responses to subcutaneous (s.c.) AMG517 (dose indicated) in mice. **b** Colonic temperature responses of mice to i.p. administration of NH_4Cl (dose indicated) or sterile water after pretreatement at -120 minutes with an s.c. administration of AMG517 (dose indicated) or vehicle (Rumbus, et al., 2024).

We also wanted to confirm that the blockade of TRPV1 leads to the augmentation of NH_4Cl-induced hypothermia not only in mice, but also in rats. We administered the same dose (210 μg/kg) of AMG 517 to rats i.v. 20 min before the i.p. injection of NH_4Cl. As expected, AMG 517 caused prompt hyperthermia, which was present also at the time of the i.p NH_4Cl injection (Figure 15a). After the injection of NH_4Cl, the deep T_b of vehicle-pretreated rats started to decrease immediately and reached a mean maximal decrease of -1.2 ± 0.2°C at 30 min ($P < 0.001$) compared to sterile water injection; it was significantly ($P < 0.05$) different between the two i.p. treatment groups (NH_4Cl vs. sterile water) between 20-100 min (Figure 15b). Importantly, in AMG 517-pretreated rats both the magnitude and the duration of the NH_4Cl-induced hypothermia was markedly exaggerated. The maximal mean T_b fall of -1.7 ± 0.2°C developed at 40 min, and the T_b of the NH_4Cl-treated rats remained lower than the T_b of the sterile water-treated rats between 20-130 min ($P < 0.05$). Accordingly, the effect of NH_4Cl on T_b was significantly different between the i.v. pretreatment groups (AMG 517 vs. vehicle) from 40 to 70 and at 110 min post-NH_4Cl injection (Figure 15b).

Similarly to our results with the genetic blockade of TRPV1, the NH_4Cl-induced hypothermia developed both with and without the pharmacological blockade of the TRPV1 channel (though to a different extent), which suggests the involvement of TRPV1-independent mechanisms.

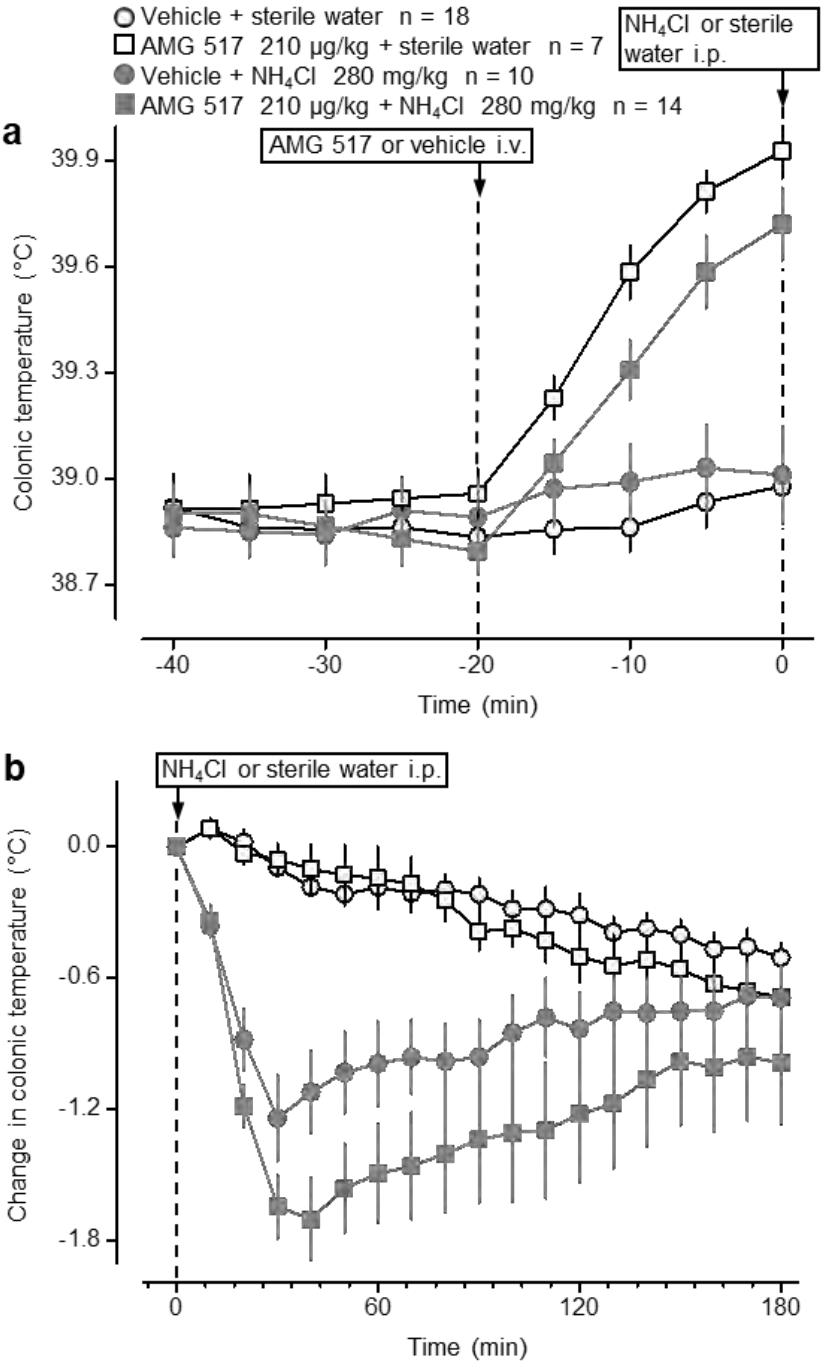

Figure 15. a Colonic temperature in responses to intravenous (i.v.) infusion AMG517 (dose indicated) in rats. **b** Colonic temperature responses of rats to i.p. administration of NH_4Cl (dose indicated) or sterile water after pretreatement at -20 minutes with an i.v. administration of AMG517 (dose indicated) or vehicle (Rumbus, et al., 2024).

IV/2.2.4. The hypothermic response to NH_4Cl is attenuated in the absence of the TRPA1 channel in mice

Because TRPV1 and TRPA1 channels are often co-expressed (Fernandes, Fernandes, & Keeble, 2012; Huang, et al., 2012; Kobayashi, et al., 2005) and crosstalk between them was also reported (Patil, Jeske, & Akopian, 2010; Staruschenko, Jeske, & Akopian, 2010), after discovering the exaggeration of NH_4Cl-induced hypothermia in different models of TRPV1 blockade, we studied whether the TRPA1 channel also plays a role in this thermal response. Similarly as in the case of TRPV1, in our first approach we used mice genetically lacking the channel (*Trpa1*$^{-/-}$) and their WT littermates (*Trpa1*$^{+/+}$). As expected, the injection of NH_4Cl (321 mg/kg, i.p.) caused marked hypothermia in *Trpa1*$^{+/+}$ mice as compared to sterile water (Figure 16). In *Trpa1*$^{+/+}$ mice, the biggest NH_4Cl-induced drop in T_b (-2.8 ± 0.3C°) developed 30 min after the injection ($P < 0.001$), and the T_b of NH_4Cl-tretaed mice was significantly lower than the T_b of the sterile-water-treated group from 20 to 110 min post-injection ($P < 0.05$). In the *Trpa1*$^{-/-}$ mice, however, the hypothermic response to NH_4Cl was greatly attenuated: the biggest mean T_b decrease was -2.5 ± 0.2°C at 20 min, and it was significantly ($P < 0.05$) lower than that of sterile water-treated mice only between 20 and 50 min (Figure 16). With regards to intergenotype difference, *Trpa1*$^{+/+}$ mice had significantly lower deep T_b than *Trpa1*$^{-/-}$ mice between 40 and 100 min after the administration of NH_4Cl with a maximal mean T_b difference of 1.0°C at 70 min ($P = 0.008$).

It should be noted that although the NH_4Cl-induced hypothermia was markedly attenuated in the genetic absence of the TRPA1 channel, it was still present in the KO mice suggesting the contribution of TRPA1-independent mechanisms to the response.

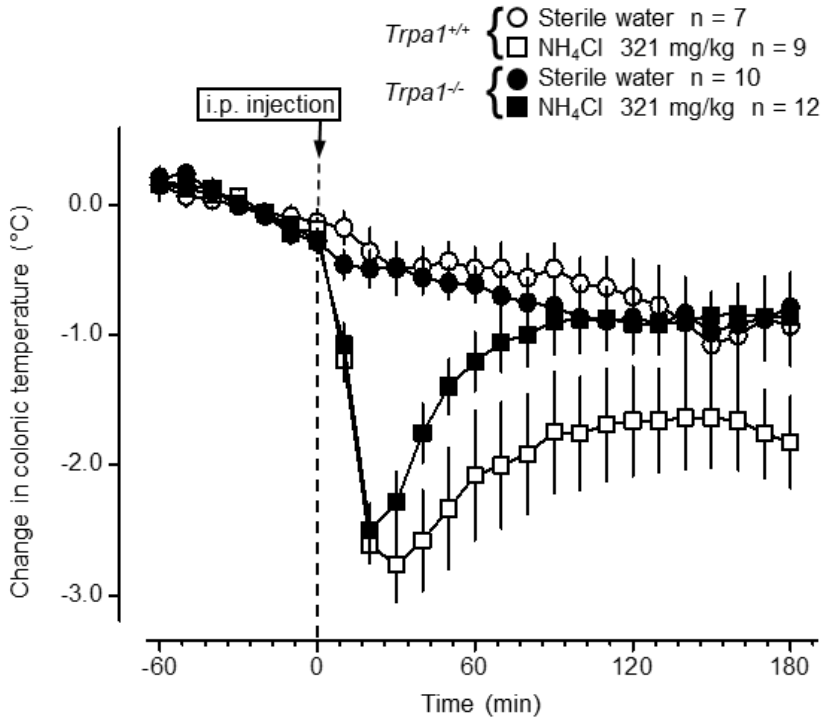

Figure 16. Colonic temperature responses of *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice to i.p. administration of NH_4Cl (dose indicated) or sterile water (Rumbus, et al., 2024).

IV/2.2.5. The hypothermic response to NH_4Cl is attenuated by the pharmacological blockade of the TRPA1 channel in rats

Similarly as in the case of TRPV1, it was important to exclude the potential presence of chronic compensatory mechanisms that may have developed in the absence of the TRPA1 channel in the *Trpa1*$^{-/-}$ mice. For that, we used pharmacological blockade of the channel with

a highly potent and selective TRPA1 antagonist, A967079 (Chen, et al., 2011). However, we could not use the mouse experimental model that was applied in case of AMG 517, because the half-life of A967079 is relatively short, ca. 49 min (Chen, et al., 2011), thus the efficacy of the drug could have been questionable 2 hours after its s.c. administration, when NH_4Cl could be injected to mice (for details, see Table 1). In addition, it was of crucial importance to test the contribution of the TRPA1 channel in another species than mice, because interspecies differences were demonstrated in the temperature-sensitivity of the channel as well as in its response to pharmacological agents (for review, see J. Chen & Hackos, 2015). Therefore, we studied the effects of i.v. administered A967079 on NH_4Cl-induced hypothermia in rats with a similar experimental design as in the case of AMG 517 (for details, Table 1). In accordance with previous studies (Chen, et al., 2011; de Oliveira, et al., 2014), A967079 did not have any meaningful effect on the colonic temperature of the rats before the i.p injection of NH_4Cl or sterile water (Figure 17). On the contrary, when the same dose (5 mg/kg) of A967079 was infused 20 min before the i.p. injection of NH_4Cl, it markedly attenuated the hypothermic response. While the T_b of NH_4Cl-treated rats was significantly ($p < 0.05$) lower at 20-150 min compared to sterile water injection after pretreatment with vehicle, in the A967079-pretreated rats the NH_4Cl-induced hypothermia was significant only between 10-90 min (Figure 17). In accordance, a statistically significant difference was also present between the two pretreatments groups in the NH_4Cl-treated rats between 40-120 min with a maximal difference of 0.7°C at 50 min ($P = 0.003$).

In line with our results with the genetic blockade of TRPA1, the NH_4Cl-induced hypothermia was substantially attenuated, but still present after the pharmacological

inhibition of the TRPA1 channel suggesting the contribution of TRPA1-independent mediators to the response.

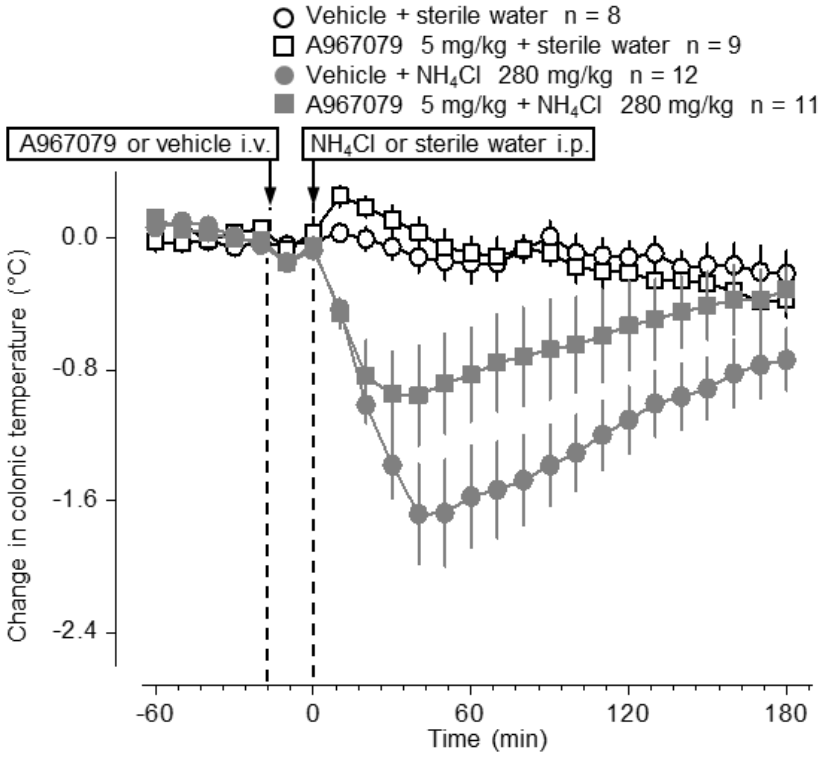

Figure 17. Colonic temperature responses of rats to i.p. administration of NH_4Cl (dose indicated) or sterile water after pretreatement at -20 minutes with an i.v. administration of A967079 (dose indicated) or vehicle (Rumbus, et al., 2024).

IV/2.2.6. I.p. administration of NH_4Cl decreases the blood pH in rats and mice

Last, we wanted to know how the applied doses of NH_4Cl affected the blood pH of the rats and the mice. In rats, the blood pH after i.p. administration of NH_4Cl was decreased at both doses (to 7.47 and 7.24) compared to sterile water treatment (pH = 7.51), however, the difference was significant only at the higher (280 mg/kg) dose ($P < 0.001$) (Figure 18).

The blood pH was also statistically different between two doses of NH_4Cl ($P< 0.001$). In mice, the i.p. injection of NH_4Cl (321 mg/kg) resulted in substantial drop in blood pH in all genotypes compared to sterile water injection ($P < 0.001$) (Figure 18). The fall in the blood pH of the mice reached a similar extent in all genotypes ranging between 7.23-7.30 after the i.p. injection of NH_4Cl.

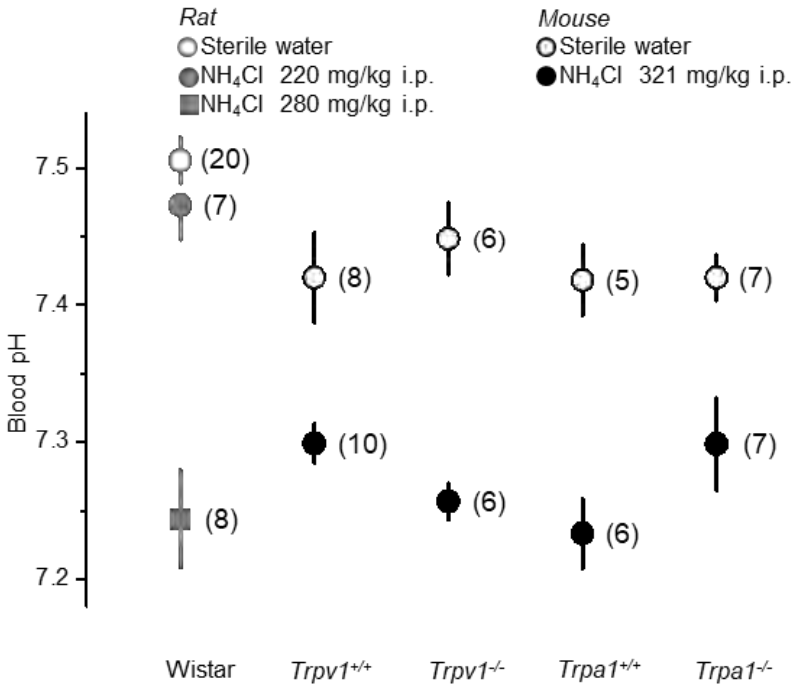

Figure 18. Blood pH of rats and *Trpv1*$^{+/+}$, *Trpa1*$^{+/+}$, *Trpv1*$^{-/-}$ and *Trpa1*$^{-/-}$ mice in response to i.p. administration of sterile water or NH_4Cl (doses indicated; numbers of animals in parentheses) (Rumbus, et al., 2024).

V. Discussion

In the present thesis, we investigated the predictive role of vital parameters (T_b and blood pH) for the outcomes of two different manifestations of systemic inflammation and the role of TRP channels in pharmacological modulation of T_b. First, we revealed a clear association between T_b and mortality in septic patients by using a detailed statistical approach which was based on an extensive literature search of previous human studies. Second, a strong association between blood pH and the outcome of AP was found with meta-analysis of human studies. Then, our meta-analysis of human-trial data has confirmed that the first-generation TRPV1 antagonists cause hyperthermia in humans, whereas the second-generation compounds may lack this effect. Finally, we showed that the i.p. administration of NH_4Cl decreases deep T_b in rats and mice, and genetic and pharmacological blockade of TRPV1 and TRPA1 channels modulates the hypothermic effect of NH_4Cl. Nevertheless, this hypothermic response occurred (to a different extent) in all of our experimental models, indicating that per se neither TRPA1 nor TRPV1 is essential for NH_4Cl-induced hypothermia.

In the first part of our work, we found that fever reduces, while hypothermia promotes mortality in septic patients as compared to normothermic subjects. From previous studies of septic patients only limited information was available to show an association between T_b and mortality in a wide temperature range. While a worse outcome was consistently found to be related to hypothermia (Arons, et al., 1999; Clemmer, et al., 1992; Drewry, et al., 2015; Kushimoto, et al., 2013), those studies compared hypothermia with

fever (Arons, et al., 1999; Clemmer, et al., 1992) or with nonhypothermic (i.e., by merging febrile and normothermic) patients (Drewry, et al., 2015; Kushimoto, et al., 2013). In one study, no association was found between hypothermia and the clinical outcome of sepsis (Megged, et al., 2006). Regarding the role of fever, different clinical trials came to controversial results in sepsis. Several authors showed that a higher T_b was beneficial (Bryant, et al., 1971; Mackowiak, Browne, Southern, & Smith, 1980; Megged, et al., 2006; Weinstein, et al., 1983), others that it was disadvantageous (Manthous, et al., 1995; Schortgen, et al., 2012), and a few found no association between fever and mortality (Kushimoto, et al., 2013; Young, et al., 2015). The discrepancy among the studies may result from the limited sample size used in the trials. Another explanation for not detecting significant association could be the insensitivity of the used statistical method. Indeed, when we first performed a commonly used regression analysis, the Pearson correlation, which did not allow us to weight the collected data for example for sample size, we found only a very weak negative correlation ($P = 0.047$) between T_b and mortality ratio in sepsis. Thus, we applied more precise statistical tools (forest plot and meta-regression analyses).

In our analyses, we used a sizeable, heterogeneous population of septic patients (n = 10,834) with a wide range of T_b (33.0–39.9°C). We showed that in sepsis, mortality rates are lower if fever is present and higher in cases of hypothermia as compared to the normothermic group. In addition, we demonstrated a strong negative correlation ($P = 0.0004$) between T_b and mortality ratio with the help of meta-regression analysis. In our statistical approach, we also included a substantial number of septic patient groups with average T_bs within the normal range (n = 3,904). Furthermore, when we calculated the mean T_bs of septic patients

in the mortality quartiles, we found that it gradually decreased from the lowest to the highest quartile and it was significantly higher in the lowest (0–25%) than in the highest quartile (75–100%) of mortality (38.1 ± 0.1 vs 37.1 ± 0.2°C for mean ± standard error of the mean, respectively). Taken together the results from all of our statistical approaches, our data strongly suggest a predictive role of T_b for the outcome of sepsis.

Sepsis continues to constitute a major challenge in critical care medicine (Esteban, et al., 2007; Fleischmann, et al., 2016; Vincent, et al., 2014; Walkey, et al., 2015). As a form of systemic inflammation, sepsis is frequently accompanied by abnormalities of T_b, like fever and hypothermia. In animal experiments, lower doses of endotoxin usually cause fever, whilst higher doses lead to the development of hypothermia (Pakai, Garami, Nucci, Ivanov, & Romanovsky, 2015; Rudaya, Steiner, Robbins, Dragic, & Romanovsky, 2005), indicating that the severity of the disease determines the change in T_b and not the way around. Based on our statistical analyses of human studies, fever seems beneficial, but hypothermia rather disadvantageous for the organism regarding the outcome. However, it has to be noted that the current analysis does not allow us to conclude that the change of T_b per se is responsible for the lower and higher mortality rates in fever and hypothermia, respectively. Instead of a cause-effect relationship, the abnormal T_b should be rather regarded as a prognostic vital parameter of the severity and progress of the inflammation, and as such, as a warning sign, which can help clinicians to assess the outcome of to the infection. However, this association does not automatically imply that fever is always beneficial and hypothermia is harmful in sepsis (Table 2) (Rumbus & Garami, 2019). The causative relationship between the

thermoregulatory manifestations and the outcome in systemic inflammation could not be assessed in our study and it deserves discussion.

Table 2. Disease coping strategies in different premorbid conditions, thermoregulatory manifestations, and potential outcomes in different severities of sepsis (Rumbus & Garami, 2019).

Infection severity	Premorbid condition	Copingstrategy	Deep body temperature	Predicted outcome	Effect on mortality
Mild	Healthy	Disease fighting	Fever	Pathogen clearance	Not applicable
Moderate	Healthy	Disease fighting	Fever	Pathogen clearance	↓
			Extreme fever	Organ failure, energy depletion	↑
	Comorbidities* or exhaustion†	Disease fighting	Fever	Organ failure, energy depletion	↑
		Energy saving	Hypothermia	Disease tolerance††	↓
Severe (e.g., septic shock)	Healthy	Energy saving	Hypothermia	Disease tolerance	↓
	Comorbidities or exhaustion		Extreme hypothermia	Organ failure	↑

Effects on mortality are marked as: ↓, decrease; ↑, increase. *E.g., pre-existing cardiovascular, pulmonary, neurological disease. †E.g., because of old age, starvation, prolonged systemic inflammation. ††The host's ability to tolerate the presence of the pathogen (for details, see Garami, Steiner, & Romanovsky, 2018).

With regards to the adaptive biological value of T_b alterations in mammals, the development of fever in systemic inflammation is considered to indicate the activation of defense mechanisms of the body to fight the intruding agent (Romanovsky, et al., 2005; Romanovsky & Szekely, 1998; Saper, et al., 2012). By enhancing immune functions and accelerating the elimination of the microorganism from the body, at the onset of the

inflammation fever is an adaptive, beneficial thermoregulatory response, although it involves a higher energy cost (Romanovsky, et al., 2005; Romanovsky & Szekely, 1998; Saper, et al., 2012). Therefore, fever itself is assumed to have a direct, advantageous effect on the mortality ratio in systemic inflammation (e.g., sepsis), when it is affordable for the host. However, T_b regulation should be considered in the framework of complex energy balance (Garami A, 2014), therefore, the beneficial value of fever as an energetically expensive defense response is doubtable when there is a risk of energy deficiency, which often develops as the severity of the disease further progresses. In support of that, the administration of antipyretics resulted in an increase of mortality rate of critically ill patients in prospective clinical trials (Lee, et al., 2012; Schulman, 2005). However, in severe sepsis or septic shock, the use of pharmacological antipyretics did not influence mortality (Bernard, et al., 1997; Mohr, et al., 2012), while fever control with external cooling decreased early mortality in human studies (Schortgen, et al., 2012).

Spontaneous hypothermia represents a distinct, adaptive mechanism to systemic inflammation in experimental animals (Liu, et al., 2012) and in septic patients (Fonseca, et al., 2016). It characteristically develops in severe cases of already progressed diseases, when—instead of actively coping with the microorganism—the organism attempts to increase survival by saving its energy resources (Romanovsky, et al., 2005; Romanovsky & Szekely, 1998). A recent study by Fonseca et al. (Fonseca, et al., 2016) revealed that spontaneous hypothermia is a transient, self-limiting, and nonterminal event in human sepsis, which underlies its biological value as an adaptive mechanism in the critically ill patients. According to this study, hypothermia can occur not only when systemic

inflammation becomes more severe. In the studies of our meta-analysis constantly monitored T_b values were not reported, thus the effect of spontaneous nonterminal hypothermia itself on mortality rate could not be analyzed. On the other hand, the effect of a febrile episode before terminal hypothermia could not be analyzed for similar reasons. However, regarding mortality, a modulating effect of initial fever cannot be ruled out.

Although the results of our analysis showed that hypothermia is associated with higher mortality, it should be noted that we can not be sure how mortality ratio of the patients would have changed if hypothermia had not developed or if the patients were rewarmed. As of today, to our knowledge, the effect of rewarming vs non-rewarming on the mortality of septic patients with spontaneous hypothermia has not been compared in randomized controlled trials. Therefore, hypothermia in itself should not be regarded harmful for the body as the associated higher mortality rate of the septic patients is presumably due to their more severe clinical condition. We suggest that the difference between the mortality rates of febrile and hypothermic patients with sepsis is due to the different severity and progression of the inflammation and not due to T_b itself. As a consequence, T_b itself serves not as a detrimental factor, but instead, as an indicative predictor for the severity of the disease and as such for mortality in sepsis.

From a clinical perspective, our results highlight the importance of precise and regular measurements of deep T_b, since its abnormalities can help physicians - especially in critical care medicine - not only in the diagnosis, but also in the follow up of the progression, and in the prognosis of sepsis. Based on our findings, it would be worth to consider that hypothermia should be weighted differently than fever and not equally as currently used in

many scoring systems (e.g., SIRS, APACHE), since hypothermia indicates a more severe stage of sepsis, and therefore it is associated with worse clinical outcome. Regarding therapeutic interventions, the T_b management of septic patients should be always carefully evaluated and perhaps guidelines could be established (e.g., for the initiation of antipyretic treatment) to improve the clinical outcome in sepsis.

Next, investigating another important vital parameter, the blood pH, our analyses showed that lower blood pH predicts higher mortality rate, longer LOS, and worsens the severity of AP. A significant negative correlation between blood pH and mortality rate in severe forms of AP was found with meta-regression analysis of human studies.

Although previous human studies indicated a link between MA and AP (Nair, et al., 2000; Sharma, et al., 2014; Shen, et al., 2016; Shinzeki, et al., 2008; Zhu, et al., 2003), we found only one, single-center prospective study which directly aimed to explore this correlation (Sharma, et al., 2014). Because of the scarcity of data available from targeted clinical trials, we aimed to clarify the association between MA and AP by systematic review of the current literature and by meta-analysis of the available data. By identifying 13 eligible studies for the analysis (Eachempati, et al., 2002; Kaya, et al., 2007; Keskinen, et al., 2007; Lei, et al., 2013; Nair, et al., 2000; Pupelis, et al., 2007; Ranson, et al., 1976; Sharma, et al., 2014; Shen, et al., 2016; Shinzeki, et al., 2008; Zhan, et al., 2015; Zhu, et al., 2003), we included 2,311 patients with AP in the analyses. In all of these studies, blood sample analysis was performed at admission or within 24 h thereafter, hence the blood pH parameters were determined with practically the same latency compared to the time when AP was diagnosed. Unavoidably however, the disease could progress to different stages in the different patients

before the diagnosis has been reached. There were huge differences between the protocols of the individual studies, but it is remarkable that no matter how the patients were grouped by the authors originally, the patient group with lower pH had always (with no exceptions) worse outcomes (mortality rate, LOS, severity scores) than the group with higher pH in AP, which suggests that in the early stages (viz., until the time of diagnosis) of AP acidosis is an important influencing factor of the outcome regardless from the actual progression of the disease. Unfortunately, the design of the studies did not allow to analyze the causative relationship between MA and AP. In most of the studies, the systemic pH status of the patients prior to or repeatedly after the diagnosis of AP was not reported, thus the dynamics in the changes of pH during the time course of AP could not be assessed in the current analysis, but it is notable that the average base deficit was markedly (4-8 fold) higher in populations of patients, who did not survive severe AP (Kaya, et al., 2007; Keskinen, et al., 2007). In the prospective trial by Sharma et al. (Sharma, et al., 2014), in those severe AP patients, who had a blood pH of less than 7.35, the mortality rate was nearly 10 times higher than in those patients whose pH was above this level (54 vs. 6.5%).

As limitations of our study, it should be mentioned that even though our meta-analysis showed a clear association between blood pH and the outcome of AP, since originally the patients were not divided into subgroups based on their blood pH by the authors, the independent effect of lower blood pH on the outcome and the cause-effect relationship between MA and AP could not be assessed. Because of the same reason and also to reduce the inter-study heterogeneity, in each of the analyzed studies we assigned one patient group as the lower pH group and the other one as the higher pH group. Since the

reported pH values differed substantially among the analyzed studies, the cut-off value between the lower and the higher pH groups was individually determined for each study. Consequently, in the present analysis we could not determine a specific cut-off pH value which would be detrimental for the outcome of AP. The most convincing method to obtain direct evidence for the role of acidosis as an independent risk factor in AP, determine a detrimental cut-off pH value, and gain insight into the cause-effect relationship in humans would be to conduct targeted clinical trials in which patients with AP are grouped based on their blood pH at admission and their acid-base status as well as the severity and the outcome of AP is continuously monitored. By collecting data of individual patients in such clinical trials, it would be possible to statistically analyze the direct (independent) effect of acid-base disturbances on AP. Until such or similar trials are conducted, we are restricted to use different (not so direct) approaches such as meta-analyses and animal experiments.

Secondly, we studied the role of TRP channels in the pharmacological modulation of T_b by conducting meta-analysis of clinical trials and animal experiments (Garami, et al., 2020; Rumbus, et al., 2024). This was important because targeted modulation of T_b may improve the outcomes of systemic inflammation.

Our meta-analysis of the human-trial data has confirmed that the first-generation TRPV1 antagonists cause hyperthermia in humans, whereas the second-generation compounds may lack this effect. In humans TRPV1 channels may modulate T_b via pH and heat signals. The location of TRPV1 channels that sense T_bs (whether shell or core) to drive thermoeffector responses in humans is unknown and can be different from the location of the channels that are tonically activated by protons. Knowing that the skin plays a prominent

thermosensory role in all species, at least some TRPV1 channels that mediate thermal signals to drive thermoeffectors in humans can be speculated to be located in the skin.

Finally, in animal experiments, we revealed that the genetic and pharmacological blockade of the TRPV1 channel exaggerates the hypothermic effect of NH_4Cl. On the contrary, the hypothermic response to NH_4Cl is attenuated by the genetic ablation and pharmacological inhibition of the TRPA1 channel. These findings suggest that TRPV1 channels are limiting regulators, whereas TRPA1 channels are potentiating signaling molecules of NH_4Cl-induced hypothermia.

The hypothermic effect of i.p. administered NH_4Cl has been shown in mice long time ago (Gordon, 1988). However, it has remained unknown whether it also develops in other species and which receptors are involved in the NH_4Cl-induced hypothermic response. NH_4Cl administration is often used to induce MA in rodents (Celotto, et al., 2016; Galicek, et al., 1981; Nowik, et al., 2010; Rothe & Schimek, 1984; Rumbus, et al., 2018). The mechanism of NH_4Cl-induced MA is consumption of bicarbonate during conversion of ammonia to urea nitrogen via the urea cycle (Matsumoto, et al., 2019). In severe cases of the NH_4Cl-induced MA, a fall in intracellular pH also developed (Tizianello, et al., 1977), although the intra- and extracellular pH levels did not always correlate in milder cases of acidosis (extracellular pH drop of 0.11-0.19) (Rothe & Schimek, 1984; Tizianello, et al., 1977). The different effects on pH can originate from the used species (rat vs. mouse), experimental model (in vitro vs. in vivo), and acid loading protocol (acute vs. chronic). In our experiments, NH_4Cl decreased the blood pH to ~7.25 in rats and mice, which was

markedly lower than in the control animals, however, in this in vivo experimental design the intracellular pH could not be measured.

The decreased blood pH could potentially serve as a direct mechanism for the development of the hypothermia through the stimulation of the TRPV1-mediated acido-antithermogenic and acido-antivasoconstrictor reflexes that originate from trunk muscles and limit the increase in deep T_b during physical exercise (for review, see Garami, et al., 2020). However, since the NH_4Cl-induced hypothermia was still detectable after genetic and pharmacological blockade of both the TRPV1 and the TRPA1 channels, it cannot be excluded that the hypothermic response to the decreased blood pH involved TRPV1- and TRPA1-independent mechanisms. Several acid-sensitive ion channels, other than TRPV1 and TRPA1, are expressed on primary sensory neurons, which are involved in a number of physiological and pathophysiological reactions to acidosis (for review, see Holzer, 2009). Whether any of these acid sensors contribute to the development of NH_4Cl-induced hypothermia remains subject for future studies. It is also possible, however, that the hypothermic effect of NH_4Cl was triggered by mechanisms that are not related solely to the decreased blood pH, because the lower dose of NH_4Cl caused hypothermia in rats, but it did not influence the blood pH significantly, which argues against a direct acid-induced effect. Unfortunately, the pH of the mice was not reported in the study by Gordon (Gordon, 1988). It should be also noted that although the acidosis clearly developed in the rats (at the higher dose) as well as in the mice of all genotypes in our experiments, the extent of decrease in blood pH was not very severe. The drop in blood pH was probably not sufficient to reach the proton activation threshold of the TRPV1 channel, because the half-maximal response

of the rat TRPV1 channel to protons occurs at a pH of ~5.78 in vitro (McIntyre, et al., 2001). However, the elevated proton concentrations could potentiate responses evoked by other agonists, resulting in increased activity of the channel (Tominaga, et al., 1998). With regards to the TRPA1 channel the pH activation is complex. It was demonstrated that weak acids activate rodent TRPA1, but this was due to intracellular acidosis and direct proton activation of the channel from the cytosolic side (Wang, et al., 2011; Wang, Chang, & Liman, 2010). Although protons can rapidly permeate through the membrane to induce an intracellular acidosis when the extracellular H^+ concentration is increased (Andersson, Chase, & Bevan, 2004), rodent TRPA1 failed to respond to extracellular acidosis, and protons even inhibited the channel in a later study (de la Roche, et al., 2013). Nevertheless, exclusively in humans, TRPA1 is an important (nociceptive) proton sensor (de la Roche, et al., 2013), which complicates the translation of results from animal experiments to humans. Moreover, in case of NH_4Cl administration the intracellular pH level does not always correlate with the extracellular pH and an increased pH within the cell was also described instead of acidosis (for review, see Roos & Boron, 1981). Ammonia and intracellular alkalization were shown to activate both the TRPV1 and the TRPA1 channels (Dhaka, et al., 2009; Fujita, et al., 2008). Hence, the rapid inward diffusion of gaseous ammonia and the resulting intracellular alkalization could have also contributed to our results. It is known that systemic (i.v.) administration of NH_4Cl leads to formation of ammonia, which can readily cross the blood-brain barrier (Rapoport & Thompson, 1974). This raises the possibility that activation of TRPA1 channels in thermoregulatory neurons in the brain triggered the hypothermic response, as shown earlier in case of another gasotransmitter, hydrogen sulfide (Olah, et al.,

2021), but the investigation of the exact molecular interaction between NH_4Cl and TRP channels, as well as finding the site of the thermoregulatory action of NH_4Cl were beyond the scope of the present study and remain subjects for future research.

The exaggeration of NH_4Cl-induced hypothermia by the blockade of TRPV1 channels was an unexpected novel finding. We can only speculate that NH_4^+ ions could inhibit TRPV1 channels located in the abdominal wall, which were shown to tonically suppress skin vasoconstriction and thermogenesis (Garami, et al., 2023). When these TRPV1 channels are blocked with genetic or pharmacological tools the hypothermia-counteracting effect is absent, hence the thermal response to NH_4Cl becomes augmented. In support of this assumption, it was found that different quaternary ammonium ions blocked the TRPV1 channel from the intracellular surface with the bigger molecules becoming slower blockers (Jara-Oseguera, Llorente, Rosenbaum, & Islas, 2008). As an alternative theory, we showed earlier that acidosis-induced vasodilation of rat and mouse tail arteries are limited by non-neuronal TRPV1 channels in the vascular wall (Ivic, et al., 2016), thus it can be assumed that the blockade of these vasodilation-limiting TRPV1 channels could result in higher heat loss and exaggerated hypothermic response to NH_4Cl. It has to be noted, however, that the abovementioned action mechanisms of NH_4Cl on TRPV1 channels remain hypothetical until directly tested in future experiments. As another limitation of our study, it should be mentioned that we used only male animals in our experiments, hence sex differences in the thermal response to NH_4Cl could not be investigated. Nevertheless, our results can also serve as an encouraging basis for designing future studies, which are required to determine the

potential presence of sex differences in the development of NH_4Cl-induced hypothermia and its relation to TRPA1 and TRPV1 channels.

VI. Conclusions

In the present work, we investigated the predictive role of two vital parameters, the T_b and the blood pH, in an infectious (sepsis) and mainly noninfectious (AP) manifestation of systemic inflammation. The deviations of deep T_b are strongly associated with mortality in sepsis. We revealed a strong negative correlation between the mortality ratio and deep T_b. In our meta-analysis, we included data from a total of 10,834 septic patients in a wide temperature range (between 33.0 and 39.9°C) thus the sample size and temperature range can be considered large enough to draw conclusions about the association of T_b and mortality rate in sepsis. Furthermore, patients with the lowest mortality quartile have significantly higher deep T_b than those who belong to the highest mortality quartile. Similar to the T_b, the correlation between blood pH and mortality showed significant negative correlation in AP. We also found that lower systemic pH predicts longer LOS, and worsens the severity of AP. Our findings suggest that systemic pH level should be closely monitored in patients with AP and normalization of the low pH of patients with AP should be considered in clinical settings.

In the second part of my work, we highlighted the importance of the nonthermal activation of TRPV1 and TRPA1 channels in thermoregulation with different methodological designs in humans and rodents. Our meta-analysis of human trials showed that the first-generation TRPV1 antagonists cause hyperthermia in humans, whereas the second-generation compounds may lack this effect. In our animal experiments we found that (i) i.p. administration of NH_4Cl induces hypothermia in rats as well as in mice; (ii) TRPA1 channels contribute to the development of NH_4Cl-induced hypothermia in both mice and

rats; (iii) TRPV1 channels play a limiting function in this process. It can be hypothesized that activation of TRPA1 channels in thermoregulation-related brain nuclei are responsible for the induction of NH_4Cl-induced hypothermia, while TRPV1 channels on the periphery, possibly in abdominal muscles or vascular smooth muscle exert a limiting effect on the response although in itself neither of them is essential for the occurrence of the response. Our findings highlight the importance of the nonthermal activation of TRPV1 and TRPA1 channels in thermoregulation. Since NH_4Cl is still used in clinical practice (e.g., as a diuretic, expectorant, and perhaps COVID-19 treatment), the present results warrant for regular T_b monitoring and careful consideration of the treatment, especially in patients with TRPA1 and TRPV1 channel function disorders.

In systemic inflammation, the pharmacological modulation of T_b may be beneficial in humans and animals, and TRPV1 and TRPA1 channels are probably good targets to modify T_b. However, future research is needed to clarify the pharmacological potential of these channels in this field.

VII. Summary of new findings

1. In our meta-analysis we revealed a strong negative correlation between the mortality ratio and deep T_b in sepsis. Patients with hypothermia have a higher predicted mortality rate than patients with fever. This finding emphasizes that hypothermia and fever should be weighted not equally as currently used in many scoring systems (e.g., SIRS, APACHE).
2. We also showed that lower blood pH or base excess is associated with higher mortality rate, elevated severity scores, and longer hospital stay in AP patients. Similar to hypothermia and fever in sepsis, acidosis and alkalosis should not be weighted equally as the APACHE II score does.
3. The mode-nonselective TRPV1 antagonists cause hyperthermia whereas NEO6860, the mode-selective TRPV1 antagonist, decreased the deep T_b. The hyperthermic effect of mode-nonselective TRPV1 antagonists is dose-dependent.
4. We described that the NH_4Cl-induced hypothermic response is not specific only for mice because the i.p. administration of NH_4Cl decreases deep T_b in rats as well.
5. TRPA1 channels contribute to the development of NH_4Cl-induced hypothermia in mice and rats whereas TRPV1 channels play a limiting function.
6. I.p. administration of NH_4Cl decreases the blood pH in rats and mice. The drop in the blood pH of the mice reached a similar extent in *Trpv1*, *Trpa1* KO and WT mice.

VIII. Acknowledgments

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IX. Appendix

Publications related to the subject of the thesis

- Number of publications related to the subject of the thesis: 5
- Number of publications not related to the subject of the thesis: 36
- Number of book chapters: 1
- Sum of all impact factors: 157.789
- Sum of impact factors from publications related to the topic of PhD thesis: 23.477
- All citations: 866
- Independent citations: 779

Publications related to the topic of the PhD thesis

Rumbus, Z., Fekete, K., Kelava, L., Gardos, B., Klonfar, K., Keringer, P., Pinter, E., Pakai, E., & Garami, A. Ammonium chloride-induced hypothermia is attenuated by transient receptor potential channel vanilloid-1, but augmented by ankyrin-1 in rodents. Life sciences, 2024;346, 122633. Advance online publication.
IF: 5.2 Q1/D1 Total citations/Independent citations: 0/0

Garami, A., Shimansky, Y. P., **Rumbus, Z**., Vizin, R. C. L., Farkas, N., Hegyi, J., Szakacs, Z., Solymar, M., Csenkey, A., Chiche, D. A., Kapil, R., Kyle, D. J., Van Horn, W. D., Hegyi, P., & Romanovsky, A. A. Hyperthermia induced by transient receptor potential vanilloid-1 (TRPV1) antagonists in human clinical trials: Insights from mathematical modeling and meta-analysis. Pharmacology & therapeutics, 2020, 208, 107474.
IF: 12.31 Q1/D1 Total citations/Independent citations: 90/84

Rumbus, Z., & Garami, A. Fever, hypothermia, and mortality in sepsis: Comment on: Rumbus Z, Matics R, Hegyi P, Zsiboras C, Szabo I, Illes A, Petervari E, Balasko M, Marta K, Miko A, Parniczky A, Tenk J, Rostas I, Solymar M, Garami A. Fever is associated with reduced, hypothermia with increased mortality in septic patients: a meta-analysis of clinical trials. PLoS One. 2017;12(1):e0170152. DOI: 10.1371/journal.pone.0170152. Temperature (Austin, Tex.), 2018. 6(2), 101–103.

Rumbus, Z., Toth, E., Poto, L., Vincze, A., Veres, G., Czako, L., Olah, E., Marta, K., Miko, A., Rakonczay, Z., Jr, Balla, Z., Kaszaki, J., Foldesi, I., Maleth, J., Hegyi, P., & Garami, A. Bidirectional relationship between reduced blood pH and acute pancreatitis: A translational study of their noxious combination. Frontiers in physiology, 2018; 9, 1360.
IF: 3.201 Q2 Total citations/Independent citations: 11/5

Rumbus, Z., Matics, R., Hegyi, P., Zsiboras, C., Szabo, I., Illes, A., Petervari, E., Balasko, M., Marta, K., Miko, A., Parniczky, A., Tenk, J., Rostas, I., Solymar, M., & Garami, A. Fever is associated with reduced, hypothermia with increased mortality in septic patients: A meta-analysis of clinical trials. Plos one, 2017; 12(1), e0170152.
IF: 2.766 Q1 Total citations/Independent citations: 110/100

Other publications, not related to the topic of the PhD thesis

Bálint, A., Hanák, L., Hegyi, P., Szakács, Z., Eitmann, S., Garami, A., Solymár, M., Márta, K., **Rumbus, Z.,** & Komócsi, A. Increased risk of adverse events in patients with low-on clopidogrel platelet reactivity after percutaneous coronary intervention: A systematic review and meta-analysis. Cardiology journal, 2023;30(3), 391–400.
IF: 2.9 Q2 Total citations/Independent citations: 3/1

Garai, J., Radnai, B., Vámos, E., Kovács, D., Vántus, V. B., **Rumbus, Z.,** Pákai, E., Garami, A., Gulyás-Fekete, G., Agócs, A., Krekó, M., Zaman, K., Prókai, L., Őrfi, L., Jakus, P. B., & Lóránd, T. Synthesis and evaluation of a new class of MIF-inhibitors in activated macrophage cells and in experimental septic shock in mice. European journal of medicinal chemistry, 2023; 247, 115050.
IF: 6.0 Q1 Total citations/Independent citations: 2/1

Jávor, P., Hanák, L., Hegyi, P., Csonka, E., Butt, E., Horváth, T., Góg, I., Lukacs, A., Soós, A., **Rumbus, Z.,** Pákai, E., Toldi, J., & Hartmann, P. Predictive value of tachycardia for mortality in trauma-related haemorrhagic shock: a systematic review and meta-regression. BMJ open, 2022; 12(10), e059271.
IF: 2.9 Q1 Total citations/Independent citations: 4/3

Kelava, L., Nemeth, D., Hegyi, P., Keringer, P., Kovacs, D. K., Balasko, M., Solymar, M., Pakai, E., **Rumbus, Z**., & Garami, A. Dietary supplementation of transient receptor potential vanilloid-1 channel agonists reduces serum total cholesterol level: a meta-analysis of controlled human trials. Critical reviews in food science and nutrition, 2022; 62(25), 7025–7035.
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Keringer, P., Furedi, N., Gaszner, B., Miko, A., Pakai, E., Fekete, K., Olah, E., Kelava, L., Romanovsky, A. A., **Rumbus, Z.**, & Garami, A. The hyperthermic effect of central cholecystokinin is mediated by the cyclooxygenase-2 pathway. American journal of physiology. Endocrinology and metabolism, 2022; 322(1), E10–E23.

IF: 5.1 Q1 Total citations/Independent citations: 4/3

Kovács, D. K., Gede, N., Szabó, L., Hegyi, P., Szakács, Z., Faludi, B., Sebők, Á., Garami, A., Solymár, M., Kósa, D., Hanák, L., Rumbus, Z., & Balaskó, M. Weight reduction added to CPAP decreases blood pressure and triglyceride level in OSA: Systematic review and meta-analysis. Clinical and translational science, 2022; 15(5), 1238–1248.

IF: 3.9 Q1/D1 Total citations/Independent citations: 5/5

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IF: 3.2 Q2 Total citations/Independent citations: 6/3

Ruzsics, I., Matrai, P., Hegyi, P., Nemeth, D., Tenk, J., Csenkey, A., Eross, B., Varga, G., Balasko, M., Petervari, E., Veres, G., Sepp, R., Rakonczay, Z., Jr, Vincze, A., Garami, A., **Rumbus, Z.** Noninvasive ventilation improves the outcome in patients with pneumonia-associated respiratory failure: Systematic review and meta-analysis. Journal of infection and public health, 2022; 15(3), 349–359.

IF: 6.7 Q1 Total citations/Independent citations: 7/7

Garai, J., Krekó, M., Őrfi, L., Jakus, P. B., **Rumbus, Z.,** Kéringer, P., Garami, A., Vámos, E., Kovács, D., Bagóné Vántus, V., Radnai, B., & Lóránd, T. Tetralone derivatives are MIF tautomerase inhibitors and attenuate macrophage activation and amplify the hypothermic response in endotoxemic mice. Journal of enzyme inhibition and medicinal chemistry, 2021; 36(1), 1357–1369.

IF: 5.756 Q2 Total citations/Independent citations: 3/1

Martonosi, Á. R., Soós, A., **Rumbus, Z.,** Hegyi, P., Izsák, V., Pázmány, P., Imrei, M., Váncsa, S., Szakács, Z., Párniczky, A. Non-invasive diagnostic tests in cystic fibrosis-related liver disease: A diagnostic test accuracy network meta-analysis. Frontiers in medicine, 2021; 8, 598382.

IF: 5.058 Q1 Total citations/Independent citations: 3/3

Olah, E., **Rumbus, Z**., Kormos, V., Tekus, V., Pakai, E., Wilson, H. V., Fekete, K., Solymar, M., Kelava, L., Keringer, P., Gaszner, B., Whiteman, M., Keeble, J., Pinter, E., & Garami, A. The hypothermic effect of hydrogen sulfide is mediated by the transient receptor potential

ankyrin-1 channel in mice. Pharmaceuticals (Basel, Switzerland), 2021; 14(10), 992.
IF: 5.215 Q1 Total citations/Independent citations: 9/3

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IF: 4.869 Q1 Total citations/Independent citations: 3/2

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IF: 4.997 Q1/D1 Total citations/Independent citations: 13/12

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IF: 5.021 Q1/D1 Total citations/Independent citations: 26/25

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IF: 5.325 Q1/D1 Total citations/Independent citations: 25/22

Kerémi, B., Márta, K., Farkas, K., Czumbel, L. M., Tóth, B., Szakács, Z., Csupor, D., Czimmer, J., **Rumbus, Z.**, Révész, P., Németh, A., Gerber, G., Hegyi, P., & Varga, G. Effects of chlorine dioxide on oral hygiene - A systematic review and meta-analysis. Current pharmaceutical design, 2020; 26(25), 3015–3025.
IF: 3.116 Q2 Total citations/Independent citations: 20/17

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IF: 4.38 Q1/D1 Total citations/Independent citations: 12/11

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IF: 3.295 Q1 Total citations/Independent citations: 26/26

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IF: 2.74 Q1/D1 Total citations/Independent citations: 32/31

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IF: 2.849 Q2 Total citations/Independent citations: 34/33

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IF: 2.751 Q1 Total citations/Independent citations: 14/13

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IF: 2.687 Q1 Total citations/Independent citations: 83/76

Keringer, P., & **Rumbus, Z.** The interaction between neurokinin-1 receptors and cyclooxygenase-2 in fever genesis. Temperature (Austin, Tex.), 2019; 6(1), 4–6.

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IF: 2.946 Q1 Total citations/Independent citations: 49/47

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IF: 3.754 Q1 Total citations/Independent citations: 37/31

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PLOS ONE

RESEARCH ARTICLE

Fever Is Associated with Reduced, Hypothermia with Increased Mortality in Septic Patients: A Meta-Analysis of Clinical Trials

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Abstract

Background

Sepsis is usually accompanied by changes of body temperature (T_b), but whether fever and hypothermia predict mortality equally or differently is not fully clarified. We aimed to find an association between T_b and mortality in septic patients with meta-analysis of clinical trials.

Methods

We searched the PubMed, EMBASE, and Cochrane Controlled Trials Registry databases (from inception to February 2016). Human studies reporting T_b and mortality of patients with sepsis were included in the analyses. Average T_b with SEM and mortality rate of septic patient groups were extracted by two authors independently.

Results

Forty-two studies reported T_b and mortality ratios in septic patients (n = 10,834). Pearson correlation analysis revealed weak negative linear correlation (R^2 = 0.2794) between T_b and mortality. With forest plot analysis, we found a 22.2% (CI, 19.2±25.5) mortality rate in septic patients with fever (T_b > 38.0ÊC),which was higher, 31.2% (CI, 25.7±37.3), in normothermic patients, and it was the highest, 47.3% (CI, 38.9±55.7), in hypothermic patients (T_b < 36.0ÊC).Meta-regression analysis showed strong negative linear correlation between T_b and mortality rate (regression coefficient: -0.4318; P < 0.001). Mean T_b of the patients was higher in the lowest mortality quartile than in the highest: 38.1ÊC(CI, 37.9±38.4) vs 37.1ÊC (CI, 36.7±37.4).

Competing Interests: The authors have declared that no competing interests exist.

Conclusions

Deep T_b shows negative correlation with the clinical outcome in sepsis. Fever predicts lower, while hypothermia higher mortality rates compared with normal T_b. Septic patients with the lowest (< 25%) chance of mortality have higher T_b than those with the highest chance (> 75%).

Introduction

Sepsis constitutes a global burden for medical care with an estimated 31 million cases per year worldwide [1]. The incidence of sepsis has remained considerable [2±4] and it is associated with high mortality rates even nowadays [3]. It also underlies the importance and actuality of the topic for clinical praxis that the definitions of sepsis and associated illnesses have been updated recently [5].

As a systemic inflammation response, sepsis is often associated with changes of deep body temperature (T_b), which can be manifested as fever or hypothermia in experimental animals [6±8], as well as in human patients [8±10]. Not surprisingly, deep T_b is regularly measured as one of the vital signs in the clinical praxis. In fact, many scoring systems (e.g., APACHE II, PIRO, SAPS II, SIRS), which help in the diagnosis or in the assessment of the progress of sepsis, include an abnormal deviation of T_b from the normal range [5, 11±14]. Usually T_bs below 36.0ÊCor above 38.0ÊCare considered equally pathological [15], which values are in accordance with the criteria of the systemic inflammatory response syndrome [5, 11]. Based mainly on experimental data from animal studies Romanovksy and colleagues [6, 8] proposed that fever and hypothermia can both develop as two distinct adaptive mechanisms in sickness syndrome. The former characteristically occurs at the onset of an infection, representing an active fight against the pathogen, while the latter is usually associated with progressed stage or severity of the disease and it aims to secure the vital systems of the host [6, 8]. The two adaptive strategies can develop sequentially (e.g., early phase fever followed by late phase hypothermia) as the severity of the disease progresses [8], but hypothermia can be also one of the earliest developing events in animal models of endotoxin shock [16], moreover, septic patients admitted to ICU develop hypothermia more frequently in the early than in the late stages of their stay [17]. Despite the different pathological background of fever and hypothermia in systemic inflammation, both the increase and the decrease of T_b are evaluated commonly as equally severe signs in the clinical praxis [15]. This can be, at least in part, due to the standpoint that fever and hypothermia both represent an adaptive (though different) biological response to infection [8]. Accordingly, beneficial effects have been shown for elevated T_b on the clinical outcome of sepsis in clinical trials [18, 19], although no association between fever and disease severity was also reported [20]. Therapeutic (i.e., induced) hypothermia has been also shown to improve the outcome of sepsis in human studies [21, 22], but in case of spontaneously occurring hypothermia usually a positive association with mortality rate was found [20, 23, 24]. The definite association of T_b and mortality rate in a large study population has remained unknown.

We hypothesized that the deviation of T_b from the normal range predicts the clinical outcome in sepsis differently, and consequently septic patients with fever have lower chances for mortality than those who develop hypothermia. We performed an extensive literature search for human studies in septic patients and collected data on their T_b and mortality rate. The data were then analyzed with multiple statistical approaches, including Pearson regression, forest

plot, and meta-regression analyses. Based on a high number of patients, we show a strong association between T_b and mortality ratio in sepsis across a wide temperature range.

Materials and Methods

Our meta-analysis was conducted in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols [25] (S1 Table). The analysis was based on the Participants, Intervention (prognostic factor), Comparison, Outcome (PICO) model: in septic population, we aimed to assess the predictive role of T_b deviations on the mortality ratio. No review protocol has been registered for the current meta-analysis.

Search strategy

A search of the PubMed, EMBASE, and Cochrane Controlled Trials Registry databases was performed with using the following Medical Subject Headings and search terms (from inception to February 2016): sepsis OR bacteremia OR "septic syndrome" AND ("body temperature" OR fever OR hypothermia OR normothermia OR hyperthermia) AND (mortality OR survival). We restricted our search to original human studies published in English without time period limitations. Publications reporting immunosuppressive conditions (e.g., cancer, transplantation, HIV infection) were not included in the analysis. As a specific example, in the EMBASE database, which identified the highest number of articles, the term [a]sepsis OR bacteremia OR "septic syndrome" AND ("body temperature" OR fever OR hypothermia OR normothermia OR hyperthermia) AND (mortality OR survival) NOT (cancer OR immunosuppressive OR aids OR hiv OR transplantation)[o] was entered, and then the following filters were selected: humans, English, article, article in press, conference abstract, conference paper, major clinical study, case control study, clinical trial, cohort analysis, comparative study, controlled clinical trial, controlled study, cross-sectional study, double blind procedure, medical record review, multicenter study, observational study, outcomes research, phase 3 clinical trial, prospective study, randomized controlled trial, retrospective study. The search was conducted separately by two authors (ZR, AG), who also assessed study eligibility and extracted data from the selected studies independently. Disagreements were resolved by consensus with the help of a third party (MR).

Study selection and data extraction

The titles and abstracts of the publications from the literature search were screened and the full text of potentially eligible articles was obtained. We included studies in which both the T_b values and the mortality ratios were reported for the same group(s) of patients with systemic inflammation accompanied by suspected or confirmed blood infection. From all included articles we extracted the sample size, the reported mean T_b value of the patients with its standard error (SEM), and the mortality ratio within the group during 28±30 days in most cases. To analyze the influence of fever, normothermia, and hypothermia on the mortality ratio in sepsis we separated the collected data into three study groups based on the mean T_b of the patients.

Statistical analysis

We have used event rates (mortality rates) as effect size data. Studies were grouped by T_b as low (up to 36.0ÊC;n = 890), medium (36.1 to 38.0ÊC;n = 3,904) and high (above 38.0ÊC; n = 6,040) and forest plots in the three groups were used to describe mortality. Selection of the T_b groups was based on the SIRS criteria [5, 11]. Another grouping was conducted by mortalities, these were split into quartiles and the means of T_bs were compared by investigating the

presence or absence of overlaps in the 95% confidence intervals (CI), just like in case of the grouping by T_b.

Between-study heterogeneity was tested with *Q* homogeneity test (*P* values of less than 0.05 were considered as indicators of significant heterogeneity) and with I^2 statistical test, where I^2 is the proportion of total variation attributable to between-study variability (an I^2 value of more than 50 was considered as indicating considerable heterogeneity). These two values were used to model selection purposes as well (fixed vs random). The tests revealed considerable heterogeneity in the overall study population ($Q = 809.509$; $I^2 = 89.25$) and also in all three T_b groups, in particular $Q = 270.447$; $I^2 = 85.58$ in the high, $Q = 373.357$; $I^2 = 90.63$ in the medium, and $Q = 36.843$; $I^2 = 70.14$ in the low T_b group. Consequently, we applied the random effect model in our forest plot and meta-regression analyses.

Publication bias was tested by inspecting the funnel plot. Meta-regression was performed to assess the overall effect of T_b to mortality. Except for the Pearson correlation analysis for which Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) was used, all analyses were performed with the Comprehensive Meta-Analysis software (Biostat, Inc., Engelwood, MJ, USA).

Results

Study selection

The flow chart of the study selection is presented in Fig 1. Until February 29, 2016 the electronic literature search identified altogether 6,083 studies from the PubMed, EMBASE, and Cochrane databases. After enabling filters for human studies and English language, 762 articles remained, which were screened on title and abstract for inclusion criteria. In 720 studies T_b or mortality rate was not suitably reported in the septic patients, these were also excluded, as a result 42 full-text publications were found eligible for statistical analysis which included data from a total of 10,834 septic patients.

Incidence of mortality in septic patients with fever, normothermia, and hypothermia

As a rude approach, first we performed a common (Pearson) correlation analysis between T_b and mortality rate of all septic patients. A weak negative linear correlation was found ($y = -0.0909x + 3.6902$; $R^2 = 0.2794$), which suggests an association between T_b and mortality in sepsis. This method, however, did not allow us to weight the collected data according to the size of the studied populations, thus a detailed meta-analysis was needed.

First, we investigated the incidence of mortality in fever associated with sepsis. We found 29 studies [18±20, 23, 26±50], in which the authors reported fever (defined as $T_b > 38.0$ÊC)in sepsis. From these studies, 40 groups of septic patients could be separated and included in the analysis with the random effect model. The meta-analysis of the mortality rates in the septic patients with fever revealed an average event rate of 22.2% (95% CI, 19.2±25.5%; Z = -13.4331) (Fig 2). This percentage was significantly (*P* = 0.000) lower than the 50% chance of mortality, which could be regarded as a random outcome.

Next, we analyzed the mortality ratios of patients who developed neither fever nor hypothermia in association with sepsis, therefore this population could be regarded as normothermic ($T_b = 36.0$±38.0ÊC).From the 25 studies, in which normal T_b was reported in the septic patients [20, 22, 24, 28, 31, 35±37, 39±41, 43, 44, 47, 50±60], 36 subgroups of patients were separated, which were then analyzed with the random effect model. We found that the average mortality ratio was 31.2% (95% CI, 25.7±37.3%; Z = -5.7089) (Fig 3), which was higher than in

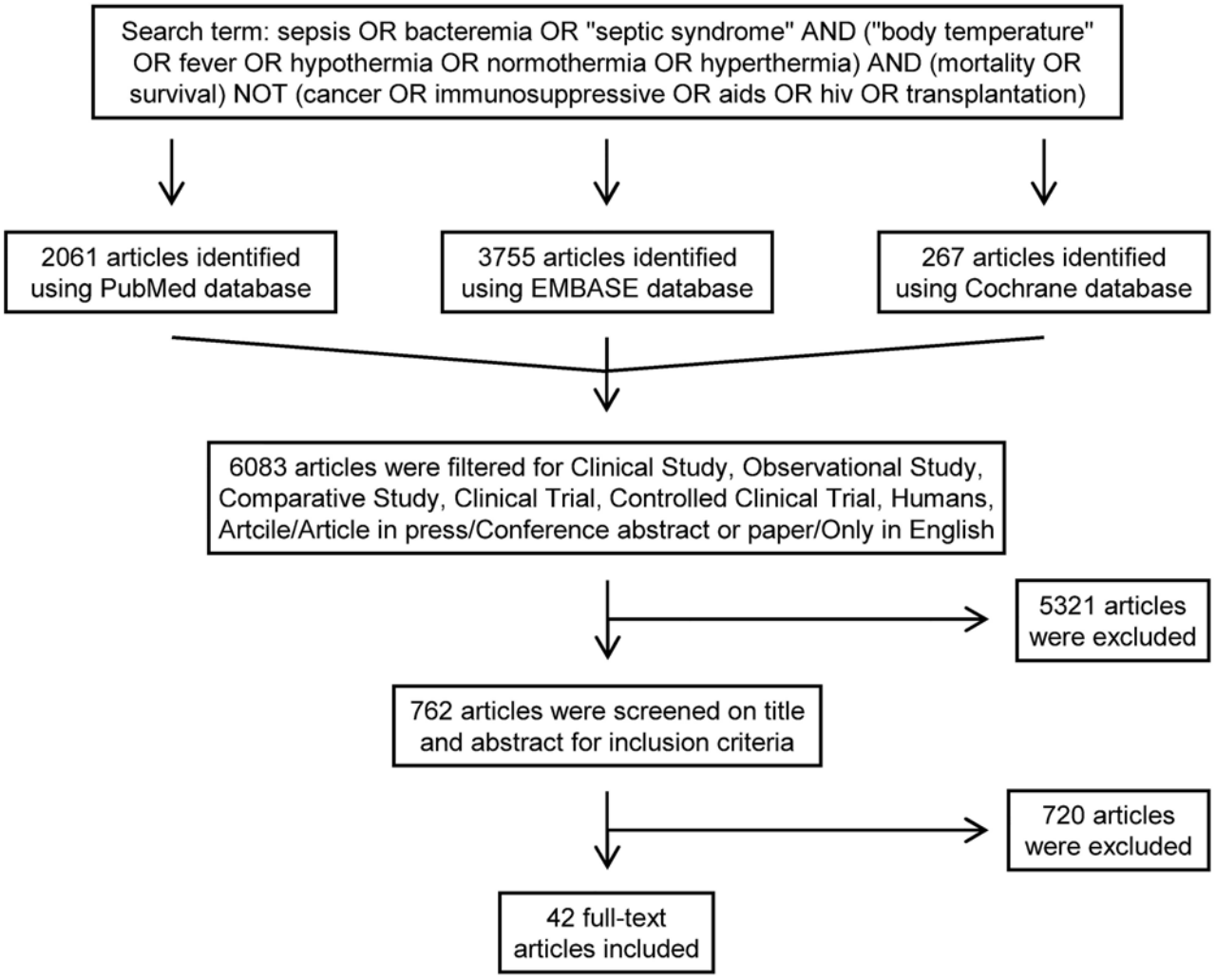

Fig 1. Flowchart of study selection and inclusion.

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the fever group. The mortality rate was significantly ($P < 0.001$) lower than 50% in this study population.

Then, we examined the incidence of mortality in hypothermic ($T_b < 36.0$ÊC)septic patients. We identified 11 studies [20, 22±24, 27, 29, 30, 35, 50, 54, 61], which included data on both T_b and mortality in septic patients. From these, the patients could be divided in 12 subgroups, which served as the basis of the meta-analysis. The random effect model revealed that the average mortality rate was the highest, 47.3% (95% CI, 38.9±55.7; Z = 0.520491) in the hypothermic patients (Fig 4), which did not significantly differ from the 50% random chance ($P = 0.603$).

As a further statistical approach, we also performed a meta-regression analysis on the collected data. We found a significant ($P < 0.001$) negative linear correlation between T_b and mortality rate (regression coefficient: -0.4318; 95% CI, -0.6699 - -0.1938) based on 51 studies included in the analysis (Fig 5).

Last, we divided the patients into quartiles (Q1-Q4) of mortality ratios (Q1: 0±25, Q2: 26±50, Q3: 51±75, and Q4: 76±100%) and calculated the average T_b for each mortality quartile.

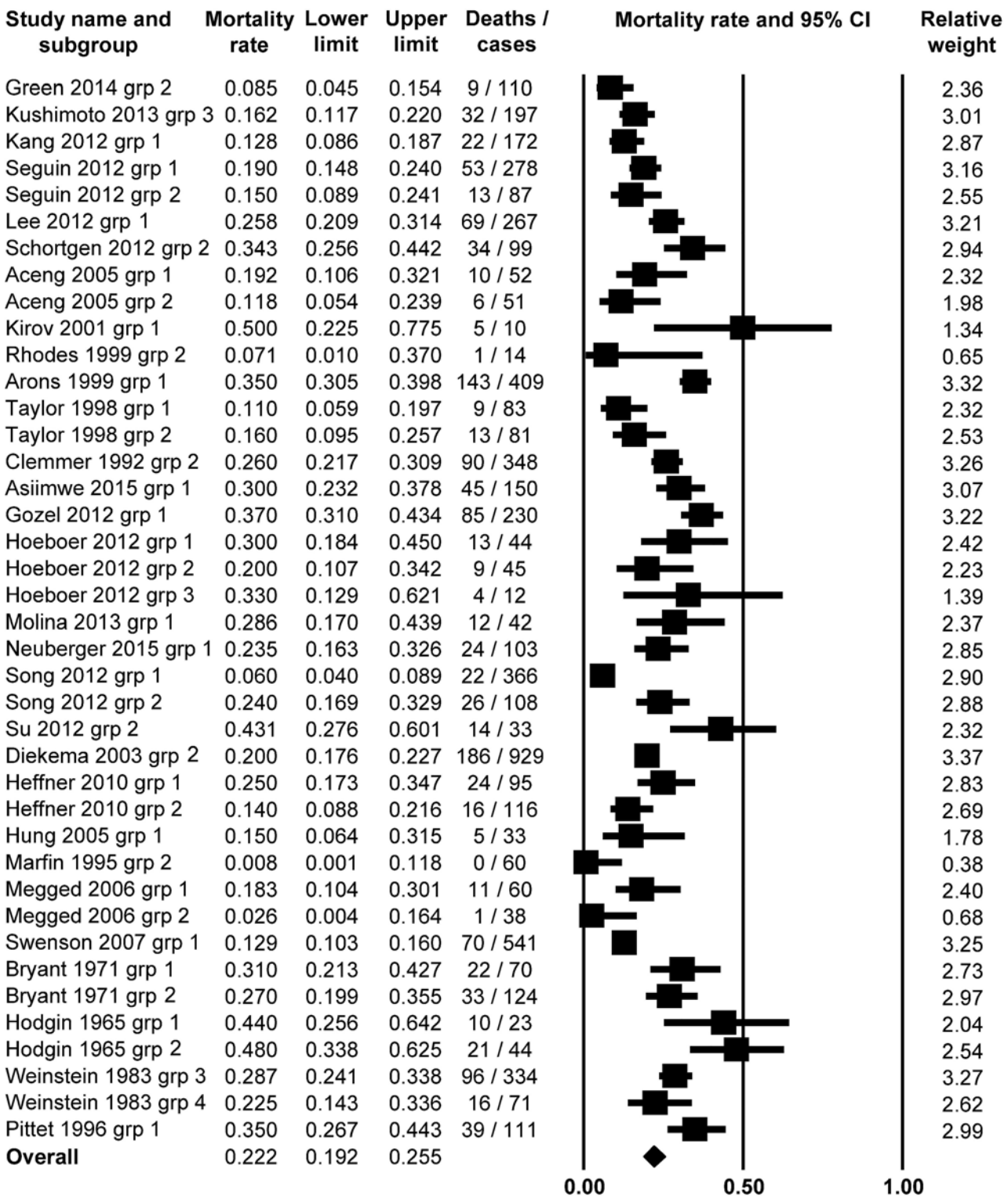

Fig 2. Forest plot analysis of mortality rate using random-effects model in septic patients with fever (body temperature above 38.0ÊC;*n* = 6,040).

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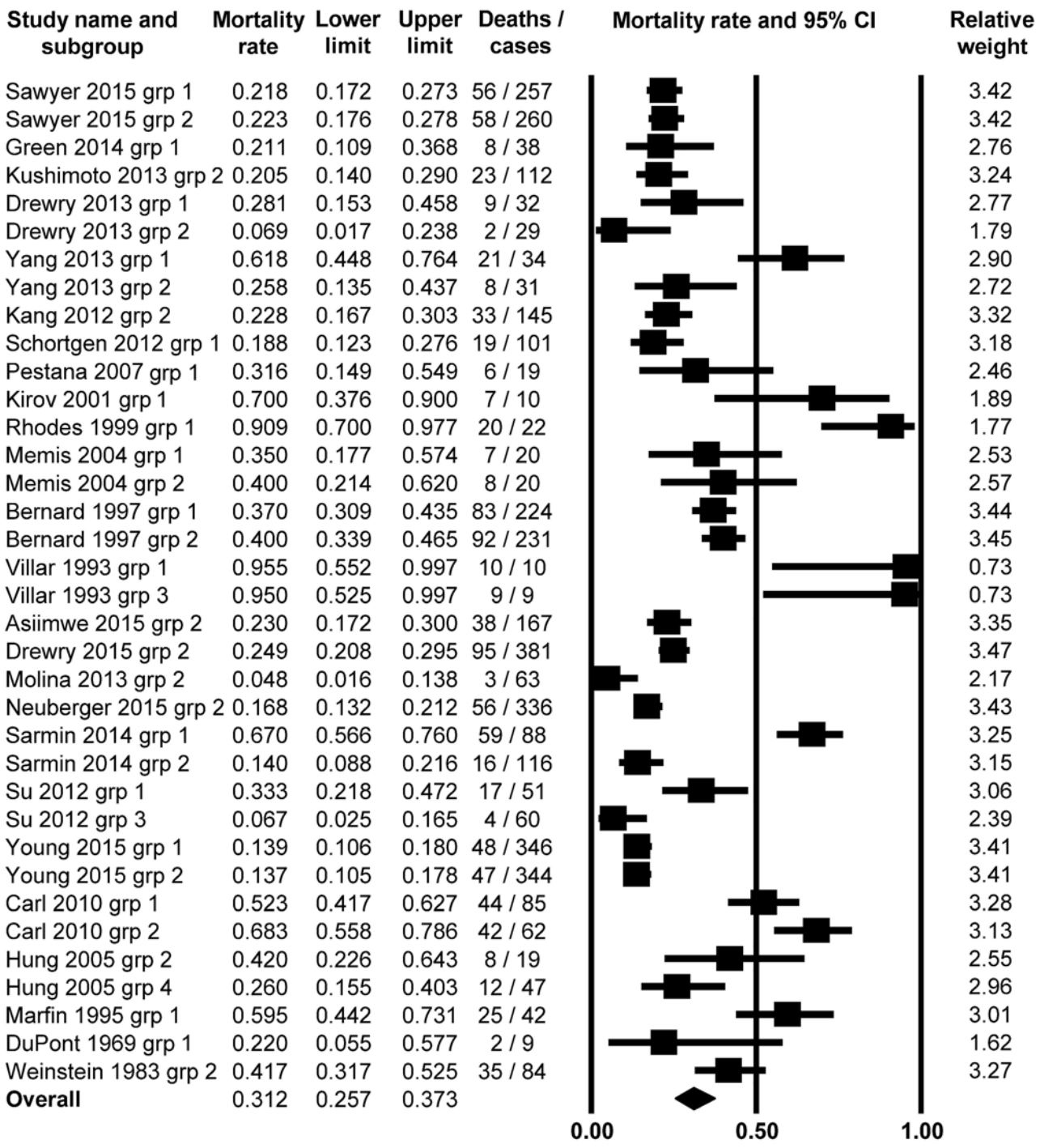

Fig 3. Forest plot analysis of mortality rate using random-effects model in septic patients with normothermia (body temperature between 36.1 and 38.0ÊC;*n* = 3,904).

doi:10.1371/journal.pone.0170152.g003

The weighted average T_bs were 38.1 (95% CI, 37.9±38.4ÊC),37.8 (95% CI, 37.5±38.2ÊC),37.6 (95% CI, 36.5±38.7ÊC),and 37.1ÊC(95% CI, 36.7±37.4ÊC)in the Q1, Q2, Q3, and Q4 groups, respectively. These results also indicate that in sepsis a higher T_b is associated with better outcome, while a lower T_b is related with higher risk of mortality. Of note, the T_bs in Q1 and Q4

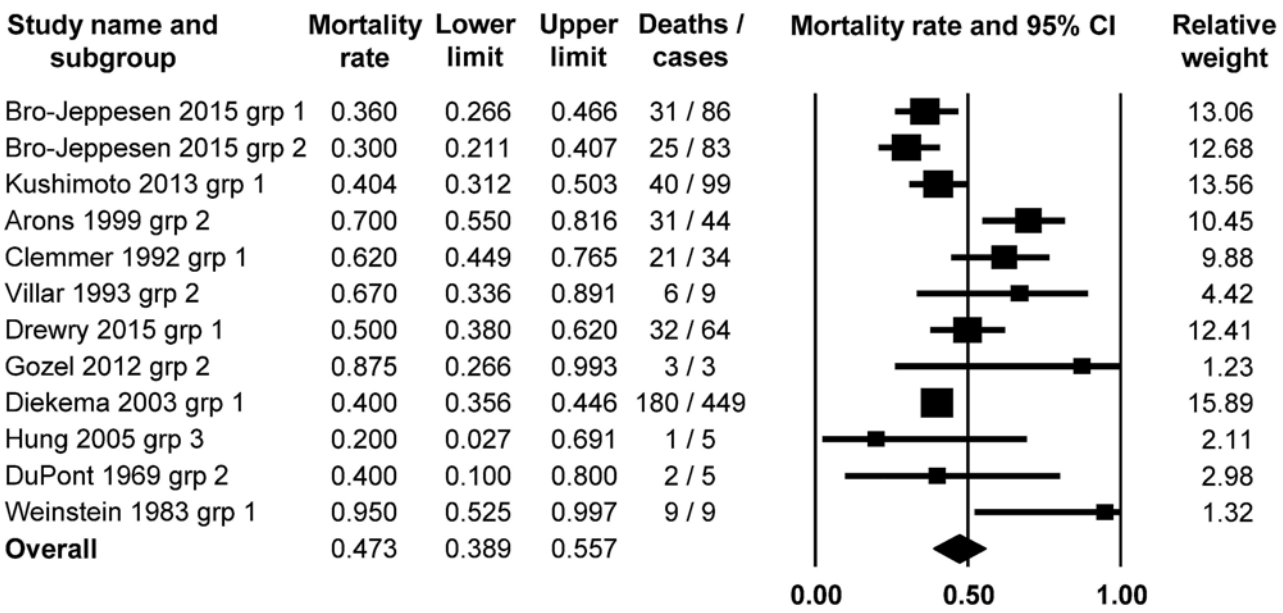

Fig 4. Forest plot analysis of mortality rate using random-effects model in septic patients with hypothermia (body temperature up to 36.0ÊC;*n* = 890).

doi:10.1371/journal.pone.0170152.g004

(i.e., in the groups with lowest and highest mortality, respectively) are clearly distinct from each other, as the 95% CIs do not overlap.

Discussion

In the current analysis we revealed a clear association between T_b and mortality in septic patients by using a detailed statistical approach which was based on an extensive literature search of previous human studies. We found that the presence of fever reduces, while that of hypothermia promotes mortality in septic patients as compared to normothermic subjects.

From previous studies of septic patients only limited information is available to show an association between T_b and mortality in a wide temperature range. While a worse outcome was consistently found to be related to hypothermia [20, 23, 24, 27], those studies compared hypothermia with fever [23, 27] or with nonhypothermic (i.e., by merging febrile and normothermic) patients [20, 24]. In one study, no association was found between hypothermia and the clinical outcome of sepsis [19]. Regarding the role of fever, different clinical trials came to controversial results in sepsis. Several authors showed that a higher T_b was beneficial [18, 19, 50, 62], others that it was disadvantageous [44, 63], and a few found no association between fever and mortality [20, 60]. The discrepancy among the studies may result from the limited sample size used in the trials. Another explanation for not detecting significant association could be the insensitivity of the used statistical method. Indeed, when we first performed a commonly used regression analysis, the Pearson correlation, which did not allow us to weight the collected data for example for sample size, we found only a very weak negative correlation ($P = 0.047$) between T_b and mortality ratio in sepsis. Thus, we applied more precise statistical tools (forest plot and meta-regression analyses).

In our analyses, we used a sizeable, heterogeneous population of septic patients ($n = 10{,}834$) with a wide range of T_b (33.0±39.9ÊC).We showed that in sepsis mortality rates are lower if fever is present and higher in cases of hypothermia as compared to the normothermic group. In addition, we demonstrated a strong negative correlation ($P = 0.0004$) between T_b and

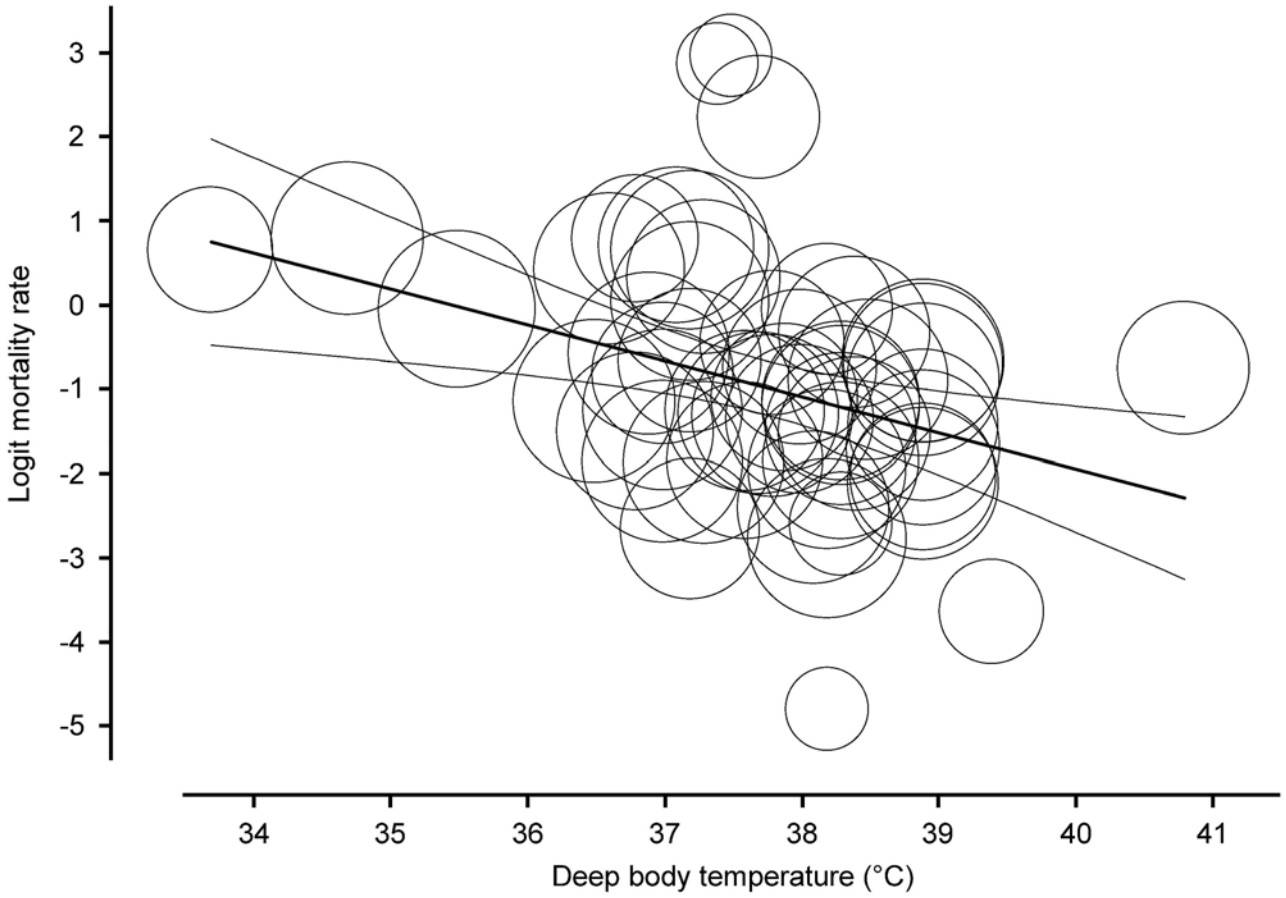

Fig 5. Meta-regression analysis of the association between body temperature and mortality ratio in septic patients (*n* = 10, 834).

doi:10.1371/journal.pone.0170152.g005

mortality ratio with the help of meta-regression analysis. In our statistical approach, we also included a substantial number of septic patient groups with average T_bs within the normal range (n = 3,904). Furthermore, when we calculated the mean T_bs of septic patients in the mortality quartiles, we found that it gradually decreased from the lowest to the highest quartile and it was significantly higher in the lowest (0±25%) than in the highest quartile (75±100%) of mortality (38.1 ± 0.1 vs 37.1 ± 0.2ÊCfor mean ± SEM, respectively). Taken together the results from all of our statistical approaches, our data strongly suggest a predictive role of T_b for the outcome of sepsis.

Sepsis continues to constitute a major challenge in critical care medicine [1±4]. As a systemic inflammation process, sepsis is frequently accompanied by abnormalities of T_b, like fever and hypothermia. In animal experiments, lower doses of endotoxin usually cause fever, whilst higher doses lead to the development of hypothermia [64, 65], indicating that the severity of the disease determines the change in T_b and not the way around. Based on our statistical analyses of human studies fever seems beneficial, but hypothermia rather disadvantageous for the organism regarding the outcome. However, it has to be noted that the current analysis does not allow us to conclude that the change of T_b per se is responsible for the lower and higher mortality rates in fever and hypothermia, respectively. Instead of a cause-effect relationship, the abnormal T_b should be rather regarded as a prognostic vital parameter of the severity and progress of the inflammation, and as such, as a warning sign, which can help doctors to asses the outcome of to the infection.

With regard to the adaptive biological value of T_b alterations in mammals, the development of fever in systemic inflammation is considered to indicate the activation of defense mechanisms of the body to fight the intruding agent [6±8]. By enhancing immune functions and accelerating the elimination of the microorganism from the body, at the onset of the inflammation fever is an adaptive, beneficial thermoregulatory response, although it involves a higher energy cost [6±8]. Therefore, fever itself is assumed to have a direct, advantageous effect on the mortality ratio in systemic inflammation (e.g., sepsis), when it is affordable for the host. However, T_b regulation should be considered in the framework of complex energy balance [66], therefore, the beneficial value of fever as an energetically expensive defense response is doubtable when there is a risk of energy deficiency, which often develops as the severity of the disease further progresses. In support of that, the administration of antipyretics resulted in an increase of mortality rate of critically ill patients in prospective clinical trials [38, 67]. However, in severe sepsis or septic shock, the use of pharmacological antipyretics did not influence mortality [51, 68], while fever control with external cooling decreased early mortality in human studies [44].

Spontaneous hypothermia represents a distinct, adaptive mechanism to systemic inflammation in experimental animals [69] and in septic patients [17]. It characteristically develops in severe cases of already progressed diseases, whenÐinstead of actively coping with the microorganismÐthe organism attempts to increase survival by saving its energy resources [6, 8]. A recent study by Fonseca et al. [17] revealed that spontaneous hypothermia is a transient, self-limiting, and nonterminal event in human sepsis, which underlies its biological value as an adaptive mechanism in the critically ill patients.

Although the results of our analysis showed that hypothermia is associated with higher mortality, it should be noted that we can not be sure how mortality ratio of the patients would have changed if hypothermia had not developed or if the patients were rewarmed. As of today, to our knowledge, the effect of rewarming vs non-rewarming on the mortality of septic patients with spontaneous hypothermia has not been compared in randomized controlled trials. Therefore, hypothermia in itself should not be regarded harmful for the body as the associated higher mortality rate of the septic patients is presumably due to their more severe clinical condition. We suggest that the difference between the mortality rates of febrile and hypothermic patients with sepsis is due to the different severity and progression of the inflammation and not due to T_b itself. As a consequence, T_b itself serves not as a detrimental factor, but instead, as an indicative predictor for the severity of the disease and as such for mortality in sepsis.

From a clinical perspective, our results highlight the importance of precise and regular measurements of deep T_b, since its abnormalities can help physiciansÐespecially in critical care medicineÐnot only in the diagnosis, but also in the follow up of the progression, and in the prognosis of sepsis. Based on our findings, it would be worth to consider that hypothermia should be weighted differently than fever and not equally as currently used in many scoring systems (e.g., SIRS, APACHE, PIRO), since hypothermia indicates a more severe stage of sepsis, and, therefore it is associated with worse clinical outcome. Regarding therapeutic interventions, the T_b management of septic patients should be always carefully evaluated and perhaps guidelines could be established (e.g., for the initiation of antipyretic treatment) to improve the clinical outcome in sepsis.

Conclusions

The abnormalities of deep T_b are strongly associated with the clinical outcome in sepsis. The mortality ratio of febrile patients is lower, while in patients with hypothermia it is markedly

higher than that of patients with normal T_b. In cases of sepsis, there is a strong negative correlation between the mortality ratio and deep T_b in a wide temperature range. Septic patients with the lowest ($< 25\%$) chance of mortality have significantly higher deep T_b than those who belong to the highest mortality quartile ($> 75\%$).

Strengths and Limitations

Our meta-analysis included data from a total of 10,834 septic patients with overall 2,724 mortality events. We believe that our search strategy was adequately broad and included the three main databases of human studies. As result, 42 full-text articles could be indentified and used in our analyses. Although the sample size and the overall event rate can be considered large enough to draw solid conclusions about the association of T_b and mortality rate in sepsis, our study has certain limitations.

First, due to the nature of the meta-analysis method, we have studied the reported mean T_bs in populations of patients, rather than the association between T_b and the outcome of sepsis in individual patients. The latter approach would certainly allow one to draw firmer conclusions about the association between T_b and mortality, but it would also necessitate access to the original data of the analyzed articles, which is not feasible. Alternatively, a well-designed clinical trial with a big sample size could also provide high-quality individual data and based on our results it can be warranted to conduct such trials.

Second, the studied population of patients is quite diverse, which diversity could also have its own impact on the results. For example, T_b measurements were performed in different ways and not at the same time points in the analyzed studies. Despite such differences, we believe that the size of the analyzed sample was big enough to mitigate the methodological differences among the studies and to allow for drawing conclusions about the association of T_b and mortality in the septic patients.

Supporting Information

S1 Table. PRISMA 2009 checklist.
(DOC)

Author Contributions

Conceptualization: ZR AG.

Data curation: RM.

Formal analysis: ZR RM AG.

Funding acquisition: PH AG.

Investigation: ZR RM CZ EP MB KM AM JT IR MS.

Methodology: ZR RM AG.

Project administration: ZR PH AG.

Resources: PH AG.

Software: RM PH.

Supervision: PH EP MB AG.

Visualization: ZR RM CZ KM AM AP JT IR AG.

Writing ± original draft: ZR AG.

Writing ± review & editing: ZR RM PH CZ IS AI EP MB KM AM AP JT IR MS AG.

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PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	4
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	4-5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	4
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	5
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	5-6

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	6
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	6
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	6; Fig. 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Figs. 2-4
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	6
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	6-8; Figs. 2-4
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	6-8; Figs. 2-4
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	6
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	7-9; Fig. 5
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	9-12
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	12-13
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	12
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	submitted to journal

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ORIGINAL RESEARCH
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Bidirectional Relationship Between Reduced Blood pH and Acute Pancreatitis: A Translational Study of Their Noxious Combination

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Acute pancreatitis (AP) is often accompanied by alterations in the acid-base balance, but how blood pH influences the outcome of AP is largely unknown. We studied the association between blood pH and the outcome of AP with meta-analysis of clinical trials, and aimed to discover the causative relationship between blood pH and AP in animal models. PubMed, EMBASE, and Cochrane Controlled Trials Registry databases were searched from inception to January 2017. Human studies reporting systemic pH status and outcomes (mortality rate, severity scores, and length of hospital stay) of patient groups with AP were included in the analyses. We developed a new mouse model of chronic metabolic acidosis (MA) and induced mild or severe AP in the mice. Besides laboratory blood testing, the extent of pancreatic edema, necrosis, and leukocyte infiltration were assessed in tissue sections of the mice. Thirteen studies reported sufficient data in patient groups with AP (n = 2,311). Meta-analysis revealed markedly higher mortality, elevated severity scores, and longer hospital stay in AP patients with lower blood pH or base excess ($P < 0.001$ for all studied outcomes). Meta-regression analysis showed significant negative correlation between blood pH and mortality in severe AP. In our mouse model, pre-existing MA deteriorated the pancreatic damage in mild and severe AP and, vice versa, severe AP further decreased the blood pH of mice with MA. In conclusion, MA worsens the outcome of AP, while severe AP augments the decrease of blood pH. The discovery of this vicious metabolic cycle opens up new therapeutic possibilities in AP.

Keywords: experimental pancreatitis, acidosis, acid-base balance, meta-analysis, mortality

INTRODUCTION

Acute pancreatitis (AP) is one of the most frequent gastrointestinal causes of hospitalization with significant morbidity and mortality in the US (Yadav and Lowenfels, 2013; Parniczky et al., 2016). Although the mortality rate in mild and moderate AP is low, this value is still unacceptably high (30%) in its severe form (Parniczky et al., 2016). Since no specific therapy is available, only prompt and accurate interventions, such as aggressive fluid therapy can be beneficial (Vinish et al., 2017).

An important function of the pancreas is bicarbonate production, which is required to maintain its constant "milieu intérieur," thereby to prevent premature activation of pancreatic proteases (Pallagi et al., 2011, 2015; Hegyi and Petersen, 2013). When pancreatic bicarbonate production is challenged by local or systemic acid load (i.e., metabolic acidosis, MA), the resulting lower pH can facilitate pancreatic enzyme activation and deteriorate cell damage (Reed et al., 2011). Furthermore, injection of acidic contrast solution either into the pancreatic duct or into the vein significantly increased the severity of AP in rats (Noble et al., 2008; Bhoomagoud et al., 2009). Beside an external acid load, the pancreatic pH balance can also be compromised by tissue injury such as AP, which can lead to acidification of local tissues, thus deteriorate cell damage (Behrendorff et al., 2010). The luminal pH of the main pancreatic duct was also lower in human patients with AP compared to controls (Takacs et al., 2013), suggesting that the development of AP is accompanied by a reduction of local pH. Multiple mechanisms have been implicated in AP which can lead to MA, including direct mechanisms such as the loss of bicarbonate-rich pancreatic juice via pancreatic fistula or drainage (Rice et al., 2014), as well as indirect ones through lactic acidosis which can sequentially occur in AP due to shock, sepsis, cardiovascular failure, or upper gastrointestinal bleeding (Zhan et al., 2015). However, the interaction between AP and systemic pH is still not fully clarified.

Acidosis is often considered as a marker of disease severity, viz., a by-product of systemic dysregulation, and as such it is a proven prognostic factor in the assessment of critically ill patients (Vincent and Moreno, 2010). Despite the fact that scoring systems, which are used to help the diagnosis and the assessment of the progression of AP, include the changes in systemic pH balance of the patients (e.g., Acute Physiology and Chronic Health Evaluation, APACHE II and Ranson scores), clinical trials aiming to reveal a correlation between the acid-base status and the outcome of AP are scarce. To our knowledge, the sole published human study, which aimed to directly answer this question showed that changes in the parameters of systemic acid-base status can predict mortality in AP (Sharma et al., 2014). On the contrary, the necessity of arterial blood gas sampling was questioned in patients with AP in another human study (Ward et al., 2008). With regards to the results obtained in experimental animals, a detailed analysis of the correlation between systemic pH and the outcome of AP would be of utmost importance, because it could establish blood pH as a predictor of the severity and the outcome of the disease and, arguably, identify acidosis as a therapeutic target in AP.

In the present study, by using a dual, translational approach, we have discovered a vicious, bidirectional interaction between blood pH and the outcome of AP. Based on our discovery, the possibility of new therapeutic approaches in AP can be suggested.

Abbreviations: AP, acute pancreatitis; APACHE, acute physiology and chronic health evaluation; CI, confidence interval; CRP, C-reactive protein; ES, estimated logit mortality rate; IL, interleukin; i.p., intraperitoneal(ly); LOS, length of hospital stay; MA, metabolic acidosis; MAP and SAP, mild and severe acute pancreatitis, respectively; SEM, standard error of mean; SMD, standardized mean difference; TNF, tumor necrosis factor.

MATERIALS AND METHODS

Study Design 1: Meta-Analysis of Clinical Trials

Search Strategy

Our meta-analysis was conducted in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (Moher et al., 2009) (**Supplementary Table 1**), similarly as in our recent study (Olah et al., 2018). The analysis was based on the Patients, Intervention (or indicator), Comparison, Outcome (PICO) model: in patients with AP, we aimed to assess the predictive role of the change in pH status (as assessed by blood pH, bicarbonate concentration, base excess, or base deficit) on disease severity (indicated by clinical scores), length of hospital stay (LOS), and mortality ratio. This meta-analysis has been registered with PROSPERO (CRD42017055396).

A search in the PubMed, EMBASE, and Cochrane Controlled Trials Registry databases was performed from inception to January 2017 using the following terms: "pancreatitis AND (mortality OR survival OR severity) AND ("arterial pH" OR "blood pH" OR "systemic pH" OR "base deficit" OR "base excess" OR bicarbonate OR HCO3- OR "anion gap" OR acidosis OR alkalosis OR acid-base)." We restricted our search to original human studies published in English without time period limitations. A manual search of the reference lists of relevant full-text articles was conducted to identify further potentially eligible articles. The search was conducted separately by two authors (ZRu, AG), who also assessed study eligibility and extracted data from the selected studies independently. Disagreements were resolved by consensus with the help of a third party (PH).

Study Selection and Data Extraction

The titles and abstracts of the publications from the literature search were screened and the full text of potentially eligible articles was obtained. We included studies in which blood pH or a related parameter (e.g., base excess, base deficit, or bicarbonate) and severity scores or LOS or mortality ratios were reported for the same group(s) of patients with AP. From all included articles we extracted the sample size, the reported mean pH or its related parameter for the studied patient groups with the corresponding standard error (SEM) or deviation, as well as the severity score, LOS, and mortality ratio within the group. To analyze the influence of the change in acid-base status on the severity and the outcome of AP, in each study we assigned the patient groups as a lower pH group and as a higher pH group,

irrespective from the original basis for grouping used by the authors of the study.

Outcomes of Interest

We used mortality ratio of the AP patients groups as the primary outcome. Regarding secondary outcomes, we used two commonly applied severity indices (i.e., APACHE II and Ranson scores) and the LOS.

Quality Assessment

We assessed the quality of each study included in the meta-analysis by using the Newcastle-Ottawa Scale (Wells et al., 2000; **Supplementary Table 2**).

Statistical Analysis

We used logit transformation of event rates for mortality ratios and standardized mean difference (SMD) for LOS and severity scores as the effect size data. The secondary outcomes were compared between the lower and higher pH groups (see above) within each study, and then the estimated pooled mean values were calculated. The relevant studies were compared with standard meta-analysis tools (e.g., forest plot) in case of each outcome.

Between-study heterogeneity was assessed by I^2 statistical test, where I^2 is the proportion of total variation attributable to between-study variability (an I^2 value of more than 50 was considered as indication of considerable statistical heterogeneity). The selection of patients, study design, and the used methods showed variability among the studies included in our analyses, which also resulted in statistical heterogeneity. Since the lack of statistical significant results on these heterogeneity tests could be also due to the lack of power because of the small number of studies eligible for the analyses, we used the random effect model in case of each forest plot, similarly to our earlier meta-analysis (Rumbus et al., 2017). Publication bias was assessed by funnel-plot analysis, Egger's test and Duval and Tweedie trim and fill method (**Supplementary Figures 1–5**). Publication bias plots were used to assess whether studies with small sizes could have been missed in our analyses, however, due to the design of these tests they do not allow to firmly rule out the possibility that some papers missed the inclusion criteria of our search.

As a different statistical approach to reveal a correlation between systemic pH and mortality in moderate and severe forms of AP, we performed meta-regression analysis of those studies in which both blood pH and mortality rate were reported within the same patient group. The meta-analyses were performed with Comprehensive Meta-Analysis (version 3.3; Biostat, Inc., Engelwood, MJ, USA) and Stata (version 11.1; StataCorp, College Station, TX, USA) software.

Study Design 2: Experimental Procedures

Animals

The experiments were performed in 40 female FVB/N mice (Charles Rivers Laboratories, Wilmington, MA, USA). This commercially available, multipurpose mouse strain is characterized by excellent reproductive performance and it was repeatedly used by our group to study the mechanisms of AP (Kui et al., 2015; Maleth et al., 2015). The mice were housed in standard plastic cages kept in a room with an ambient temperature of 24°C on a 12-h light-dark cycle in the animal facility of the First Department of Medicine at the University of Szeged. The mice were allowed free access to water and standard laboratory chow for rodents (Biofarm, Zagyvaszanto, Hungary).

Ethics

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Szeged and also by an independent committee assembled by national authorities (XII/3773/2012.). All experiments were conducted in compliance with the European Union Directive (2010/63/EU) and the Hungarian government decree (40/2013, II.14.).

Experimental Modeling of Chronic MA in Mice

To develop a mouse model of chronic MA, the mice were randomly divided into the following 4 groups for a 12-day treatment: (i) ammonium chloride (NH_4Cl) administration with drinking water (8.2 ± 0.5 ml/day/mouse) as reported in earlier studies (Galicek et al., 1981; Nowik et al., 2010); (ii) intraperitoneal (i.p.) injections of NH_4Cl (0.5 ml, 0.28 M) on days 1 and 6; (iii) administration of NH_4Cl with drinking water (as in group 1) and i.p. injections (as in group 2); and (iv) controls, receiving NH_4Cl-free tap water and 2 i.p. injections of saline on days 1 and 6.

Experimental Modeling of AP

Two different types of AP were used in this study. Mild acute pancreatitis (MAP) was induced by alcohol and fatty acid as described earlier (Huang et al., 2014; Maleth et al., 2015). Severe acute pancreatitis (SAP) was induced by the injections of cerulein (50 μg/kg, i.p.) at start time, and then at every hour for 9 h (Mareninova et al., 2006). In the chronic MA model, MAP and SAP were induced on day 12 of the acidifying treatment.

Laboratory Measurements

Animals were euthanized by i.p. injection of sodium pentobarbital (50 mg/kg). Blood samples were collected by cardiac puncture. Serum amylase activity was measured by using a colorimetric kinetic method (Diagnosticum, Budapest, Hungary). Serum concentrations of creatinine and glucose as well as urea concentration in urine were measured with commercially available laboratory kits (Institute of Laboratory Medicine, University of Szeged). Arterial blood samples were collected in sealed plastic capillaries (170 μl), which were previously treated with lithium and heparin. Analysis of the arterial blood samples was performed by a blood gas analyzer (Cobas b221 system; Roche Ltd., Basel, Switzerland) within 1 min after blood collection at room temperature (22°C).

Histology

Histological evaluations were performed as described earlier (Kui et al., 2015). In brief, the extent of pancreatic edema (0: none; 1: patchy interlobular; 2: diffuse interlobular; 3: diffuse interlobular and intraacinar), necrosis (%), and leukocyte infiltration (0: none; 1: rare patchy interlobular; 2: patchy interlobular; 3: diffuse

interlobular; 4: diffuse interlobular and intraacinar) were assessed in pancreatic tissue sections stained with haematoxylin and eosin under a light microscope (Zeiss Axio scope A1 microscope) at 40x magnification by an investigator who was expert in pancreas histology, however blinded to the animal's treatment group. The percentage of acinar cell necrosis was evaluated by ImageJ software (NIH, Bethesda, MD, USA).

Statistical Analysis

Data were compared by one-way ANOVA followed by Holm-Sidak test, two-way ANOVA followed by Fischer Least Significant Difference test, or two-tailed Student's *t* test, as appropriate. SPSS 23.0 (IBM, Armonk, NY, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) software was used for statistical analysis. The effects were considered significant when $P < 0.05$. In the experimental part of the study, data are reported in the Mean ± SEM format.

RESULTS

Meta-Analysis

Study Selection

The flow chart of the study selection is presented in **Figure 1**. Until January 2017 the electronic literature search identified altogether 1,076 studies from the PubMed, EMBASE, and Cochrane databases. After enabling filters for human studies and English language and removal of duplicates, 793 articles remained, which were screened on title and abstract for inclusion criteria. Full texts of the remaining 122 articles were reviewed in detail. In 109 studies pH parameters or outcomes were not suitably reported in the patients with AP, therefore these were also excluded. As a result, 13 full-text publications were found eligible for statistical analysis which included data from a total of 2,311 patients (Ranson et al., 1976; Nair et al., 2000; Eachempati et al., 2002; Zhu et al., 2003; Kaya et al., 2007; Keskinen et al., 2007; Pupelis et al., 2007; De Campos et al., 2008; Shinzeki et al., 2008; Lei et al., 2013; Sharma et al., 2014; Zhan et al., 2015; Shen et al., 2016). The characteristics of these studies are summarized in **Supplementary Table 3**.

Reduction of Blood pH Is Associated With Higher Mortality Rate in AP

First, we investigated the association between systemic (blood) pH status and our strongest endpoint, viz., the mortality. Our meta-analysis revealed a logit event rate of −0.09 (95% CI, −0.79, 0.61), corresponding to an average mortality rate of 51.0% (95% CI, 31.5, 70.1) in the more acidotic patient groups, while in the patient groups with higher pH or bicarbonate level the logit event rate was −3.68 (95% CI, −4.81, −2.55), which corresponds to an average mortality rate of 3.0% (95% CI, 1.2, 7.1) (**Figure 2**). The mortality ratios were significantly different between the two groups ($P < 0.001$).

Lower pH or Bicarbonate Concentration Worsens the Severity of AP

We wanted to know whether the change in acid-base status can also predict the severity of AP as assessed by clinical scores. Thus, we studied the association between blood pH and clinical severity scores. We found two scores, the Ranson and the APACHE II scores, which were reported in suÿ cient number of studies for statistical analysis (Ranson et al., 1976; Nair et al., 2000; Eachempati et al., 2002; Zhu et al., 2003; Kaya et al., 2007; Keskinen et al., 2007; Pupelis et al., 2007; De Campos et al., 2008; Shinzeki et al., 2008; Lei et al., 2013; Sharma et al., 2014; Zhan et al., 2015; Shen et al., 2016). Meta-analysis revealed that the pooled SMDs of the Ranson score (0.92, 95% CI, 0.58, 1.26) and the APACHE II score (1.38, 95% CI, 0.95, 1.81) were significantly positive between the patient groups with lower pH or bicarbonate levels and the less acidotic groups of patients ($P < 0.001$) (**Figures 3A,B**). These standardized values correspond to 1.60 (95% CI, 0.77, 2.42) higher Ranson score and 7.40 (95% CI, 5.05, 9.75) higher APACHE II score in the more acidotic patients with AP. The correlation found between lower blood pH and higher clinical scores could be expected as these scores also include blood pH in their calculation (Vincent and Moreno, 2010), nevertheless, these results confirm the feasibility of our meta-analysis approach to reveal an interaction between systemic pH and the outcome of AP.

Acidosis Is Associated With Longer Hospitalization in AP

Next, we analyzed the LOS in patients with AP by using the same grouping of acid-base status as used for mortality and severity scores. For the meta-analysis, LOS was expressed as SMD between the patient groups. We found that the pooled difference was significantly positive between the more acidotic patient groups and the groups with higher pH or bicarbonate concentrations (0.89, 95% CI, 0.73, 1.04; $P < 0.001$) (**Figure 4**), which difference corresponds to 15.05 days (95% CI, 10.84, 19.19) longer LOS in the more acidotic AP patient group.

Meta-Regression Analysis

As a further statistical approach to determine a correlation between blood pH and mortality in the more progressed forms of AP, we also performed a meta-regression analysis on the collected data. For that, we used those study groups, in which pH and mortality rate was reported in moderately severe or severe manifestations of AP for the same patient groups (Zhu et al., 2003; Lei et al., 2013; Shen et al., 2016). We found a significant correlation between pH and mortality rate with a regression slope of −55.4 (95% CI, −97.9, −12.9; $P = 0.011$) (**Figure 5**). The potential reason for statistical heterogeneity, as revealed in the forest plots, could not be evaluated in the meta-regression analysis because of the small number of eligible studies.

Experimental Animal Model

Acidosis Is Augmented in Severe Form of AP

Analyses of data from 2,311 patients showed strong association between acidosis and the outcome of AP. Therefore, we moved from the "bedside to the bench" to clarify their causative relationship. First, we developed a new experimental model to mimic chronic MA by comparing different types (oral or i.p. or both) of acidifying treatments in mice. We

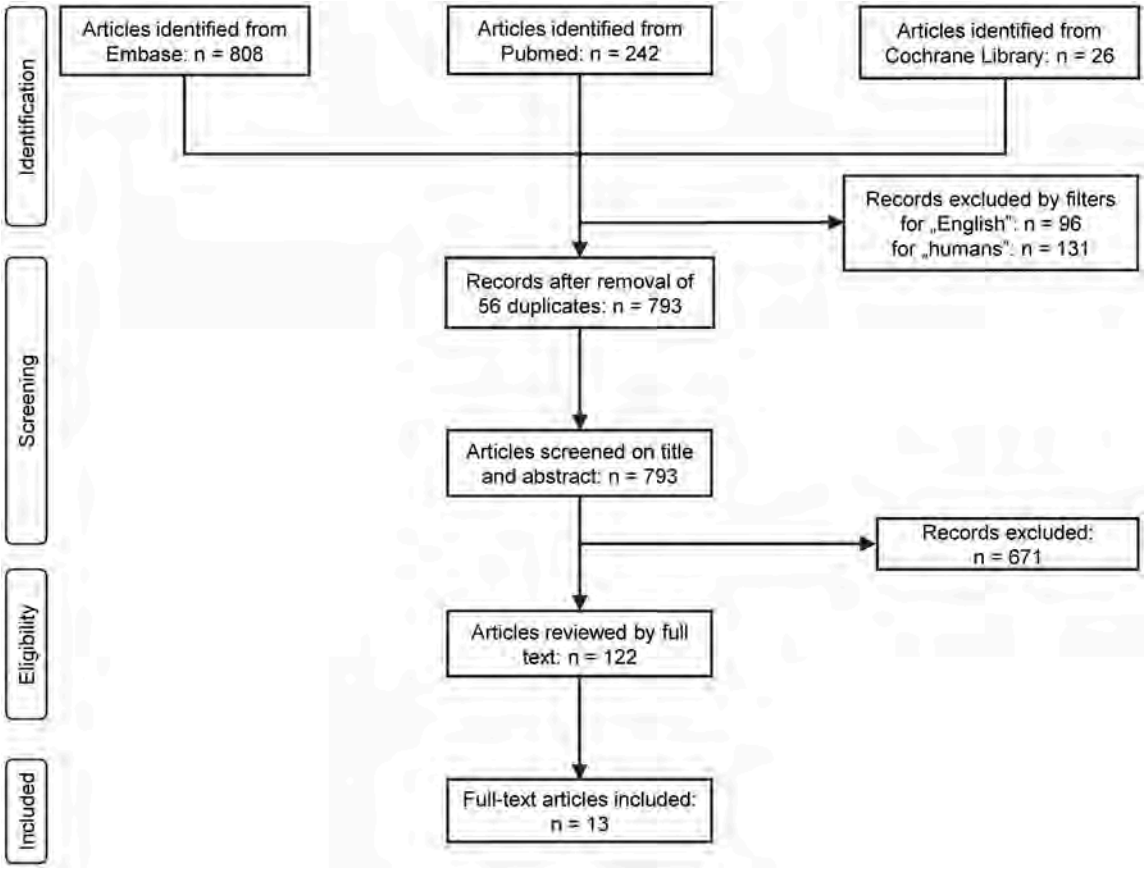

FIGURE 1 | Flow chart of study selection and inclusion.

found that MA can be induced in mice by the combined administration of oral and i.p. NH_4Cl, which decreased blood pH to 6.80 ± 0.04, but it did not cause any pancreatic damage (**Supplementary Figure 6**), nor did it change serum glucose and urine urea levels (**Supplementary Figure 7**). Similarly to pH, arterial blood bicarbonate level decreased most significantly ($P < 0.001$) in the combination (oral and i.p.) treatment group as compared to controls (16.5 ± 0.9 vs. 26.4 ± 0.8 mmol/l) (**Supplementary Figure 7**). We detected no significant differences in the serum concentrations of creatinine, sodium, and potassium among the different treatment groups (data not shown). We used this MA model to study the interaction between acidosis and AP. For that, mice with or without pre-existing MA were assigned to MAP, SAP, and control (no AP) groups and their arterial blood pH were compared. As expected, pre-existing MA induced by dual (oral and i.p.) acidifying treatment resulted in significantly decreased blood pH in the mice without AP, as well as in mice with either MAP or SAP (**Figure 6**). In the mice with pre-existing MA, the extent of the pH reduction was similar in the sham AP and MAP groups (7.08 ± 0.04 and 7.11 ± 0.03, respectively), while in the mice with SAP the arterial pH decreased to 6.97 ± 0.05, which was significantly lower than in the sham AP and MAP groups ($P < 0.05$ compared to both), suggesting that SAP further deteriorates MA (**Figure 6**). As expected, MA also resulted in decreased arterial blood bicarbonate levels, which reached the level of significance in the MAP group (16.7 ± 1.4 mmol/l; $P < 0.01$) (**Supplementary Figure 8**). The levels of urea in the urine were markedly decreased in mice with SAP (3.6 ± 0.2 mmol/l) regardless of their pH status as compared to the urine urea levels in the sham AP groups without and with pre-existing MA (7.4 ± 0.2 and 7.5 ± 0.1 mmol/l, respectively; $P < 0.001$ for both). Importantly, MA significantly lowered urine urea levels in mice with MAP compared to mice with MAP without pre-existing MA (5.9 ± 0.7 vs. 8.9 ± 1.0 mmol/l; $P < 0.05$) (**Supplementary Figure 8**). We did not detect significant difference in the serum concentrations of glucose (**Supplementary Figure 8**), and in the levels of creatinine, sodium, and potassium among the different treatment groups (data not shown).

Pre-existing Acidosis Deteriorates Both Mild and Severe Forms of AP in Mice

To determine whether the presence of pre-existing MA has any effects on the pancreatic damage during AP, pancreatic edema, necrosis, and leukocyte infiltration scores were assessed in pancreatic sections of mice without AP, or with MAP or SAP in the presence and the absence of pre-existing MA. The

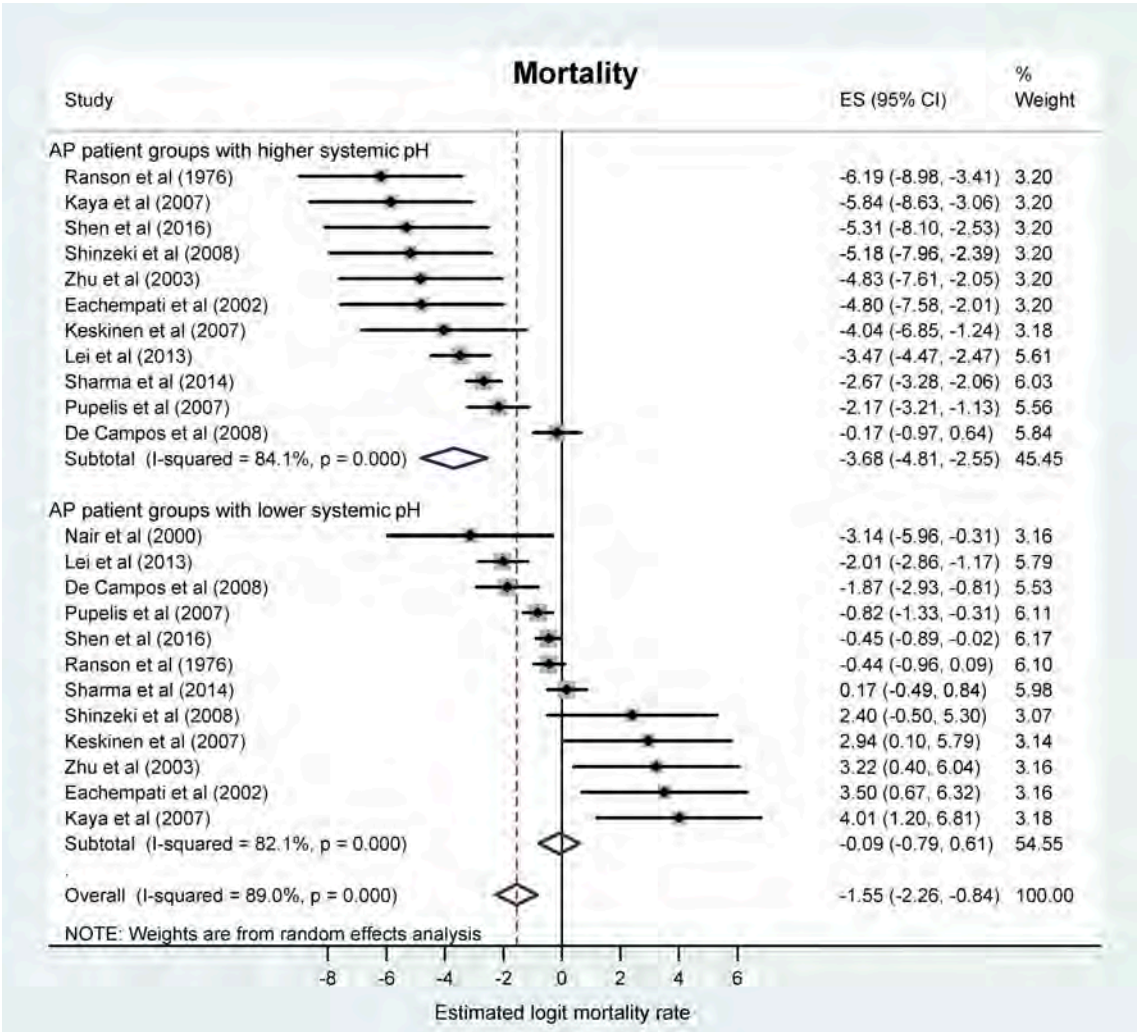

FIGURE 2 | Forest plot of mortality rate using random-effects model in different systemic pH groups of patients with acute pancreatitis (AP). For each patient group, black circles and horizontal lines represent the estimated logit mortality rate (ES) and the corresponding confidence interval (CI), respectively. Lower ES corresponds with lower mortality rate and vice versa. Gray squares indicate the relative statistical weight of a given patient group. Open diamonds show the average ES and CI of patient groups with higher systemic pH (top), lower systemic pH (middle), and all patient groups (bottom).

acidifying treatment caused no pancreatic damage in the control (no AP) mice (**Figure 7**), which is line with our previous results (**Supplementary Figure 6**). On the contrary, in mice with pre-existing MA, MAP resulted in significantly larger edema (2.0 ± 0.3 vs. 1.4 ± 0.2; $P < 0.05$), increased necrosis (21.0 ± 2.4 vs. 10.0 ± 2.2%; $P < 0.05$), and elevated leukocyte infiltration (2.5 ± 0.4 vs. 1.6 ± 0.2; $P < 0.05$) compared to MAP in mice with normal blood pH (**Figure 7**). Pancreatic damage was also markedly more pronounced in SAP in mice with pre-existing MA compared to SAP in mice with normal blood pH as indicated by increased edema (3.6 ± 0.2 vs. 2.4 ± 0.2; $P < 0.05$), necrosis (38.6 ± 5.0 vs. 25 ± 2.2%; $P < 0.01$), leukocyte infiltration (3.6 ± 0.4 vs. 2.6 ± 0.2; $P < 0.05$), and serum amylase activity (12,730 ± 384 vs. 11,362 ± 106 Unit/l; $P < 0.05$) (**Figure 7**). These results suggest that MA further deteriorates pancreatic damage in both MAP and SAP.

DISCUSSION

In the present study, we revealed a strong association between blood pH and the outcome of AP with meta-analysis of human studies. Our analyses showed that lower blood pH predicts higher mortality rate, longer LOS, and worsens the severity of AP. A significant negative correlation between blood pH and mortality rate in severe forms of AP was found with meta-regression analysis of the human studies. To better clarify how MA can interact with AP, we developed a mouse model of chronic MA and showed that SAP worsens the MA in the mice. In the same model we also demonstrated that pre-existing MA further deteriorates the tissue damage in both mild and severe forms of AP.

Although previous human studies indicated a link between MA and AP (Nair et al., 2000; Zhu et al., 2003; Shinzeki et al., 2008; Sharma et al., 2014; Shen et al., 2016), we found only one,

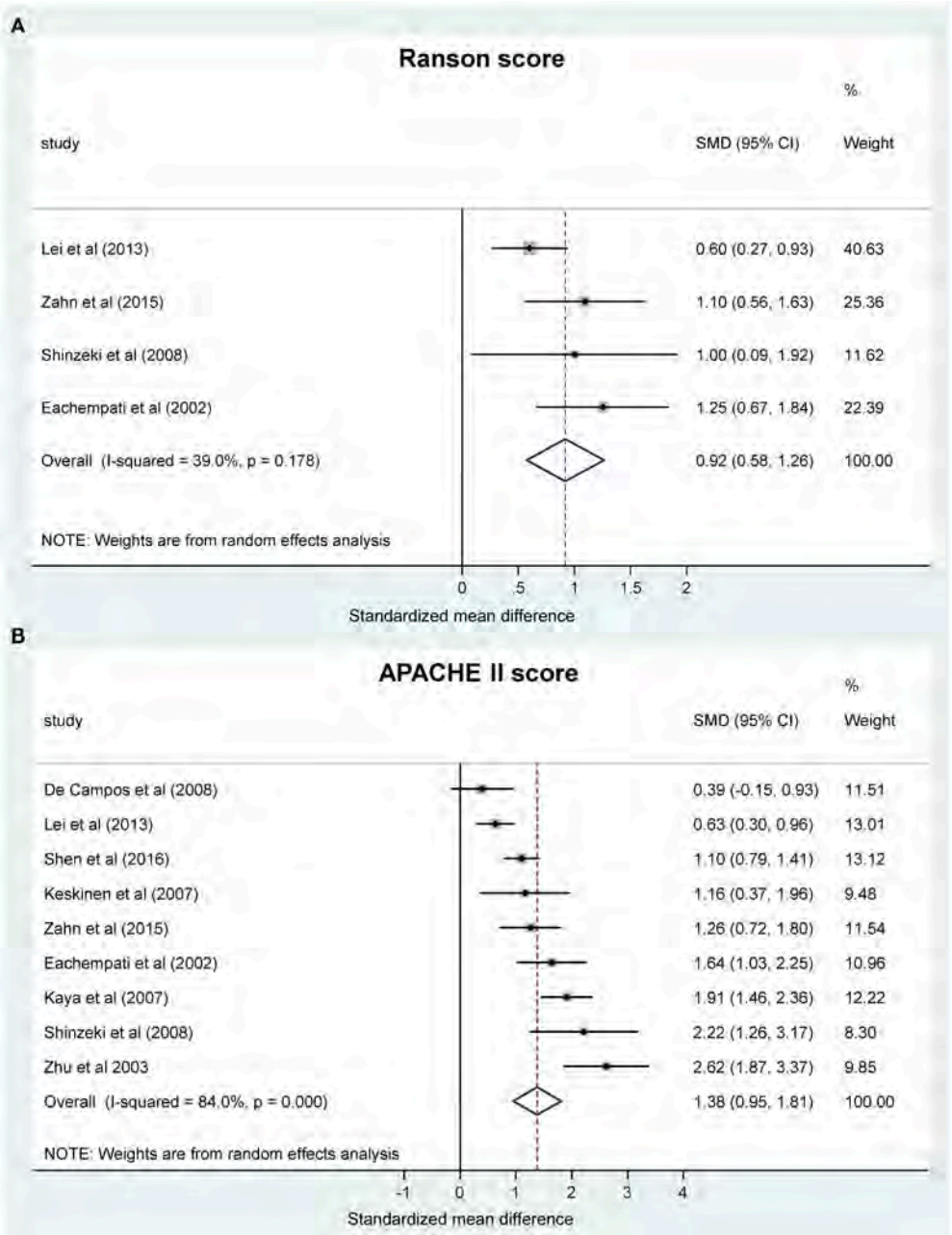

FIGURE 3 | Forest plot of **(A)** Ranson scores and **(B)** Acute Physiology and Chronic Health Evaluation (APACHE II) scores using random-effects model in different systemic pH groups of patients with acute pancreatitis. Here and in **Figure 4**, in each study the standardized mean difference (SMD) of the outcome was calculated between the patient group with lower and higher pH. Black circles represent the SMD for each study, while the left and right horizontal arms of the circles indicate the corresponding 95% confidence intervals (CI) for the SMD for each study. The size of the gray box is proportional to the sample size of the study; bigger box represents larger sample size, thus bigger relative weight of the study, and vice versa. Circles close to zero represent smaller SMD between the lower and higher pH groups in the given study. A positive SMD means higher score (**Figure 3**) or longer hospital stay (**Figure 4**) in the patient group with lower pH compared to the patient group with higher pH. The diamond on the bottom represents the averaged SMD calculated from the SMDs of all the individual studies. The vertical dashed line is determined by the two vertical points of the diamond and indicates the value of the averaged SMD of all studies. The horizontal points of the diamond represent the 95% CI of the averaged SMD.

single-center prospective study which directly aimed to explore this correlation (Sharma et al., 2014). Because of the scarcity of data available from targeted clinical trials, we aimed to clarify the association between MA and AP by systematic review of the current literature and by meta-analysis of the available data. By identifying 13 eligible studies for the analysis (Ranson et al.,

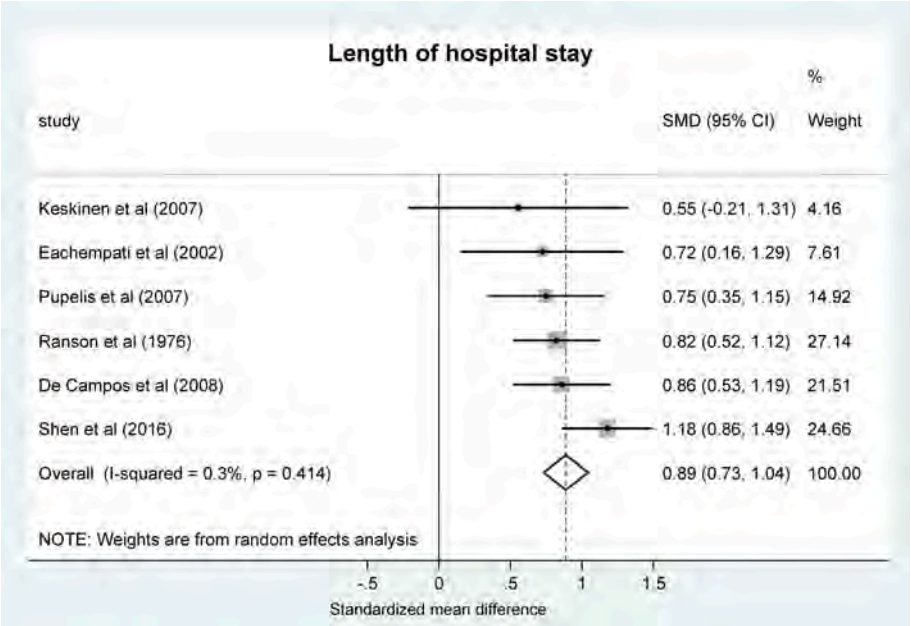

FIGURE 4 | Forest plot analysis of the length of hospital stay using random-effects model in different systemic pH groups of patients with acute pancreatitis. For explanation, see the legend of **Figure 3**. SMD, standardized mean difference; CI, confidence interval.

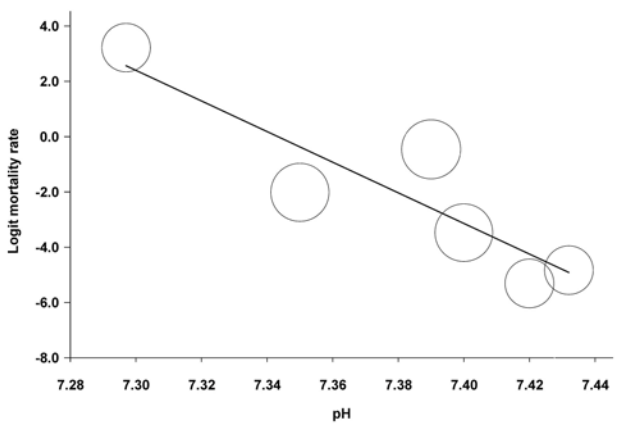

FIGURE 5 | Meta-regression analysis of the association between blood pH and mortality rate in patients with moderately severe and severe forms of acute pancreatitis. The circles indicate estimated logit mortality rate calculated for each patient group. A lower calculated value corresponds with lower mortality rate and vice versa. The circle size is proportional to the precision of the estimated logit mortality rate. The solid black line represents the weighted regression line based on variance-weighted least squares.

1976; Nair et al., 2000; Eachempati et al., 2002; Zhu et al., 2003; Kaya et al., 2007; Keskinen et al., 2007; Pupelis et al., 2007; De Campos et al., 2008; Shinzeki et al., 2008; Lei et al., 2013; Sharma et al., 2014; Zhan et al., 2015; Shen et al., 2016), we included 2,311 patients with AP in the analyses. In all of these studies, blood sample analysis was performed at admission or within 24 h thereafter, hence the blood pH parameters were determined with practically the same latency compared to the time when AP was diagnosed. Unavoidably however, the disease could progress to different stages in the different patients before the diagnosis has been reached. There were huge differences between the protocols of the individual studies, but it is remarkable that no matter how the patients were grouped by the authors originally, the patient group with lower pH had always (with no exceptions) worse outcomes (mortality rate, LOS, severity scores) than the group with higher pH in AP, which suggests that in the early stages (viz., until the time of diagnosis) of AP acidosis is an important influencing factor of the outcome regardless from the actual progression of the disease. Unfortunately, the design of the studies did not allow to analyze the causative relationship between MA and AP. In most of the studies, the systemic pH status of the patients prior to or repeatedly after the diagnosis of AP was not reported, thus the dynamics in the changes of pH during the time course of AP could not be assessed in the current analysis, but it is notable that the average base deficit was markedly (4-8 fold) higher in populations of patients, who did not survive SAP (Kaya et al., 2007; Keskinen et al., 2007). In the prospective trial by Sharma et al. (2014), in those SAP patients, who had a blood pH of less than 7.35, the mortality rate was nearly 10 times higher than in those patients whose pH was above this level (54 vs. 6.5%).

As limitations of our study, it should be mentioned that even though our meta-analysis showed a clear association between blood pH and the outcome of AP, since originally the patients were not divided into subgroups based on their blood pH by the authors, the independent effect of lower blood pH on the outcome and the cause-effect relationship between MA and AP could not be assessed. Because of the same reason and also to reduce the inter-study heterogeneity, in each of the analyzed

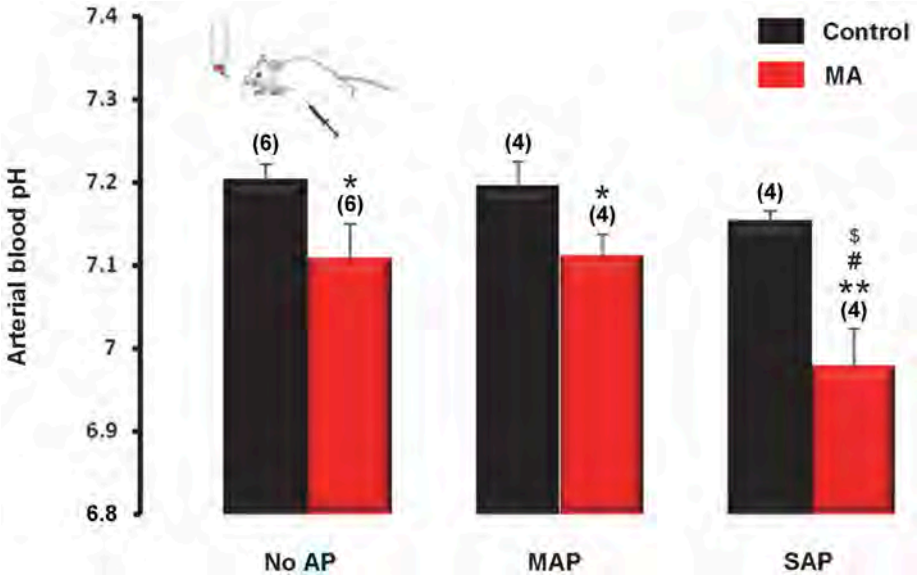

FIGURE 6 | Arterial pH of mice with metabolic acidosis (MA) induced by combination of oral and i.p. NH_4Cl administration and without acidifying treatment (control). On day 12 of the acidifying treatment, mild acute pancreatitis (MAP) or severe acute pancreatitis (SAP) was induced by alcohol and fatty acid or cerulein, respectively. Mice in the sham pancreatitis group (no AP) were injected i.p. with saline. Statistically significant differences are marked with *between MA and control (non-acidotic) groups, with $^{\#}$between no AP and MAP groups in MA, and with $^{\$}$between no AP and SAP groups in MA, as follows: $^{*}P < 0.05$ and $^{**}P < 0.01$ for MA vs. control in no AP, MAP, and SAP; $^{\#}P < 0.05$ for no AP in MA vs. SAP in MA; $^{\$}P < 0.05$ for MAP in MA vs. SAP in MA.

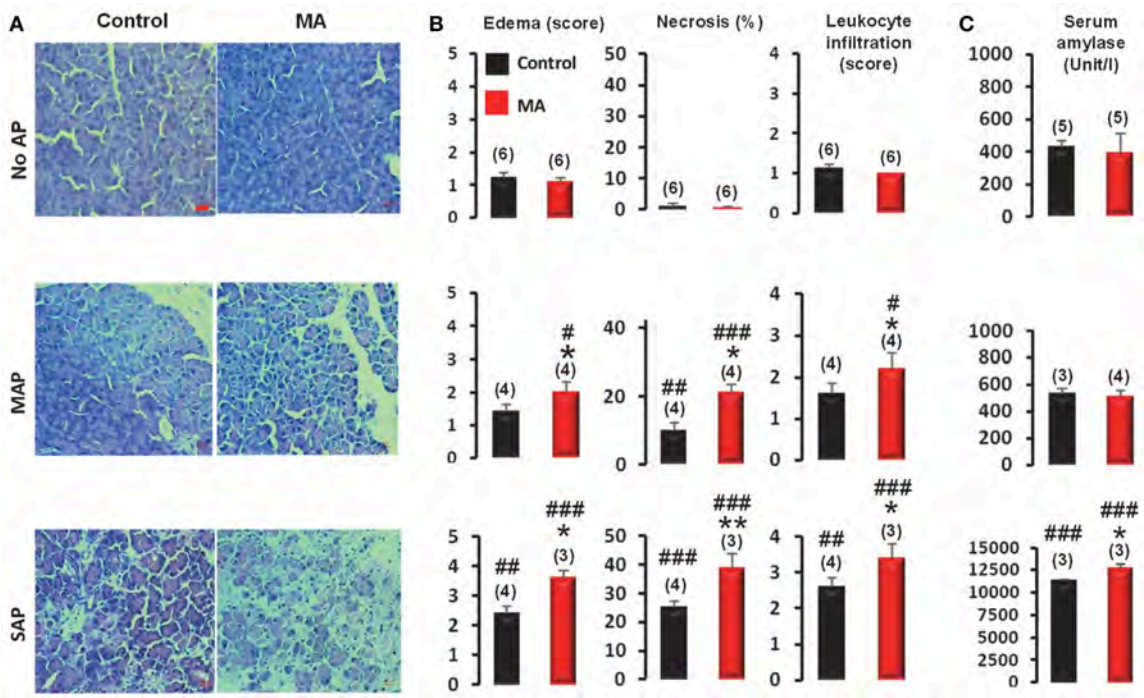

FIGURE 7 | Assessment of the severity of acute pancreatitis (AP) in mice with and without metabolic acidosis (MA and control, respectively). **(A)** Representative microphotographs of pancreatic sections, **(B)** histological evaluation of edema scores, necrosis, and leukocyte infiltration scores, and **(C)** serum amylase levels of MA and control mice with mild acute pancreatitis (MAP), severe acute pancreatitis (SAP) or without acute pancreatitis (no AP). Scale bar represents 20 μm. Statistically significant differences are marked with *between MA and control (non-acidotic) groups and with $^{\#}$between no AP and either MAP or SAP groups, as follows: $^{*}P <$ 0.05; $^{**}P < 0.01$ for MA vs. control in MAP and SAP; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ for no AP vs. MAP and SAP.

studies we assigned one patient group as the lower pH group and the other one as the higher pH group. Since the reported pH values differed substantially among the analyzed studies, the cut-off value between the lower and the higher pH groups was individually determined for each study. Consequently, in the present analysis we could not determine a specific cut-off pH value which would be detrimental for the outcome of AP. The most convincing method to obtain direct evidence for the role of acidosis as an independent risk factor in AP, determine a detrimental cut-off pH value, and gain insight into the cause-effect relationship in humans would be to conduct targeted clinical trials in which patients with AP are grouped based on their blood pH at admission and their acid-base status as well as the severity and the outcome of AP is continuously monitored. By collecting data of individual patients in such clinical trials, it would be possible to statistically analyze the direct (independent) effect of acid-base disturbances on AP. Until such or similar trials are conducted, we are restricted to use different (not so direct) approaches such as meta-analyses and animal experiments.

To discover whether a pre-existing MA worsens the outcome of AP or MA is rather the result of the progression of AP, we moved from the "bedside to the bench." Gorelick's workgroup has discovered that low extracellular pH induces pathophysiological changes in acinar cells (Bhoomagoud et al., 2009; Reed et al., 2011). They described that reduced pH sensitizes the acinar cell to secretagogue-induced pancreatitis responses in rats, and enhances connexin32 degradation and ryanodine receptor-mediated calcium signaling in the basolateral region of the acinar cell which mechanisms are responsible for the injurious effects of low extracellular pH on the exocrine pancreas (Bhoomagoud et al., 2009; Reed et al., 2011, 2014). However, the authors have not investigated the causative relationship between low pH and pancreatitis. Since no mouse model of chronic MA was available in the literature, first we designed a set of experiments to develop the most suitable MA model. Dual administration (oral and i.p.) of acidic fluid induced a marked pH drop in the blood without damaging the pancreas. By supplementing the oral treatment with i.p. acidification, our model also accounted for such conditions, when primarily the pH of the peritoneal fluid is reduced such as bacterial peritonitis (Glinska-Suchocka et al., 2016), carbon dioxide insufflation during laparoscopy (Duerr et al., 2008), or peritoneal dialysis (Farhat et al., 2008). Notably, in our model, MA developed gradually and persisted for several days in the mice which is very similar to the development of MA in human patients. Indeed, there is evidence that in human patients AP can develop in pre-existing MA, for instance in diabetic ketoacidosis either via hyperlipidemia (Nair and Pitchumoni, 1997; Nair et al., 2000) or through distinct mechanisms (Gianfrate and Ferraris, 1998). It should be noted however that in clinical settings MA typically occurs as a consequence of AP and in many cases it does not pre-exist. The shown reverse relationship between MA and AP, namely that the presence of a pre-existing acidosis can influence the severity of AP, warrants for careful pH management in such clinical situations.

Sodium bicarbonate therapy is widely accepted for the treatment of MA in conditions associated with the loss of bicarbonate (e.g., renal tubular acidosis, diarrhea), however its use to increase pH in diseases associated with acidosis not due to bicarbonate loss is questionable because of its adverse effects, for example, intracellular acidosis, hypokalemia, and decreased serum ionized calcium concentration (for reviews, see Adeva-Andany et al., 2014; Hopper, 2017). In AP, bicarbonate production becomes impaired due to the damage of the pancreatic tissue, hence when systemic pH decreases bicarbonate administration can be beneficial to maintain normal pH, thereby to improve the outcome based on our results. It has to be noted that, to our knowledge, currently there is no evidence for the benefits of bicarbonate administration in AP. In contrast with sodium bicarbonate therapy, a growing body of evidence supports the beneficial effects of lactated Ringer's solution in the treatment of AP. Since lactate is metabolized to bicarbonate in the liver, lactated Ringer's solution was successfully used to lessen the metabolic acidosis by elevating blood bicarbonate levels and to attenuate the systemic inflammation response as assessed by lower C-reactive protein (CRP) levels in patients with AP (Wu et al., 2011; de-Madaria et al., 2018). Administration of lactated Ringer's solution resulted in lower mortality rate in critically ill patients with AP (Aboelsoud et al., 2016) and it lead to transiently reduced systemic inflammation in patients with MAP (Choosakul et al., 2018), although it had no therapeutic benefits in AP in a retrospective study (Lipinski et al., 2015). For the initial management of AP, the American College of Gastroenterology guideline recommends lactated Ringer's solution as the preferred isotonic crystalloid fluid replacement with moderate quality of evidence (Tenner et al., 2013). Future clinical trials are warranted to confirm the beneficial effects of tightly controlled pH management and to identify the optimal type of fluid resuscitation in patients with AP and pre-existing MA.

Our experiments clearly showed a strong bilateral link between pH and AP. We showed that pre-existing MA worsens the outcome of AP, whereas AP reduces pH in the blood which vicious cycle could be one of the main reasons for the high mortality rate in AP. The exact mechanism of how MA can deteriorate AP remains subject for future studies, but it can be assumed that complex regulatory mechanisms, such as the pancreatic damage and zymogen activation, neurogenic inflammation, and activation of inflammatory cells and mediators, are involved; for a comprehensive review, see Gorelick and Thrower (2009). Similarly, the question of whether the augmented acidosis is a direct or an indirect consequence (e.g., through impaired kidney and/or lung functions) or a combination of these in AP remains to be answered. Indeed, several complications of AP such as renal, pulmonary, and cardiovascular failure can cause disturbances in the acid-base balance. The development of acute renal dysfunction was reported in several of the analyzed studies (Keskinen et al., 2007; Pupelis et al., 2007; Lei et al., 2013; Sharma et al., 2014; Shen et al., 2016), and the impaired kidney function (determined by increased serum creatinine and blood urea nitrogen levels) was associated with significantly worse outcome, including higher mortality rates in AP (Talamini et al., 1999; Eachempati et al., 2002). Moreover, the frequency of renal failure increased by 5-10 times if acidosis (i.e., blood pH $<$ 7.35, base deficit $>$ 4 mEq/l, or bicarbonate $<$ 22 mEq/l) occurred in AP (Sharma et al., 2014). Since we found only one clinical trial which directly

investigated the relationship between renal failure and acidosis in AP (Sharma et al., 2014), the available data in humans were not suÿ cient for meta-analysis. In our experimental model, acute renal dysfunction occurred in mice with SAP regardless of their pH status, moreover the presence of pre-existing MA significantly impaired the kidney functions in mice with MAP, which is in harmony with the observations in humans and provides direct experimental support to the association between acidosis and renal failure in AP.

Cytokines are important mediators in the whole process of AP. A number of proinflammatory mediators, such as interleukin (IL)-1, 6, 8, and tumor necrosis factor (TNF)-α, were shown to play a role in AP in experimental animals (Norman et al., 1997; Liu et al., 2003; Meng et al., 2005) and in human patients (de Beaux et al., 1996; McKay et al., 1996; Brivet et al., 1999; Mayer et al., 2000). Cytokine production occurs in the pancreas first, and then with the progression of the disease in distant organs like the lungs, liver, and spleen (Norman et al., 1997). The levels of IL-6, 8, and TNF-α are even more increased in SAP than in MAP (McKay et al., 1996; Pooran et al., 2003). Active digestive enzymes which are released from injured pancreatic cells can potently stimulate proinflammatory cytokine production in macrophages (Desser et al., 1994; Lundberg et al., 2000), moreover the pancreatic acinar cells can also produce proinflammatory cytokines (Gukovskaya et al., 1997; Brady et al., 2002). For example, amylase can induce the production of IL-1, 6, and TNF-α in human peripheral blood mononuclear cells and in dermal fibroblasts (Desser et al., 1994; Malpass et al., 2013). Lipase markedly induced TNF-α production in rat macrophages (Jaffray et al., 2000), while CRP was shown to strongly correlate with IL-6 levels in patients with AP (Viedma et al., 1992). In our mouse model, we found that amylase level was elevated in SAP, and it was further increased in the presence of a pre-existing acidosis, therefore it can be expected that circulating cytokine levels are also higher in the co-existence of SAP and MA than in SAP without acidosis. In patients with SAP, the serum lipase and CRP levels were higher when their blood pH was lower (Pupelis et al., 2007), thus suggesting higher levels of circulating cytokines.

The production of pro- and anti-inflammatory cytokines was repeatedly shown to depend from the extracellular pH (for reviews, see Kellum et al., 2004b; Okajima, 2013; Casimir et al., 2018). Although the different forms and severities of acidosis can differently influence cytokine production (Kellum et al., 2004b), a proinflammatory effect, including enhanced TNF-α synthesis and augmented nuclear factor-κB activation, of hyperchloremic acidification was shown in activated macrophages by independent groups (Bellocq et al., 1998; Heming et al., 2001; Kellum et al., 2004a). Also, decreasing extracellular pH caused increasing IL-8 expression and nuclear factor-κB activation in human pancreatic tumor cells (Shi et al., 2000). In addition to the recruitment of immune cells by the low pH-induced cytokine release, extracellular acidosis *per se* promotes the activation of neutrophils (Martinez et al., 2006), which is in line with the increased leukocyte infiltration in MAP and SAP with pre-existing acidosis compared to MAP and SAP with initially normal blood pH, as observed in the present study (**Figure 7B**). Here, we revealed a bidirectional relationship constituting a vicious circle between AP and acidosis and developed a mouse model for studying the underlying mechanisms of the progression of AP in pre-existing MA. However, the experimental conformation of the dynamics of tissue and circulating cytokine concentrations and other potential processes (e.g., calcium signaling) in this model remains subject for future studies.

In summary, by the meta-analysis of literature data available from human studies we found a significant correlation between low systemic pH and the outcome of AP, indicating that lower pH level is associated with higher mortality rates, longer LOS, and more severe AP. With regards to the mechanism, in experimental animals we showed the existence of a bidirectional interaction between MA and AP, in which pre-existing MA deteriorates AP and, vice versa, AP further increases the severity of MA. Our findings suggest that systemic pH level should be closely monitored in patients with AP and that interventions to normalize the low pH of patients with AP should be considered in clinical settings. Well-designed, targeted clinical trials are warranted to evaluate the effects of the therapeutic interventions of acidosis in patients with AP.

AUTHOR CONTRIBUTIONS

ZRu and AG conducted the literature search of meta-analysis and the quality assessment of included studies, and extracted data from the articles. ET, JM, ZB, and PH performed the experiments. PH and AG conceived and supervised the meta-analysis and the experimental procedures and obtained funding. ZRu, ET, LP, JM, AG, and PH analyzed and interpreted the data. ZRu, ET, LP, PH, and AG wrote the paper. LP, EO, AV, GV, LC, KM, AM, ZRa, ZB, JK, IF, and JM reviewed and contributed to the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01360/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

Supplementary Table 1. PRISMA checklist.

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	not applicable
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	2
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	2
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	2
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	2
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	2
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	2
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	2
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	2
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	2-3
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	3

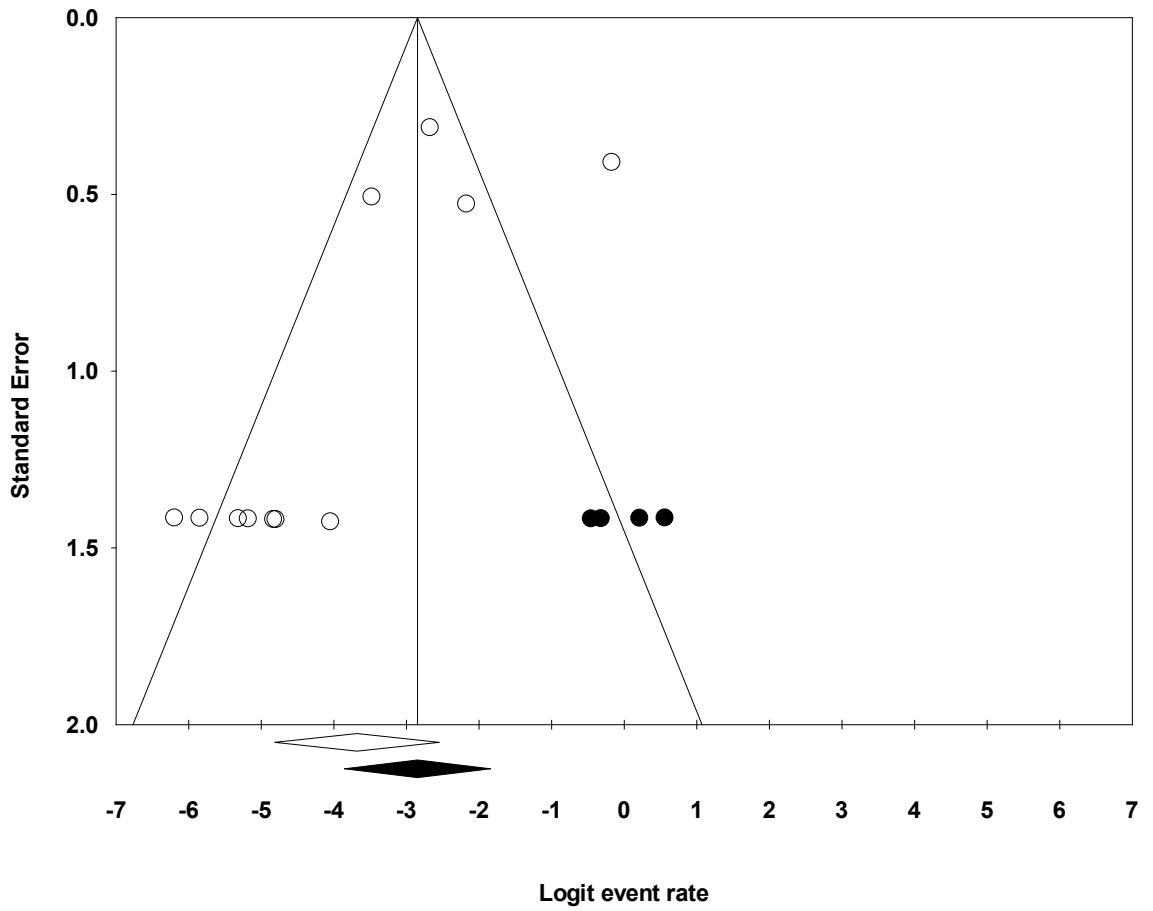
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	3
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	3
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	3
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	3
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	4; Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	4; Figure 1; Supplementary table 3
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	3; Supplementary table 2
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	4; Figures 2-4
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	4
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	4; Supplementary figures 1-5
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	4; Figure 5
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	6
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	8-10
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	11
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	11

Supplementary Table 2. Quality assessment of the studies included in the meta-analysis with the Newcastle-Ottawa Scale. A score of 7 to 9 indicates high methodological quality, a score of 4 to 6 indicates moderate quality and score of 0 to 3 indicates low quality.

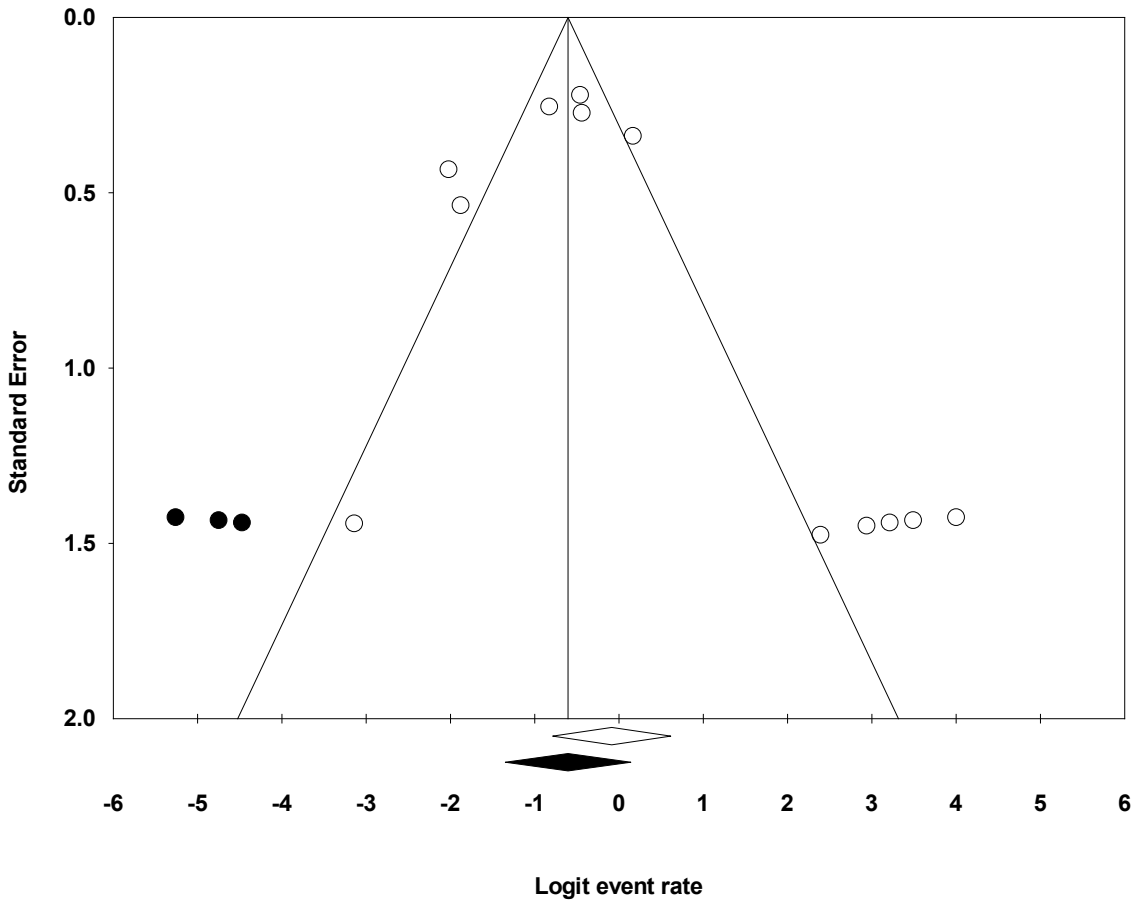
First author	Year	Selection	Comparability	Outcome/Exposure	Total score
De Campos	**2008**	***	**	***	**8**
Eachempati	**2002**	***	**	***	**8**
Kaya	**2007**	***	**	***	**8**
Keskinen	**2007**	**	**	*	**5**
Lei	**2013**	****	**	***	**9**
Nair	**2000**	***	**	***	**8**
Pupelis	**2007**	***	**	***	**8**
Ranson	**1976**	***	**	***	**8**
Sharma	**2014**	***	*	**	**6**
Shen	**2016**	***	**	***	**8**
Shinzeki	**2008**	**	**	**	**6**
Zahn	**2015**	***	**	***	**8**
Zhu	**2003**	**	**	*	**5**

Supplementary Table 3. Summary of study characteristics for publications included in the meta-analyses.

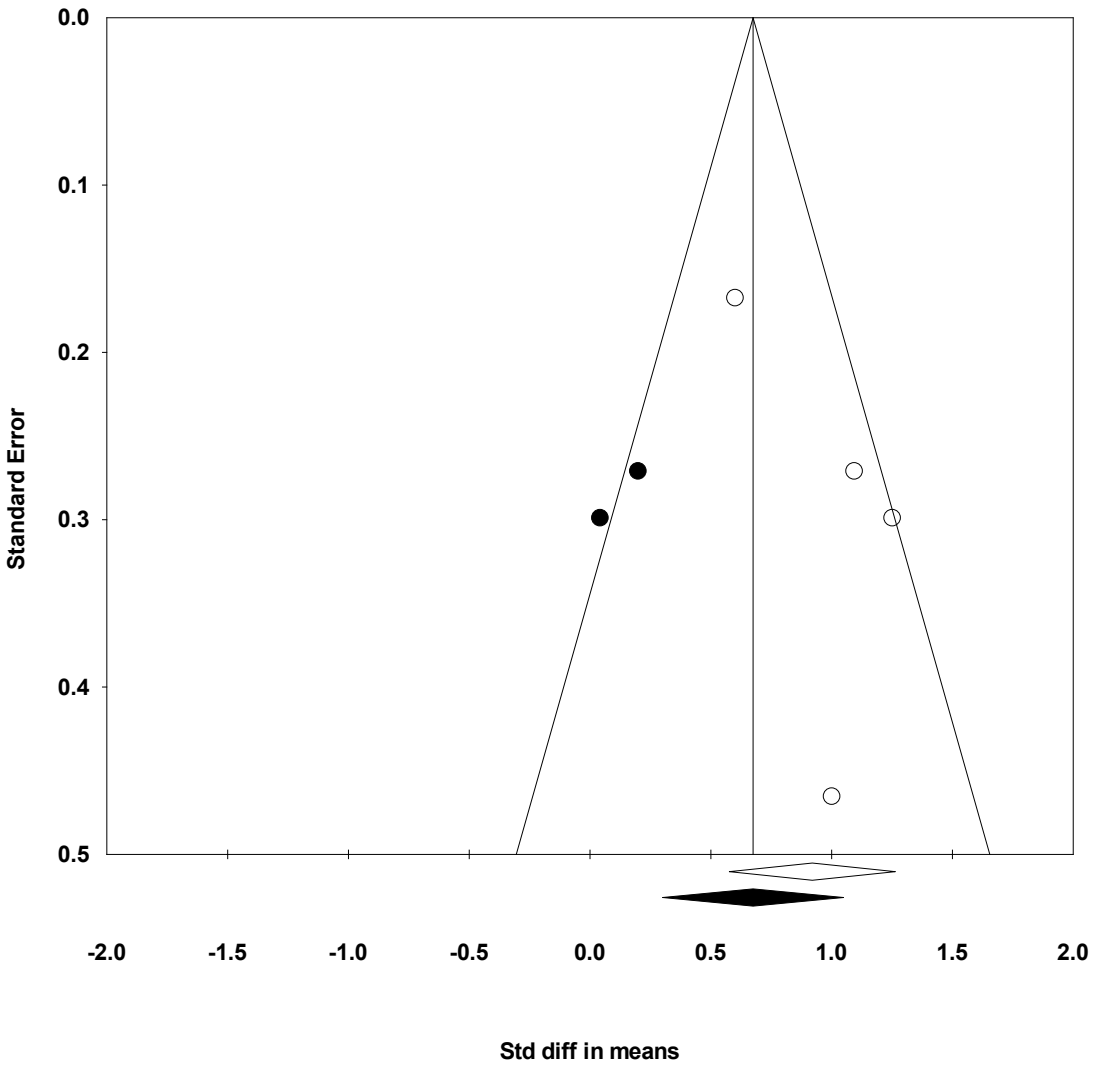
First author	Year	Design	Country	Study period	Sample size (lower pH/ higher pH)	Newcastle-Ottawa score
Ranson	1976	Retro- and prospective	USA	01/1971-02/1975	300 (56/244)	8
Nair	2000	Prospective observational	USA	01/1998-02/1999	90 (11/-)	8
Eachempati	2002	Prospective	USA	01/1993-05/2001	76 (16/60)	8
Zhu	2003	Retrospective	China	01/1993-12/2002	74 (12/62)	5
Kaya	2007	Prospective	Turkey	1998-2002	199 (27/172)	8
Keskinen	2007	Retrospective	Finland	2001-2003	59 (9/28)	5
Pupelis	2007	Prospective	Latvia	2000-2005	111 (72/39)	8
De Campos	2008	Retro- and prospective	Brazil	01/1999-11/2005	71 (47/24)	8
Shinzeki	2008	Prospective	Japan	07/1995-06/2006	93 (5/88)	6
Lei	2012	Retrospective	China	04/2007-07/2010	184 (51/133)	9
Sharma	2014	Prospective single center	India	01/2012-11/2013	205 (35/170)	6
Zhan	2015	Retrospective	China	07/2006-06/2010	101 (18/83)	8
Shen	2016	Retrospective cohort	China	11/2010-06/2014	186 (85/101)	8



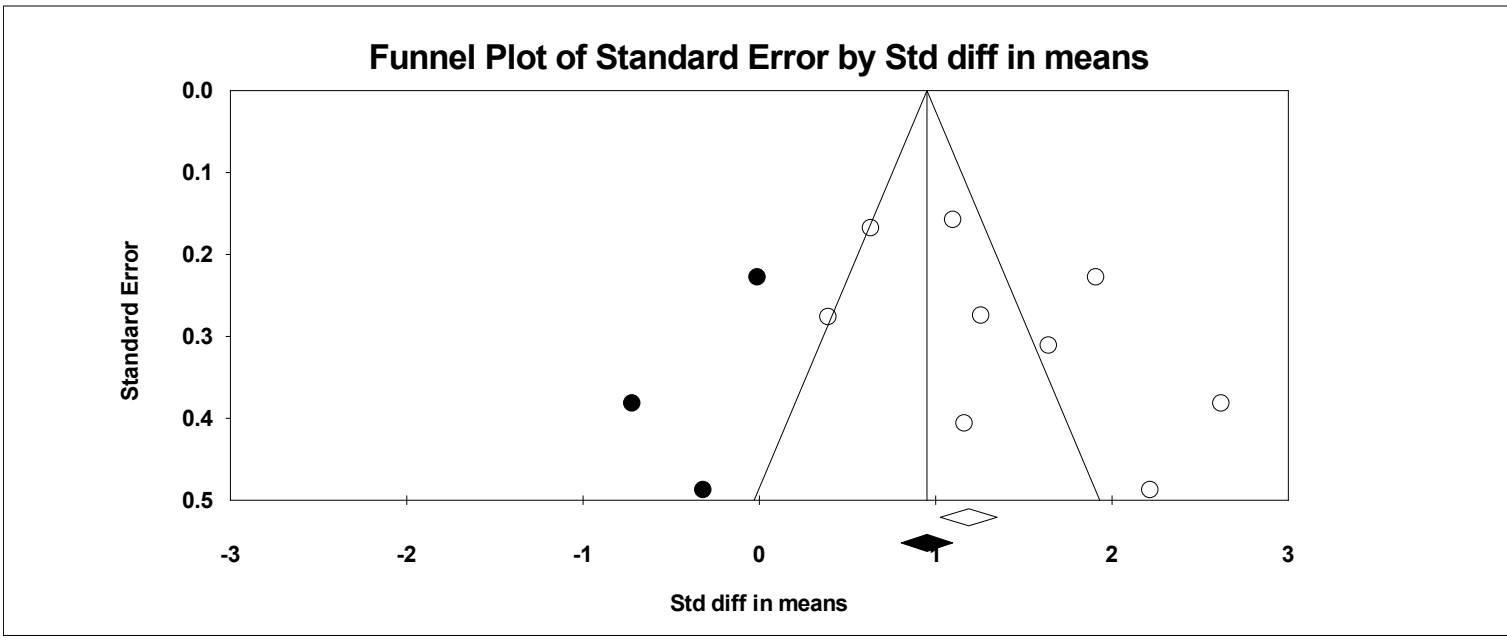
Supplementary Figure 1. Funnel plot of the studies that were included in the forest plot of mortality rate and reported higher systemic pH in patient groups with acute pancreatitis (AP). Here and in Supplementary Figures 2-5, open symbols represent results from studies included in the forest plot, while closed symbols indicate studies that appeared to be missing according to the trim and fill method of Duval and Tweedie. Open and closed diamonds represent the average estimated effect size without (open diamond) and with trim and fill correction (closed diamond). Duval and Tweedie corrected value: -2.85 (95% CI, -3.85, -1.84); $P = 0.029$ with Egger's test.



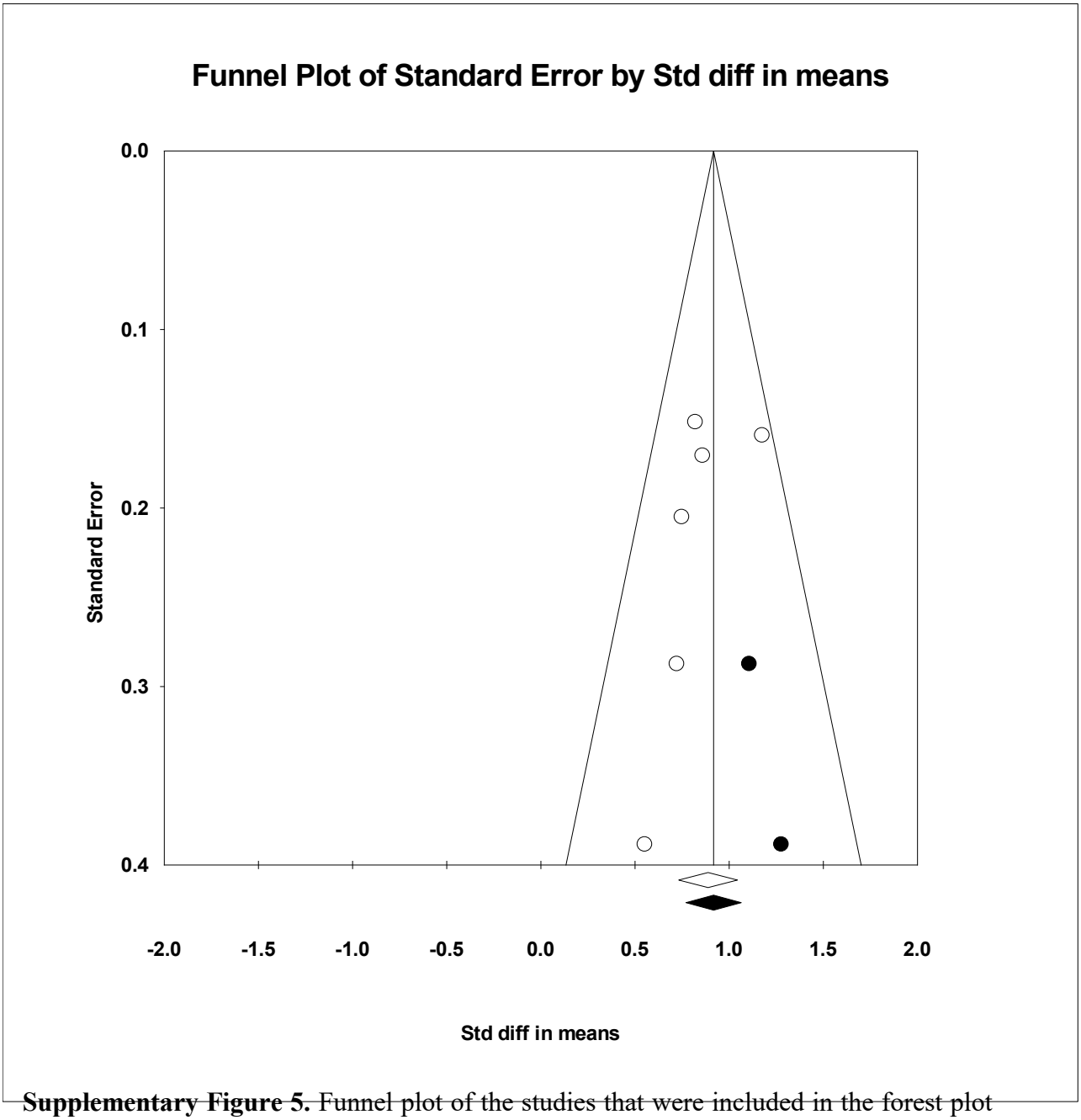
Supplementary Figure 2. Funnel plot of the studies that were included in the forest plot of mortality rate and reported lower systemic pH in patient groups with AP. Duval and Tweedie trim and fill corrected value: -0.615 (95% CI, -1.36, 0.134); P = 0.154 with Egger's test.



Supplementary Figure 3. Funnel plot of the studies that were included in the forest plot of the Ranson score in different systemic pH groups of patients with AP. Duval and Tweedie trim and fill corrected value: 0.675 (95% CI, 0.30, 2.06); $P = 0.234$ with Egger's test.

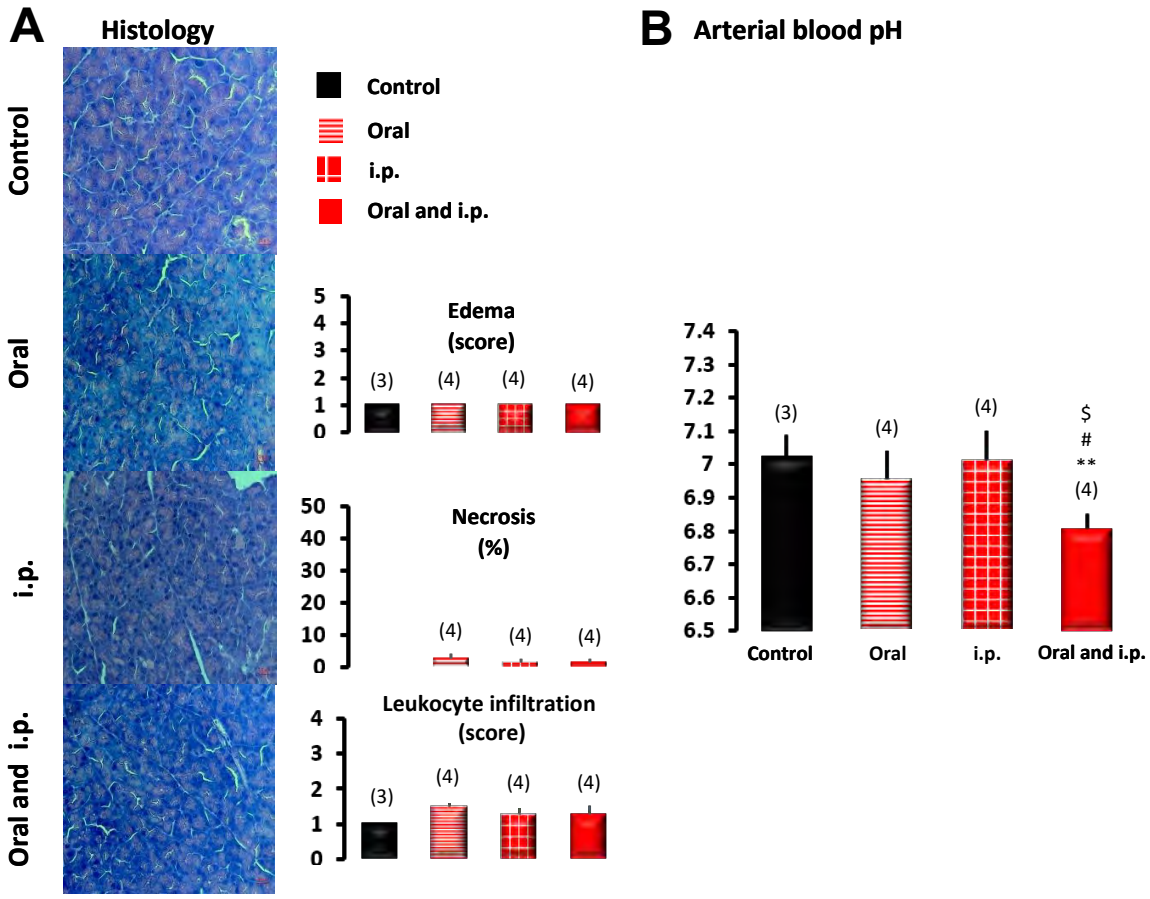


Supplementary Figure 4. Funnel plot of the studies that were included in the forest plot of the Acute Physiology and Chronic Health Evaluation (APACHE II) score in different systemic pH groups of patients with AP. Duval and Tweedie trim and fill corrected value: 0.99 (95% CI, 0.52, 1.46); $P = 0.138$ with Egger's test.

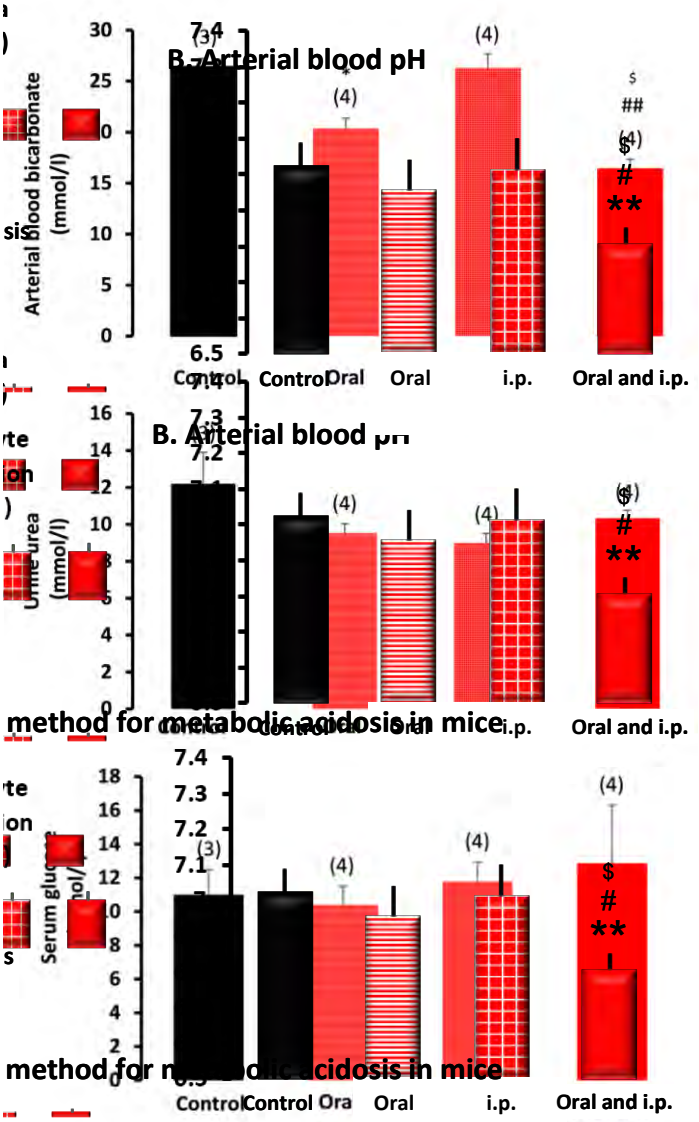


Supplementary Figure 5. Funnel plot of the studies that were included in the forest plot of the length of hospital stay (LOS) in different systemic pH groups of patients with AP. Duval and Tweedie trim and fill corrected value: 0.917 (95% CI, 0.771, 1.064); $P = 0.231$ with Egger's test.

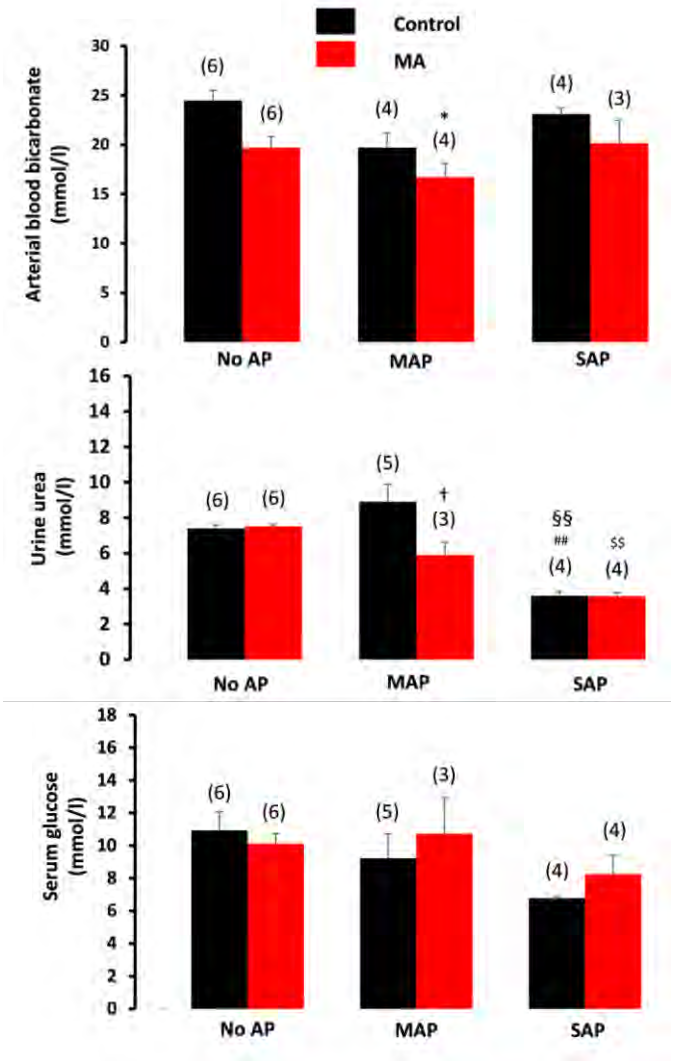
Supplementary Figure



Supplementary Figure 6. Induction of metabolic acidosis with different types (oral, i.p. or both) of NH_4Cl administration in mice. (**A**) None of the treatments caused pancreatic edema or necrosis, and only minimal (not significant) increase of leukocyte infiltration was observed in all three NH_4Cl treatment groups compared to the controls. (**B**) Arterial blood pH decreased minimally in the oral or i.p. treatment groups, while the combined (oral and i.p.) treatment significantly decreased arterial pH. **, $P < 0.01$ for the control group versus the oral and i.p. group; $^{\#}$, $P < 0.05$ for the oral group versus the oral and i.p. group; and $^{\$}$, $P < 0.05$ for the i.p. group versus the oral and i.p. group. Scale bar represents 20 μm. Here and in Supplementary Figures 7 and 8, numbers in parentheses indicate the number of animals in the corresponding groups.



Supplementary Figure 7. Arterial blood bicarbonate, urine urea, and serum glucose levels after different types (oral, i.p. or both) of NH_4Cl administration in mice. Arterial blood bicarbonate level decreased minimally in the i.p. treatment group, while the oral and the combined (oral and i.p.) treatment significantly decreased arterial bicarbonate level. Serum glucose and urine urea levels were not changed significantly in the treatment groups. *, $P < 0.05$ for the control group versus the oral group; $^{\#\#}$, $P < 0.001$ for the control group versus the oral and i.p. group; and $^{\$}$, $P < 0.05$ for the oral group versus the oral and i.p. group.



Supplementary Figure 8. Arterial blood bicarbonate, urine urea, and serum glucose levels of mice with and without metabolic acidosis (MA) in sham acute pancreatitis (No AP) and after induction of mild acute pancreatitis (MAP) or severe acute pancreatitis (SAP). Arterial blood bicarbonate and urine urea levels decreased significantly in the MAP and MA group compared to controls. Urine urea levels also decreased in SAP regardless of the pH status. Serum glucose level was not changed significantly. *, $P < 0.05$ for the No AP and control group versus the MAP and MA group; $^{\#\#}$, $P < 0.001$ for the No AP and control group versus the SAP and control group; $^{\$\$}$, $P < 0.001$ for the No AP and MA group versus the SAP and MA group; §§, $P < 0.001$ for the MAP and control group versus the SAP and control group, and †, $P < 0.05$ for the MAP and control group versus the MAP and MA group.

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Hyperthermia induced by transient receptor potential vanilloid-1 (TRPV1) antagonists in human clinical trials: Insights from mathematical modeling and meta-analysis

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ABSTRACT

Antagonists of the transient receptor potential vanilloid-1 (TRPV1) channel alter body temperature (T_b) in laboratory animals and humans: most cause hyperthermia; some produce hypothermia; and yet others have no effect. TRPV1 can be activated by capsaicin (CAP), protons (low pH), and heat. First-generation (polymodal) TRPV1 antagonists potently block all three TRPV1 activation modes. Second-generation (mode-selective) TRPV1 antagonists potently block channel activation by CAP, but exert different effects (*e.g.*, potentiation, no effect, or low-potency inhibition) in the proton mode, heat mode, or both. Based on our earlier studies in rats, only one mode of TRPV1 activation – by protons – is involved in thermoregulatory responses to TRPV1 antagonists. In rats, compounds that potently block, potentiate, or have no effect on proton activation cause hyperthermia, hypothermia, or no effect on T_b, respectively. A T_b response occurs when a TRPV1 antagonist blocks (in case of hyperthermia) or potentiates (hypothermia) the tonic TRPV1 activation by protons somewhere in the trunk, perhaps in muscles, and – *via* the acido-antithermogenic and acido-antivasoconstrictor reflexes – modulates thermogenesis and skin vasoconstriction. In this work, we used a mathematical model to analyze T_b data from human clinical trials of TRPV1 antagonists. The analysis suggests that, in humans, the hyperthermic effect depends on the antagonist's potency to block TRPV1 activation not only by protons, but also by heat, while the CAP activation mode is uninvolved. Whereas in rats TRPV1 drives thermoeffectors by mediating pH signals from the trunk, but not T_b signals, our analysis suggests that TRPV1 mediates both pH and thermal signals driving thermoregulation in humans. Hence, in humans (but not in rats), TRPV1 is likely to serve as a thermosensor of the thermoregulation system. We also conducted a meta-analysis of T_b data from human trials and found that polymodal TRPV1 antagonists (ABT-102, AZD1386, and V116517) increase T_b, whereas the mode-selective blocker NEO6860 does not. Several strategies of harnessing the thermoregulatory effects of TRPV1 antagonists in humans are discussed.

Abbreviations: A, ankyrin (as in TRPA1); CAP, capsaicin; CI(s), 95% confidence interval(s); CPZ, capsazepine; h, human (like in hTRPV1); IC_{50}, 50% inhibitory concentration of an antagonist (produces 50% of the maximum inhibitory response); i.p., intraperitoneal(ly); i.v., intravenous(ly); M, melastatin (as in TRPM8); p.o., peroral (*per os*); r, rat (like in rTRPV1); RTX, resiniferatoxin; SD, standard deviation; SDM(s), standardized difference(s) in means; T_a, ambient temperature; T_b(s), body temperature(s); TRP, transient receptor potential (channel); V, vanilloid (as in TRPV1).

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Contents

1. Introduction

Since it was first cloned by Caterina et al. (1997), the transient receptor potential (TRP) vanilloid-1 (V1) channel, formerly known as either the capsaicin receptor or VR1, has remained in the focus of pain research and drug development (Kaneko & Szallasi, 2014). Interestingly, the effects of pharmacological inactivation of this channel were studied since the 1950s (Jancso & Santha, 2015; Szolcsanyi, 2015). At that time, high doses of capsaicin (CAP), a pungent constituent of chili peppers (genus *Capsicum*), were shown to desensitize a subset of sensory nerves with consequent effects on many physiological functions. CAP is a TRPV1 agonist, and the term desensitization refers to the state of a decreased neuronal sensitivity to stimuli that normally activate TRPV1-expressing neurons, *e.g.*, noxious heat (for review, see Holzer, 1991; Szallasi & Blumberg, 1999). Studies of the desensitizing effects of CAP and other vanilloids, *e.g.*, resiniferatoxin [(RTX), an ultrapotent TRPV1 agonist naturally found in plants of the genus *Euphorbia* (Szallasi & Blumberg, 1989)], paved the way for using TRPV1 agonists to treat pain (Craft & Porreca, 1992; Szallasi & Blumberg, 1999). For example, epidermal Qutenza (CAP-containing patch) was developed for treating neuropathic pain, while intrathecal RTX has been proposed for treating pain in some forms of cancer (Chung & Campbell, 2016; Moran & Szallasi, 2018). In the late 1990s, while continuing to work on analgesic treatments based on the desensitizing property of TRPV1 agonists, multiple pharmaceutical companies had started developing TRPV1 antagonists (Holzer, 2008; Kyle & Tafesse, 2006; Lee et al., 2015). (Throughout this review, we use the terms "antagonist" and "blocker" interchangeably.) Highly potent and selective TRPV1 antagonists were, and perhaps still are, hoped to usher in a new generation of non-opioid analgesics. However, during the *in-vivo* testing of TRPV1 antagonists, adverse effects on body temperature (T_b), primarily hyperthermia, were repeatedly observed in animal studies and human clinical trials alike (*vide infra*). This review examines the thermal effects of TRPV1 antagonists and reports the results of a mathematical-modeling analysis and meta-analysis of T_b data from human trials.

In addition to vanilloids, many other stimuli are known to activate (open) the TRPV1 channel (Jordt, Tominaga, & Julius, 2000). Traditionally, when studying the TRPV1-antagonizing property of compounds, pharmaceutical companies use the following stimuli to activate this channel: CAP, low extracellular pH (< 6), and sometimes heat (> 42°C) (Fig. 1). Hence, in this review, we discuss three modes of TRPV1 activation: CAP, proton, and heat, respectively. It is now known that TRPV1 antagonists can affect these three modes differentially (for review, see Blumberg, Pearce, & Lee, 2011; Romanovsky et al., 2009). For example, a compound can potently block TRPV1 activation by CAP, but potentiate TRPV1 activation by protons (Garami, Pakai, et al., 2018; Lehto et al., 2008). In the present work, we attempted to determine the activation-mode profiles of TRPV1 antagonists that induce hyperthermia, have no effect on T_b, or even cause hypothermia in humans. Based on the results of our analyses, presented herein, we examine the strategies of minimizing the thermoregulatory effects of TRPV1 antagonists, but also of using these effects for therapeutic purposes, in humans.

2. The hyperthermic effect of TRPV1 antagonists in laboratory animals

2.1. Phenomenology

Upon systemic administration, whether intravenous (i.v.), intraperitoneal (i.p.), or peroral (p.o.), many TRPV1 antagonists cause hyperthermia in a variety of laboratory animal species, including the mouse, rat, guinea pig, dog, and cynomolgus monkey (*Macaca fascicularis*) (Fig. 2). This hyperthermia appears to be independent of chemical structure. It has been shown to develop in response to small-molecule TRPV1 antagonists belonging to different chemotypes, *viz.*, cinnamides (AMG0347 and AMG9810), pyrimidines (AMG 517), ureas (JYL 1421 and A-425619), and piperazines (BCTC), thus suggesting an on-target action (for review, see Romanovsky et al., 2009). Indeed, the on-target nature of the hyperthermic effect of TRPV1 antagonists was determined definitively, when Steiner et al. (2007) and Garami et al. (2011, 2010) showed that TRPV1 knockout mice did not increase T_b in response to either AMG0347 or AMG 517, whereas wild-type mice responded to either compound with hyperthermia.

However, not all TRPV1 antagonists are made equal as far as their ability to cause hyperthermia. While most TRPV1 antagonists produce the hyperthermic response at systemic doses in the mg/kg range (Gavva, Bannon, Surapaneni, et al., 2007; Swanson et al., 2005), others (*e.g.*, AMG0347) are effective already at 10 μg/kg (Gavva et al., 2008; Steiner et al., 2007), and yet others (*e.g.*, AS1928370) do not seem to affect T_b at all (Watabiki, Kiso, Kuramochi, et al., 2011). Some TRPV1 antagonists have been shown to be hyperthermic in several mammalian species, whereas others cause hyperthermia only in particular species. For example, AMG0347 and AMG 517 increase T_b in both rats and mice (Garami et al., 2010; Steiner et al., 2007), whereas capsazepine (CPZ) increases T_b in guinea pigs but has no thermal effect in rats (Garami et al., 2010). Intriguingly, some TRPV1 antagonists (*e.g.*, A-1165901, A-425619, AMG7905, and AMG8562) cause hypothermia instead of hyperthermia (Garami, Pakai, et al., 2018; Lehto et al., 2008; Mills et al., 2008). And yet other compounds appear to affect T_b regulation in a species-specific fashion, *e.g.*, JYL1421 was shown to cause hyperthermia in dogs and cynomolgus monkeys [Fig. 2D and E; also see Gavva, Bannon, Surapaneni et al. (2007)] but hypothermia in rats [Fig. 3D; also see Garami et al. (2010)]. Similar to the hyperthermic effect, the hypothermic effect of TRPV1 antagonists is also independent of the chemotype, as hypothermia occurs in response to small molecules with diverse chemical structures, *e.g.*, A-1165901, AMG7905, JYL1421 (Fig. 3), or AbbVie's Compound 3 (Gomtsyan et al., 2015). Two polypeptide TRPV1 antagonists, APHC1 and APHC3, have also been reported to cause hypothermia in rats (Andreev et al., 2013). In agreement with this, the hypothermic effect of TRPV1 antagonists is absent in TRPV1 knockout mice (Garami, Pakai, et al., 2018). Hence, both the hyper- and hypothermic effects of TRPV1 antagonists occur by acting on the same receptor, TRPV1, which is a highly unusual scenario. When a compound causes two opposite responses, these responses are typically mediated by different receptors (Garami, Pakai, et al., 2018). This paradox of a dual thermoregulatory action mediated by

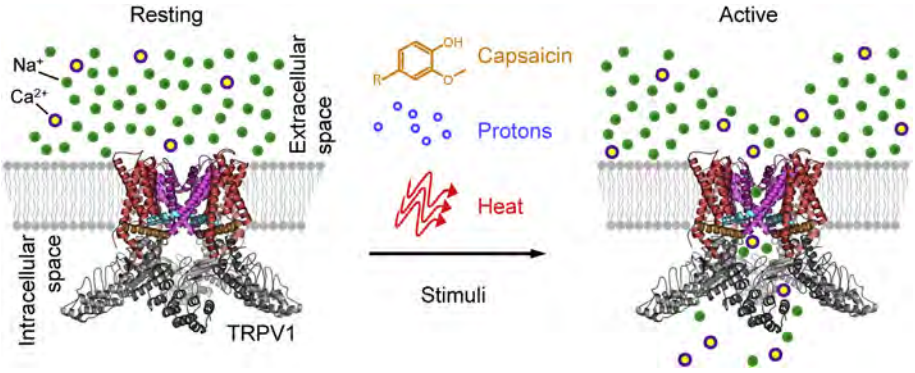

Fig. 1. TRPV1 activation: a schematic. Upon stimulation by CAP, protons, or heat, the TRPV1 channel opens, and Na^+ and Ca^{2+} cations from the extracellular space enter the cell.

the same receptor is just the tip of the iceberg – the thermoregulatory effects of TRPV1 antagonists have several other surprising physiological features.

2.2. Physiological mechanisms

Those TRPV1 antagonists that induce hyperthermia do so (at least in rats) by recruiting autonomic cold-defense effectors, *i.e.*, triggering tail-skin vasoconstriction and activating nonshivering thermogenesis in brown adipose tissue (Garami et al., 2010; Steiner et al., 2007). The same thermoeffectors – but working in reverse – bring about the hypothermic response to TRPV1 antagonists, when it occurs. In the latter case, tail-skin vasoconstriction is replaced by vasodilation, while thermogenesis is suppressed (Garami, Pakai, et al., 2018). Because the TRPV1 channel is highly sensitive to temperature (for review, see Zheng & Wen, 2019), it is often assumed that temperature signals transmitted by TRPV1 drive effector responses of the thermoregulation system, or, in other words, that the TRPV1 channel serves as a thermosensor for the thermoregulation system. Accordingly, it is further assumed that the thermoregulatory effects of TRPV1 antagonists are due to the blockade of the channel's thermosensory function (McGaraughty et al., 2009; Seebacher et al., 2010; Szolcsanyi, 2015; Vriens, Nilius, & Voets, 2014). This, however, appeared not to be the case (Romanovsky et al., 2009).

If the activity of thermoeffectors (and, consequently, the deep T_b) were to depend on TRPV1-mediated thermal signals, the magnitude of the hyperthermic effect of TRPV1 antagonists would depend on tissue temperatures in different areas of the body – deep (if the sensors are located inside the body), superficial (if the sensors are in the skin), or both. High T_bs represent strong warmth signals (that inhibit cold defenses, activate heat defenses, and – through a negative feedback loop – suppress T_b); under such conditions, TRPV1 antagonists would remove this T_b suppression and be expected to bring about a strong hyperthermic effect. Low T_bs are equivalent to the lack of warmth signals affecting thermoregulation; under such conditions, the removal of the nonexistent warmth signals by TRPV1 antagonists would be expected

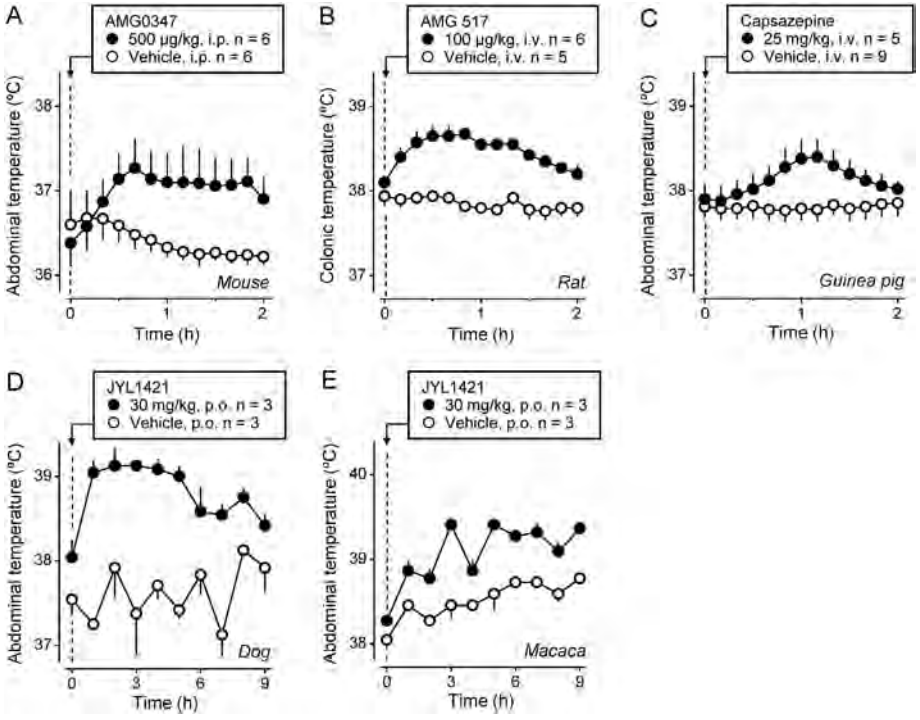

Fig. 2. Hyperthermic responses to TRPV1 antagonists in laboratory mammals. A) Effect of AMG0347 (500 µg/kg, i.p.) or its vehicle on abdominal temperature in mice at a neutral ambient temperature (T_a) of 31°C. B) Effect of AMG 517 (100 µg/kg, i.v.) or its vehicle on colonic temperature in rats at a neutral T_a of 26°C. C) Effect of capsazepine (CPZ; 25 mg/kg, i.v.) or its vehicle on abdominal temperature of guinea pigs at a neutral T_a of 27°C. D) Effect of JYL1421 (30 mg/kg, p.o.) or its vehicle on T_b (location not specified) in dogs at room temperature. E) Effect of JYL1421 (30 mg/kg, p.o.) or its vehicle on T_b (location not specified) in cynomolgus monkeys at room temperature. Modified from Steiner et al. (2007) (A); modified with permission from Gavva et al. (2008) (B); modified from Garami et al. (2010) (C) and Gavva, Bannon, Surapaneni, et al. (2007) (D, E).

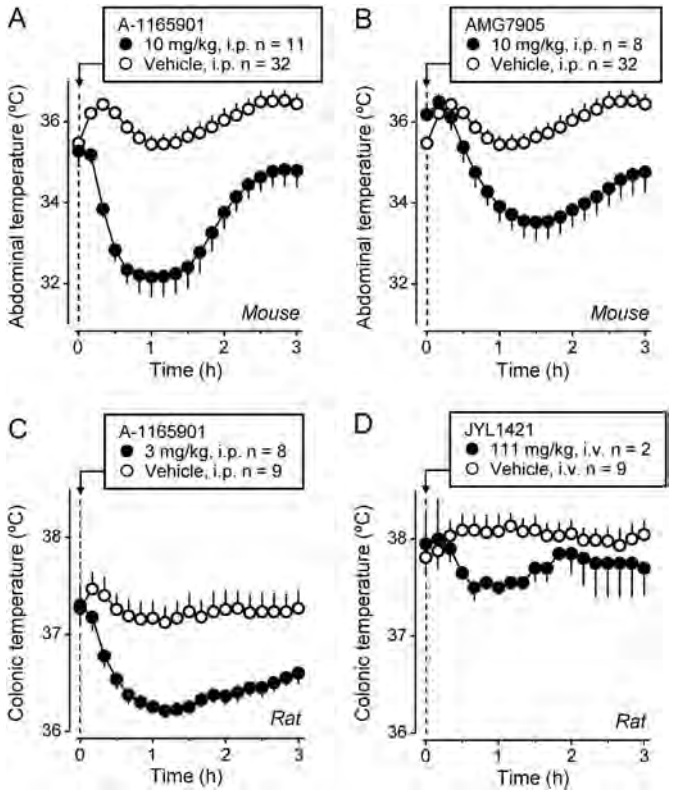

Fig. 3. Hypothermic responses to different TRPV1 antagonists in rats and mice. A) Effect of A-1165901 (10 mg/kg, i.p.) or its vehicle on abdominal temperature in mice at a subneutral T_a of 20°C. B) Effect of AMG7905 (10 mg/kg, i.p.) or its vehicle on abdominal temperature in mice at a subneutral T_a of 20°C. C) Effect of A-1165901 (3 mg/kg, i.p.) or its vehicle on colonic temperature in rats at a T_a of 26°C (the low end of the thermoneutral zone). D) Effect of JYL1421 (111 mg/kg, i.p.) or its vehicle on colonic temperature in rats at a T_a of 26°C (the low end of the thermoneutral zone). Graphs are reprinted from Garami, Pakai, et al. (2018) (A, B, C) or plotted for this work using data from Garami et al. (2010) (D).

not to affect T_b at all. *Mutatis mutandis*, these assumptions were shown to be true for the cold-sensitive TRP channel melastatin-8 (TRPM8). TRPM8 antagonists cause hypothermia in rats (Almeida et al., 2012; de Oliveira et al., 2014), and the magnitude of the hypothermic response increases with a decrease in the ambient temperature (T_a) and T_bs, including skin temperatures – when the thermal (*i.e.*, cold) activation of TRPM8 is stronger, the blockade of this activation with an antagonist causes a stronger T_b response (Almeida et al., 2012). Hence, TRPM8 is a physiologically important temperature sensor that drives thermoeffector responses in the rat.

For the TRPV1 channel, the assumptions described above turned out to be incorrect, at least in the case of young male rats. Steiner et al. (2007) have analyzed whether the extent of hyperthermia depends on the initial (preinjection) temperatures (deep T_b, tail-skin temperature, and T_a) in response to an i.v. injection of the potent TRPV1 antagonist AMG0347. The authors found no positive correlation between the magnitude of the hyperthermia and any of the initial temperatures measured (Fig. 4). The lack of a positive correlation indicates that the tonic activation of TRPV1 channels, which maintains the tonic suppression of T_b, is nonthermal in nature. *A priori*, such nonthermal factors may include a low pH, inorganic cations, or endovanilloids. The fact that TRPV1 activation by heat plays no role in the thermoregulatory effects of TRPV1 antagonists (or in thermoregulation in general), at least in male rats, was confirmed in later studies [for more detail, see section 2.4 and Garami et al. (2010), Garami, Pakai, et al. (2018), Lehto et al. (2008)].

The highest levels of TRPV1 expression are found in the primary sensory neurons of dorsal-root and trigeminal ganglia, both in rodents

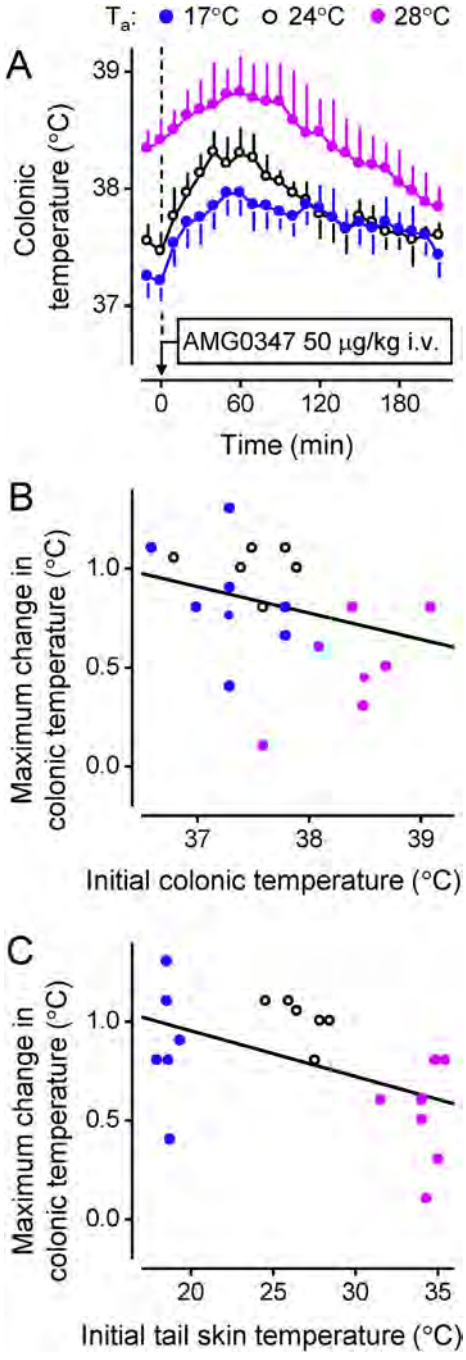

Fig. 4. In rats, the magnitude of the hyperthermic response to a TRPV1 antagonist is independent of both the initial (at the time of drug administration) colonic temperature or initial tail-skin temperature in a wide range of temperatures. A) AMG0347 (50 µg/kg, i.v.) was injected in rats at a T_a of 17, 24, or 28°C. At all T_a values tested, the drug induced hyperthermic responses of similar magnitude. No positive correlation was found when the response magnitude (the maximal increase in colonic temperature) for each rat was plotted either against the initial colonic temperature (B) or against the initial tail-skin temperature (C). Reprinted with permission from Romanovsky et al. (2009); the figure uses data from Steiner et al. (2007).

(Jang et al., 2012; Sanchez, Krause, & Cortright, 2001) and in humans (Cortright et al., 2001). However, TRPV1 channels are widely distributed throughout the body and expressed abundantly on neural elements both within and outside of the central nervous system; they are also found in non-neural tissues (reviewed by Romanovsky et al., 2009). Where are the TRPV1 channels that mediate the T_b effects of nonthermal tonic stimuli (and, consequently, the thermoregulatory effects of TRPV1 antagonists) located? To answer this question, Steiner

Table 1
Sites of TRPV1 desensitization in RTX- or vehicle-pretreated rats

Pre-treatment	Compartment					Desensitization pattern	Desensitization extent
	Abdominal cavity	Eyes	Skin	Thoracic cavity	Brain		
Vehicle	0	0	0	0	0		None
RTX 0.2 mg/kg i.p.	X	X	X	X	X		Systemic
RTX 0.02 mg/kg i.p.	X	0	0	0	0		Localized intra-abdominal

The state of TRPV1 channels in different bodily compartments is marked as follows: X, desensitized; 0, non-desensitized. In schematics of the desensitization pattern, the desensitized compartments are shown in grey; the non-desensitized compartments are shown in white. Reprinted with permission from Romanovsky et al. (2009); the table was built based on the data reported by Steiner et al. (2007).

et al. (2007) compared the thermoregulatory effects of AMG0347 administered directly into the central nervous system (*viz.*, intracerebroventricularly or intrathecally) and systematically (i.v.). If a central administration were to cause hyperthermia at much lower (10-100 times) doses than a peripheral administration, this would indicate a central action. This, however, was not the case. The authors found that the threshold hyperthermic dose of AMG0347, when administered i.v., was ~6 μg/kg (a significant effect was observed at 10 μg/kg), but that the drug did not cause any changes in T_b when the dose of 6 μg/kg was administered centrally (either intracerebroventricularly or intrathecally). These findings allowed the authors to exclude a central origin of the hyperthermic response to AMG0347.

To obtain a more precise location of the channels responsible for the hyperthermic effect of TRPV1 antagonists, Steiner et al. (2007) administered AMG0347 i.v. in rats that had TRPV1 channels desensitized in different compartments of the body. As explained above (Introduction), desensitization means a decreased neuronal sensitivity to exogenous or endogenous vanilloids or other stimuli that normally activate TRPV1-expressing neurons, *e.g.*, noxious heat (Craft & Porreca, 1992; Szallasi & Blumberg, 1999). Steiner et al. (2007) administered repeated, escalating i.p. doses of RTX to rats to achieve different desensitization patterns (Table 1). At higher doses (~200 μg/kg), RTX impairs the function of TRPV1 channels throughout the entire body (systemic desensitization). When lower doses are used (~20 μg/kg), the desensitizing effect is limited to the abdominal cavity (localized, intra-abdominal desensitization). In the latter case, TRPV1-mediated reflexes triggered from the abdominal cavity (*e.g.*, RTX-induced writhing) are suppressed, while TRPV1 sensitivity in all other body compartments remains intact (Table 1). It was found that the hyperthermic response to either AMG0347 [Fig. 5; also see Steiner et al. (2007)] or another TRPV1 antagonist, A-889425 (McGaraughty et al., 2009), was absent in rats with localized, intra-abdominal desensitization. More recently, we have shown that the hypothermic effect of the TRPV1 antagonist A-1165901 is also abolished following the intra-abdominal TRPV1 desensitization in rats (Garami, Pakai, et al., 2018). The results in RTX-desensitized rats show that both the hyper- and hypothermic responses to TRPV1 antagonists are triggered from the abdomen, perhaps the intra-abdominal viscera or abdominal-wall muscles (for further discussion, see Garami, Pakai, et al., 2018).

To summarize this section, the following picture has emerged. TRPV1 antagonists produce their thermoregulatory effects by acting on TRPV1 channels located somewhere in the abdomen: in the abdominal viscera or abdominal-wall muscles. The abdominal TRPV1 channels are tonically activated by some nonthermal stimuli. The most common thermoregulatory effect of TRPV1 antagonists – hyperthermia – results from the blockade of this nonthermal TRPV1 activation and, consequently, from disinhibition of cold defenses. What stimuli tonically activate the abdominal TRPV1 channels under normal conditions? Why would thermoregulatory responses be triggered by nonthermal, TRPV1-mediated stimuli from the trunk? Before we answer these questions, we will take a more careful look at different ways to activate the TRPV1 channel.

2.3. Mode selectivity of TRPV1 activation and differential TRPV1 pharmacology

Rat (r) TRPV1 is the most studied TRPV1 ortholog, for which six cryo-electron microscopy structures with a resolution varying from 3.0 to 4.2 Å have been obtained, thus providing direct insight into the polymodal regulation of this channel (Cao, Liao, Cheng, & Julius, 2013; Gao, Cao, Julius, & Cheng, 2016; Liao, Cao, Julius, & Cheng, 2013). Derived from the structural biology studies, the molecular architecture of TRPV1 shows that the channel is a tetramer with six transmembrane helices (S1-S6). The transmembrane domain is further divided into two structural (sub-)domains: the voltage-sensing-like (sub-)domain (helices S1-S4) and the pore (sub-)domain (S5-S6), as illustrated in Fig. 6. The pore domain houses the upper and lower gates that open and close in response to diverse stimuli, including CAP, protons, and heat (Yang et al., 2018).

The combined data from a variety of methods paint the clearest picture of the molecular mechanisms associated with rTRPV1 activation by CAP and related vanilloid compounds (Yang & Zheng, 2017). Early comparative studies of TRPV1 orthologs with different CAP sensitivity identified the vanilloid-binding pocket, of which there are four per a

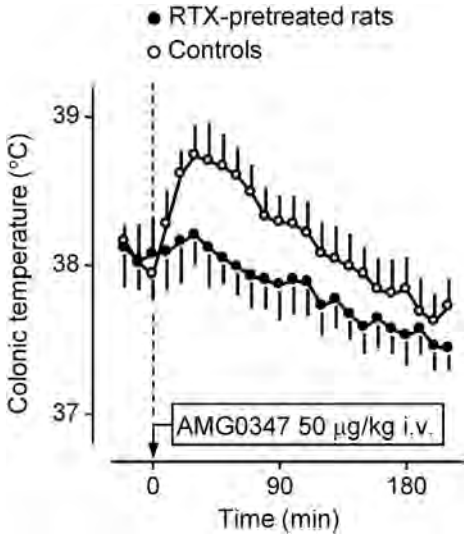

Fig. 5. The hyperthermic response to TRPV1 antagonists does not occur in rats with localized intra-abdominal TRPV1 desensitization. Shown is the abolished hyperthermic effect of AMG0347 (50 μg/kg, i.v.) in rats pretreated with RTX (20 μg/kg, i.p.). Reprinted with permission from Romanovsky et al. (2009); the figure uses data from Steiner et al. (2007).

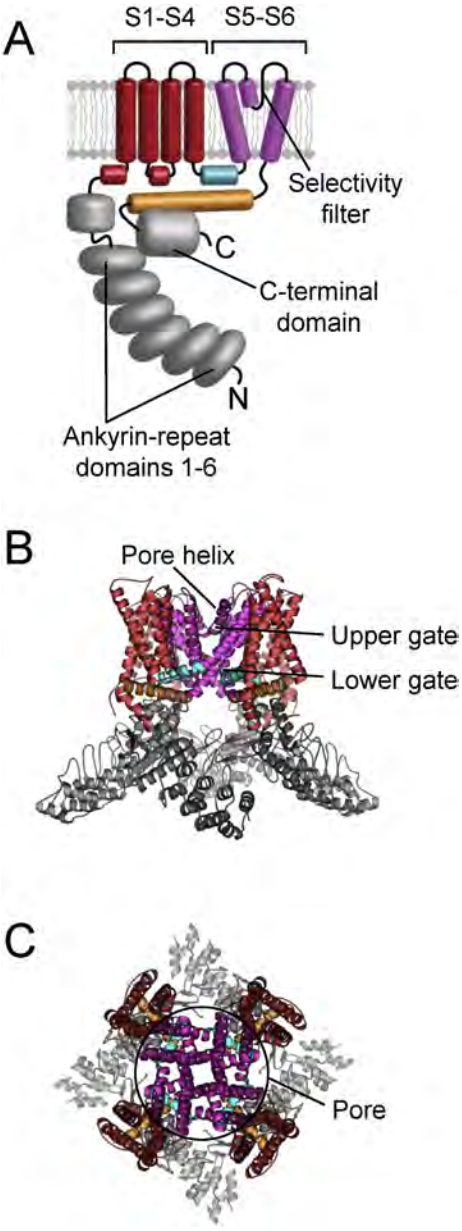

Fig. 6. Molecular architecture of TRPV1. A) A color-coded schematic of a TRPV1 monomer shows the voltage-sensing-like subdomain (S1-S4; red) and the pore subdomain (S5-S6; purple) of the transmembrane domain, as well as the S4-S5 helix linker (cyan) and the TRP helix (orange). The intracellular N- and C-termini include a series of six ankyrin-repeat domains and a C-terminal domain, respectively. The so-called selectivity filter of TRPV1 is formed from the loop that links the pore helix with the S6-helix. B) The tetrameric structure of TRPV1 (pdb ID: 3J5P) is shown with the same color-coding. The pore subdomain (purple), in its tetrameric form, includes the upper and lower gates that regulate channel activation. C) An extracellular view of the TRPV1 structure shows how the tetrameric pore subdomain forms an ion-conductive pathway (pore), which is regulated by CAP, protons, and heat.

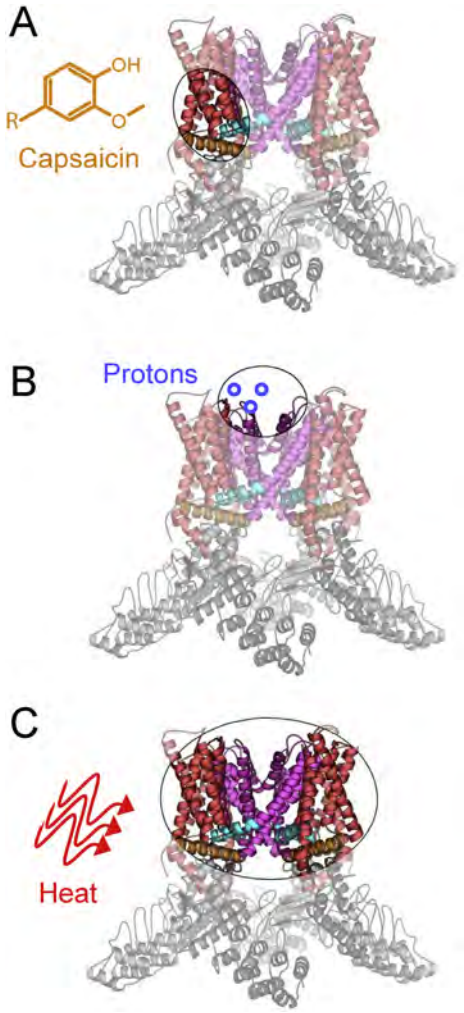

Fig. 7. Activation of TRPV1 by distinct stimuli. TRPV1 is activated by a variety of diverse stimuli, including protons, CAP, and heat, *via* spatially distinct mechanisms. A) The canonical vanilloid agonist CAP binds to the voltage-sensing-like membrane subdomain in the intracellular leaflet. B) The proton-sensing mechanism is localized primarily on the extracellular loops of the TRPV1 pore subdomain. C) The heat-activation mechanism is not well-understood. The current data suggest that the core heat-sensing architecture is harbored by the transmembrane region.

functional TRPV1 channel (Gavva et al., 2004; Jordt & Julius, 2002). The vanilloid-binding pocket (Fig. 7A) was further validated by functional, computational, and structural studies (Cao, Liao, et al., 2013; Yang et al., 2015). Activation of TRPV1 by CAP is initiated at the intracellular side of the membrane, where CAP binds to the voltage-sensing-like domain within the pocket. The vanilloid-binding pocket is energetically coupled with the pore domain, and CAP binding causes the lower gate at the S6 helix bundle crossing to open, followed by further conformational rearrangements and coupling that are propagated to the selectivity filter on the extracellular side of the membrane (*i.e.*, the upper gate), resulting in channel activation (Yang et al., 2018). While TRPV1 is the only TRPV family member that is inherently activated by vanilloids, the mechanismic understanding of TRPV1 proved to be sufficient for engineering vanilloid sensitivity into the TRPV2 and TRPV3 channels (Yang, Vu, Yarov-Yarovoy, & Zheng, 2016; Zhang et al., 2016), hence further validating the proposed model of CAP sensitivity.

Proton activation of rTRPV1 is also relatively well-studied (Boukalova, Teisinger, & Vlachova, 2013; Jordt et al., 2000). The key regions that impart proton sensitivity are located in the extracellular loops of the pore domain, across the membrane from the vanilloid-binding pocket (Fig. 7B). Canonically, two glutamate residues (E600 and E648) function as putative proton sensors, where the magnitude and range of TRPV1 pH sensitivity depend on the chemical nature of these side chains. Proton activation is initiated in the extracellular pore domain loops and then propagated to the pore helix and the upper gate (Ryu, Liu, Yao, Fu, & Qin, 2007). From there, the activation is spread to the lower gate, thus resulting in proton-dependent channel opening, *i.e.*, gating. Given the distinct spatial origins of CAP (vanilloid) and proton activation, it is interesting to note that other modes of TRPV1 regulation and activation are thought to occur extracellularly, with overlapping mechanisms to proton activation (Bohlen et al., 2010; Cao, Liao, et al., 2013; Jara-Oseguera, Bae, & Swartz, 2016). Specifically, a sodium-binding site is known to stabilize the rTRPV1 closed state in this region

(Jara-Oseguera et al., 2016), whereas a spider toxin (*i.e.*, the tarantula double-knot toxin) activates TRPV1 in the pore domain extracellular loops (Bohlen et al., 2010).

The mechanism of heat activation of rTRPV1 is still debated, as is the exact location of where heat is sensed within the channel, or even if a discreet "heat-sensor" location exists at all (Voets et al., 2004; Zheng & Wen, 2019). Indeed, virtually all areas of TRPV1 have, at one point or another, been ascribed some participation in heat activation (Hilton, Rath, Helsell, Beckstein, & Van Horn, 2015; Voets, 2014). Nonetheless, it is clear that thermosensitivity is an inherent feature of TRPV1 (Cao, Cordero-Morales, Liu, Qin, & Julius, 2013). Based on the current literature, the transmembrane region is emerging as central to thermosensitivity (Hilton, Kim, & Van Horn, 2019; also see Fig. 7C). Recent studies have shown that the pore domain of rTRPV1 is sufficient to endow a non-thermosensitive channel with heat activation, indicating that this domain is crucial for thermosensitivity (Zhang et al., 2018). There is also recent evidence that the human (h) TRPV1 voltage-sensing-like domain contributes to thermosensitivity (Kim et al., 2019). This domain undergoes a temperature-dependent conformational change that has been implicated in channel activation through the S4 helix to the pore domain, with some similarities to the mechanism of vanilloid activation. While there is strong evidence for species-specific phenotypes in TRPV1 and other TRP channels (Garcia-Avila & Islas, 2019; Hilton et al., 2015), the available data suggest that the transmembrane domain is central to heat activation, with extramembrane domains potentially modulating thermal responses. For the TRP ankyrin-1 (TRPA1) channel, a distinct thermosensitive channel, which shares the transmembrane architecture and ankyrin-repeat-based intracellular domain structures with TRPV1 (Saito & Tominaga, 2017), the temperature-sensitive region has been narrowed down to the transmembrane and C-terminal regions (Moparthi et al., 2014). More recently, temperature sensing in the TRPV3 channel has also been localized to the transmembrane domain (Singh, McGoldrick, & Sobolevsky, 2018).

Species differences, especially between rTRPV1 and hTRPV1, deserve a separate discussion. Given that the two orthologs originate from a common evolutionary ancestor and share ~85% sequence identity, as well as most general structural features, including the conserved transmembrane domain, they are expected to function similarly (Hilton et al., 2019). Indeed, the concentration of CAP that produces a half-maximal response *in vitro* is similar for the two channels (McIntyre et al., 2001), and they also have similar heat activation thresholds (McIntyre et al., 2001) and thermosensitivities (Kim et al., 2019). However, the sensitivity to protons differs between the two channels. *In vitro*, the half-maximal response occurs at the pH of ~5.8 in rTRPV1 but at the pH of ~5.5 in hTRPV1 (McIntyre et al., 2001), with the difference between the two pH values (~0.3) being very large from the physiological point of view. Hence, rTRPV1 is substantially more sensitive to protons than hTRPV1.

To summarize, it is clear that TRPV1 activation by diverse mechanisms can be spatially distinct, as evidenced by activation by CAP and protons. It is important that the spatial separation of different activation modes of TRPV1 can be exploited pharmacologically. Some TRPV1 antagonists block activation of TRPV1 in all modes (*i.e.*, by CAP, heat, and protons) with similarly high potency; these antagonists are called polymodal, mode-nonspecific (or mode-nonselective) and represent the first generation of TRPV1 antagonists. Examples of the first-generation blockers include AMG1629, AMG3731, AMG 517, AMG0347, and ABT-102 (Table 2). Yet other compounds affect TRPV1 activation in different modes differentially; they are called mode-specific (or mode-selective) and represent the second generation of TRPV1 antagonists. Second-generation compounds potently block the CAP and heat activation modes (*e.g.*, AMG2820 and AMG7988) or just solely the CAP mode (*e.g.*, SB-366791), while not affecting the remaining modes, blocking them with lower potency, or even potentiating TRPV1 activation in these modes. Examples of compounds that potentiate TRPV1 activation by protons include A-1165901, AMG8562, and JYL1421, while the TRPV1 antagonist AMG7905 potentiates TRPV1 activation by both heat and protons (Table 2). It should be noted, however, that different scientists may assign the same compound to a different generation, because the potencies of a TRPV1 antagonist in all three main activation modes are never identical, and a big difference for one author may look insignificant to another. It is also important to note that, to the best of our knowledge, all TRPV1 antagonists block TRPV1 activation by CAP with reasonable potency. This is due to the fact that pharmaceutical companies, while working on TRPV1 antagonists (at least at the early stages of their TRPV1 programs), often "discarded" any molecules that did not block TRPV1 activation by CAP – such compounds would not be considered TRPV1 antagonists. Of interest, according to R. Kapil and D. J. Kyle (personal communication), the TRPV1 program at Purdue Pharma, one of the pioneers in the field of TRPV1 antagonists, never found a molecule that blocked TRPV1 activation by protons without also blocking CAP activation.

2.4. Modeling: which modes of TRPV1 activation contribute to the hyperthermic response in rats?

We now know (Table 2) that TRPV1 antagonists can affect T_b in several ways, even in the same species (*e.g.*, the rat): many of them readily produce hyperthermia (sometimes, they are called "hyperthermic" compounds); some have no thermal effect at comparable doses ("thermally neutral" compounds); and yet others can cause hypothermia ("hypothermic" compounds). Can the ability of TRPV1 antagonists to cause different thermoregulatory responses be ascribed to their different effects on different modes of TRPV1 activation? This question was asked by scientists at Amgen (and later, among others, by scientists at AbbVie, formerly Abbott Laboratories), who synthesized a variety of compounds with differential effects on TRPV1 activation in different modes. Lehto et al. (2008) observed that, in rats, TRPV1 antagonists that potently blocked channel activation by protons (many examples are given in Section 2.3 above; also see Table 2) typically caused hyperthermia, whereas compounds that potentiated proton activation (*e.g.*, AMG8562 and AMG7905) caused hypothermia instead of hyperthermia (Table 2). However, without an advanced quantitative analysis, it is difficult to identify with certainty the relationship between the potency of a compound to block TRPV1 activation in any given mode *in vitro* and the effect of this compound on T_b *in vivo*. This uncertainty is due to, among other factors, the fact that both the thermoregulatory effect and the effect on channel activation are dose-dependent. For example, if a compound does not affect T_b, it can be inherently incapable of affecting it or, alternatively, it might have been used at a subthreshold dose for the hyperthermic effect. Furthermore, if a compound is a moderately potent blocker of TRPV1 activation in a certain mode, *e.g.*, by heat, and causes hyperthermia at moderate doses, it can be interpreted that blockers of TRPV1 activation by heat cause hyperthermia, even though the administration of a relatively low dose of this, relatively weak, blocker of TRPV1 activation by heat, could have resulted in the *in-vivo* concentrations that were insufficient to block heat activation. Not surprisingly, therefore, some studies based on comparing thermoregulatory effects of a small number of compounds administered at a couple of doses resulted in unfounded conclusions. For example, at one point, a conclusion was made that hyperthermic TRPV1 antagonists are those that block TRPV1 activation by both CAP and heat, regardless of their effect on proton activation (Gavva, Bannon, Surapaneni, et al., 2007). This conclusion was then adopted in many reviews and original-research articles (see, for example, Alawi & Keeble, 2010; Rawls & Benamar, 2011; Wong & Gavva, 2009). Yet, the subsequent research showed that this conclusion had to be modified (Garami, Pakai, et al., 2018). Clearly, a comprehensive quantitative analysis was needed.

A quantitative analysis of the contribution of the blockade of different activation modes of the TRPV1 channel to the hyperthermic response to TRPV1 antagonists was performed by our group (Garami

Table 2
TRPV1 antagonists: Their effects on deep T_b upon systemic administration in rats and their potencies at different activation modes of rat TRPV1 in vitro

Compound	Effect(s) on deep T_b		*In vitro*: IC_{50} (nM) for different activation modes			
	Effect(s)	Reference(s)	CAP (10 nM-3 μM)	pH (5.0-6.2)	Heat (45-53°C)	Reference(s)
1	↑	Gomtsyan et al., 2015			Not studied	Gomtsyan et al., 2015
74	↑	Norman et al., 2007			Not studied	Norman et al., 2007
A-1098807	↑	Reilly et al., 2012		Not studied	Not studied	Reilly et al., 2012
A-1098808	↑	Reilly et al., 2012		Not studied	Not studied	Reilly et al., 2012
A-1106625	↑	Reilly et al., 2012			Not studied	Reilly et al., 2012
A-1153818	↑	Reilly et al., 2012		Not studied	Not studied	Reilly et al., 2012
A-1241407	↑	Reilly et al., 2012		Not studied	Not studied	Reilly et al., 2012
A-889425[†]	↑	McGaraughty et al., 2009; Reilly et al., 2012		Not studied	Not studied	McGaraughty et al., 2009; Reilly et al., 2012
A-993610	↑	Reilly et al., 2012		Not studied	Not studied	Reilly et al., 2012
ABT-102	↑	Honore et al., 2009; Voight et al., 2014				Surowy et al., 2008
AMG1629	↑	Gavva, Bannon, Surapaneni, et al., 2007				Gavva, Bannon, Surapaneni, et al., 2007
AMG2820	↑	Gavva, Bannon, Surapaneni, et al., 2007		X		Gavva, Bannon, Surapaneni, et al., 2007
AMG3731	↑	Gavva, Bannon, Surapaneni, et al., 2007				Gavva, Bannon, Surapaneni, et al., 2007
AMG7988	↑	Gavva, Bannon, Surapaneni, et al., 2007		X		Gavva, Bannon, Surapaneni, et al., 2007
AMG8563	↑	Lehto et al., 2008		X		Lehto et al., 2008
C	↑	Gavva, Bannon, Surapaneni, et al., 2007				Gavva, Bannon, Surapaneni, et al., 2007
D	↑	Gavva, Bannon, Surapaneni, et al., 2007				Gavva, Bannon, Surapaneni, et al., 2007
E	↑	Gavva, Bannon, Surapaneni, et al., 2007				Gavva, Bannon, Surapaneni, et al., 2007

Compound	Effect(s) on deep T_b		*In vitro*: IC_{50} (nM) for different activation modes			
	Effect(s)	Reference(s)	CAP (10 nM-3 μM)	pH (5.0-6.2)	Heat (45-53°C)	Reference(s)
G	↑	Gavva, Bannon, Surapaneni, et al., 2007				Gavva, Bannon, Surapaneni, et al., 2007
H	↑	Gavva, Bannon, Surapaneni, et al., 2007				Gavva, Bannon, Surapaneni, et al., 2007
JNJ-39729209	↑	Maher et al., 2011			Not studied	Maher et al., 2011
V116517	↑	Tafesse et al., 2014			Not studied	Tafesse et al., 2014
A-425619	↔, ↑	Garami et al., 2010; Gavva, Bannon, Surapaneni, et al., 2007; Mills et al., 2008				El Kouhen et al., 2005; Gavva, Bannon, Surapaneni, et al., 2007; McDonald et al., 2008; Neelands, Jarvis, Han, Faltynek, & Surowy, 2005
AMG 517	↔, ↑	Garami et al., 2017; 2010; Gavva, Bannon, Hovland Jr., et al., 2007; 2008; Nash et al., 2012; Tamayo et al., 2008				Gavva, Bannon, Hovland Jr., et al., 2007; Tamayo et al., 2008; Wang, Katon, et al., 2007; Wang, Chakrabarti, et al., 2007
AMG0347	↔, ↑	Garami et al., 2010; Steiner et al., 2007				Steiner et al., 2007
AMG8163	↔, ↑	Garami et al., 2010; Gavva, Bannon, Hovland Jr., et al., 2007; Gavva, Bannon, Surapaneni, et al., 2007; Lehto et al., 2008				Gavva, Bannon, Surapaneni, et al., 2007; Lehto et al., 2008
BCTC	↔, ↑	Gavva, Bannon, Surapaneni, et al., 2007; Watabiki, Kiso, Kuramochi, et al., 2011				Correll, Phelps, Anthes, Umland, & Greenfeder, 2004; Gavva, Bannon, Surapaneni, et al., 2007; 2005; Kanai, Nakazato, Fujiuchi, Hara, & Imai, 2005; Papakosta et al., 2011; Valenzano et al., 2003; Watabiki, Kiso, Kuramochi, et al., 2011
BCTP	↔, ↑	Nash et al., 2012				Nash et al., 2012
JNJ-17203212	↔, ↑	Kelly et al., 2015; Swanson et al., 2005			Not studied	Swanson et al., 2005

Compound	Effect(s) on deep T_b		*In vitro*: IC_{50} (nM) for different activation modes			
	Effect(s)	Reference(s)	CAP (10 nM-3 μM)	pH (5.0-6.2)	Heat (45-53°C)	Reference(s)
JNJ-39439335	↔, ↑	Parsons et al., 2015		Not studied	Not studied	Parsons et al., 2015
JTS-653	↔, ↑	Kitagawa et al., 2012				Kitagawa et al., 2012
2	↔	Gomtsyan et al., 2015		Not studied	Not studied	Gomtsyan et al., 2015
A-1105512	↔	Reilly et al., 2012		Not studied	Not studied	Reilly et al., 2012
A-1165442	↔	Reilly et al., 2012; Voight et al., 2014		Not studied	Not studied	Reilly et al., 2012
A-1165746	↔	Reilly et al., 2012		Not studied	Not studied	Reilly et al., 2012
A-1208747	↔	Reilly et al., 2012		Not studied	Not studied	Reilly et al., 2012
A-1233371	↔	Reilly et al., 2012		Not studied	Not studied	Reilly et al., 2012
A-1233372	↔	Reilly et al., 2012		Not studied	Not studied	Reilly et al., 2012
A-1241797	↔	Reilly et al., 2012		Not studied	Not studied	Reilly et al., 2012
CPZ†	↔	Garami et al., 2010; Steiner, et al., 2011	X	X	X	Appendino et al., 2003; Bevan et al., 1992; Correll et al., 2004; Dickenson & Dray, 1991; Gavva et al., 2005; Jakab et al., 2005; Kanai, Hara, & Imai, 2006; Kirschstein, Greffrath, Busselberg, & Treede, 1999; Maione et al., 2007; McDonald et al., 2008; McIntyre et al., 2001; Park et al., 2003; Phillips, Reeve, Bevan, & McIntyre, 2004; Price, Patwardhan, Akopian, Hargreaves, & Flores, 2004; Rigoni et al., 2003; Savidge et al., 2002; Seabrook et al., 2002; Swanson et al., 2005; Valenzano et al., 2003
SB-366791	↔	Garami et al., 2010		X	X	Gavva et al., 2005; Varga et al., 2005
3*	↓	Gomtsyan et al., 2015		Not studied	Not studied	Gomtsyan, et al., 2015
A-1165901	↓	Garami, Pakai, et al., 2018		Potentiation	Not studied	Garami, Pakai, et al., 2018
AMG7905		Lehto et al., 2008		Potentiation	Potentiation	

Compound	Effect(s) on deep T_b		*In vitro*: IC_{50} (nM) for different activation modes			
	Effect(s)	Reference(s)	CAP (10 nM-3 μM)	pH (5.0-6.2)	Heat (45-53°C)	Reference(s)
	↓					Garami, Pakai, et al., 2018; Lehto et al., 2008
AMG8562	↓	Lehto et al., 2008		Potentiation	X	Lehto et al., 2008
I-RTX*	↓	Dogan et al., 2004			Not studied	Appendino et al., 2003; Correll et al., 2004; Johnson et al., 2006; Kanai et al., 2006; Price et al., 2004; Rigoni et al., 2003; Seabrook et al., 2002; Shimizu et al., 2005
AS1928370	↔, ↓	Watabiki, Kiso, Kuramochi, et al., 2011		X	Not studied	Watabiki, Kiso, Kuramochi, et al., 2011
JYL 1421	↔, ↓	Garami et al., 2010; Gavva, Bannon, Surapaneni, et al., 2007; Suh et al., 2003		Potentiation	X	Gavva, Bannon, Surapaneni, et al., 2007; Jakab et al., 2005; Papakosta et al., 2011; Suh et al., 2003
AMG9810	↔, ↑, ↓	Barrett, Roy, Rivard, Wilson, & Scantlebury, 2018; Garami et al., 2010; Gavva, Bannon, Surapaneni, et al., 2007; Patrone et al., 2019				Gavva, Bannon, Surapaneni, et al., 2007; Gavva et al., 2005; Norman et al., 2007

The effects on deep T_b are marked as follows: ↑, an increase; ↓, a decrease; ↔, none.
The range of IC_{50} values in the three activation modes is color-coded as follows: 1-9 nM: (CAP), (proton), (heat); 10-99 nM: (CAP), (proton), (heat); 100-999 nM: (CAP), (proton), (heat); >1,000 nM: X (any activation mode). These ranges roughly correspond to the following inhibition potencies: strong, moderate, weak, and none, respectively. When different potencies are reported for the same compound, they are shown (as different colors) within the same cell. When potentiation occurs instead of inhibition, the effect is marked as such.
Other notes: *Compound 3 and I-RTX are partial agonists (Gomtsyan et al., 2015; Shimizu et al., 2005); †CAP concentration used in the study of A-889425 by McGaraughty et al. (2009) and in the study of CPZ by Phillips, Reeve, Bevan, and McIntyre (2004) was not specified.

et al., 2010). We developed a mathematical model and used it to analyze a set of data obtained from 49 groups of rats, where each group was treated with either a distinct dose of a TRPV1 antagonist (eight antagonists were used) or vehicle. Of the antagonists studied, seven caused dose-dependent hyperthermia, whereas one (JYL1421) caused dose-dependent hypothermia. The analysis revealed that the hyperthermic response to a TRPV1 antagonist is highly sensitive to changes in the compound's *in-vitro* potency to block TRPV1 activation by protons, but is completely insensitive to the potency to block either heat or CAP activation of the channel (Fig. 8). In other words, only potent antagonists of proton activation of rTRPV1 cause hyperthermia in young male rats, and this effect does not depend on the antagonist's potency to block other modes of rTRPV1 activation. We later confirmed that those antagonists that potentiate the proton activation of TRPV1 cause hypothermia (Garami, Pakai, et al., 2018).

As explained elsewhere (Garami, Pakai, et al., 2018), the results with the CAP activation mode were initially not as clear as described in the paragraph above. In fact, the Garami et al. (2010) model produced two different outcomes, depending on whether the hypothermic antagonist JYL1421 was included in the analysis or not. The results described above and shown in Fig. 8 were obtained with a complete data set, *i.e.*, with JYL1421. If JYL1421 was excluded from the analysis, the hyperthermic response to a TRPV1 antagonist became somewhat sensitive to changes in the antagonist's potency to block CAP activation of TRPV1 – in addition to being highly sensitive to changes in the proton mode and insensitive to changes in the heat mode. At the time when the rat study (Garami et al., 2010) was conducted, we did not know whether the hyper- and hypothermic responses to TRPV1 antagonists had two distinct mechanisms (*e.g.*, one represented an off-target action) or, alternatively, whether they were brought about by the same mechanism,

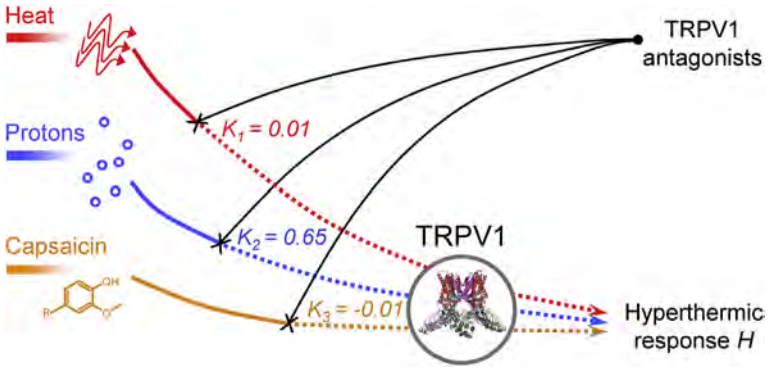

Fig. 8. Schematic presentation of the mathematical modeling results to show the contribution of different modes of TRPV1 activation to the development of TRPV1 antagonist-induced hyperthermia in rats. Signals activating TRPV1 in the heat mode (red line), proton mode (blue line), and CAP mode (orange line) are differentially blocked by TRPV1 antagonists (black lines) to cause the hyperthermic response H (see Supplementary Methods). The k_1, k_2, and k_3 values are relative sensitivities of H to the extent of TRPV1 blockade in the heat, proton, and CAP modes, respectively. Reprinted from Garami et al. (2010).

just working in reverse. In the former case, the results with JYL1421 should have been excluded from the analysis; in the latter case, they should have been included. In a more recent work (Garami, Pakai, et al., 2018), we studied the hypothermia-inducing TRPV1 antagonists A-1165901 and AMG7905 and found that the hyper- and hypothermic responses are similar from the mechanismic point of view. Both represent an on-target action (do not occur in TRPV1 knockout animals); both recruit the exact same thermoeffectors (but working in reverse); both are characterized by the same dependence of thermoeffectors on T_a; and both depend on the proton mode of TRPV1 activation (potently block *vs.* potentiate it). Hence, we concluded that TRPV1 antagonists cause hypothermia by engaging the same mechanisms as the hyperthermic TRPV1 antagonists but in reverse (Garami, Pakai, et al., 2018). Therefore, our earlier mathematical modeling study (Garami et al., 2010) should have been interpreted using a complete data set (with JYL1421), and the results obtained on the complete data set are described herein (Fig. 8).

2.5. Putting it all together: "illogical" acidification-induced anti-hyperthermic reflexes

A concept has emerged (Garami, Pakai, et al., 2018; Romanovsky et al., 2009) proposing that, at least in young male rats, TRPV1 does not serve as a thermosensor that drives thermoeffector responses – even though the thermosensing function of the channel is involved in pain in this species (Jhaveri, Elmes, Kendall, & Chapman, 2005; McGaraughty, Chu, Faltynek, & Jarvis, 2006; Vandewauw et al., 2018). Instead, TRPV1 participates in thermoregulation by sensing protons (or other stimuli that activate the channel through the same mechanism as protons). The TRPV1 channels that are tonically activated by protons and drive thermoeffectors are located somewhere in the abdominal viscera or muscles. TRPV1 antagonists affect thermoregulation, at least in young male rats, by affecting the activation of these TRPV1 channels. This picture has been drawn based on the robust experimental support (reviewed herein and elsewhere). Furthermore, as evident from the consensus paper (Garami, Pakai, et al., 2018), this view is now accepted not only by some academic scientists, but also by colleagues from Amgen and AbbVie – two companies that carried out a great amount of pioneering work with TRPV1 antagonists. And yet, this picture is rarely mentioned in the literature, while the alternative view (*i.e.*, that TRPV1 plays a thermosensory role in mammalian thermoregulation, and that TRPV1 antagonists affect T_b by blocking thermal activation of TRPV1) is widely spread. To the best of our knowledge, the thermal nature of the effector-driving TRPV1 signals – the cornerstone of this alternative view – has not been demonstrated so far, not even in a single study. Perhaps the main reason for the slow acceptance of the new reflexes, which can be called acido-antithermogenic and acido-antivasoconstrictor, is related to fact that they do not "make sense" at the first glance. Indeed, what is the biological significance of bringing the T_b down when the environment in the trunk is acidified?

Those reflexes that seem to make no sense are called "illogical" (Partridge, 1982; Romanovsky et al., 2009). The autonomic regulation, including thermoregulation, is executed by multiple, independent effector loops using both humoral and neural signals; the latter are called reflexes (Romanovsky, 2018). These reflexes are quite diverse, and only a small subset of them is active under any given set of external and internal conditions; when conditions change, a different subset of loops is recruited. We readily understand those reflexes that are vital, often engaged in everyday life of the organism, and well-studied, *e.g.*, various baroreflexes used in the cardiovascular control. Such reflexes seem "logical". Yet, there are many other reflexes that we do not understand, and some thermoregulatory reflexes that are triggered by nonthermal stimuli belong to this group. For example, skin vasoconstriction is affected by colorectal distension (Laird, Carrive, & Waite, 2006), while nonshivering thermogenesis is modulated by gastric stretching (Petervari, Garami, Pakai, & Szekely, 2005), the level of intraportal glucose (Sakaguchi & Yamazaki, 1988), and the osmolarity of the content in different parts of the gastrointestinal tract (Boschmann et al., 2007; Osaka, Kobayashi, & Inoue, 2002). All of the abovementioned reflexes seem illogical – but only until we study them, find the conditions under which they are expressed, or just start thinking about them. For example, the concentration of glucose in the portal blood, as well as gastric stretching, can be viewed as indices of energy intake, whereas nonshivering thermogenesis is a major mechanism of energy expenditure in rodents; it should not be a surprise that the latter is modulated by the former.

What could be the biological significance of the unusual TRPV1-mediated reflexes that link pH and T_b? Because polymodal TRPV1 antagonists induce robust hyperthermia in different species (Fig. 2), it is probably related to some basic physiological interactions. Initially (Steiner et al., 2007), we thought that interactions between the feeding status, gastrointestinal pH, and T_b were involved. However, in view of our recent results showing that the hyperthermic response to TRPV1 antagonists is affected neither by vagotomy (A. Garami, A. A. Steiner, and A. A. Romanovsky, unpublished observations) nor by the transection of the greater splanchnic nerves (A. Garami and A. A. Romanovsky, unpublished observations), we dismissed the visceral location of the TRPV1 channels of interest and the entire "gastrointestinal" scenario (Garami, Pakai, et al., 2018).

Instead, we propose that interactions between the acid-base homeostasis, T_b, and physical activity can be relevant. Strenuous physical activity is well-known to cause metabolic acidosis, including marked acidemia (Robergs, Ghiasvand, & Parker, 2004), and it increases deep T_b and often peripheral temperatures. Based on the tight co-expression of TRPV1 with acid-sensing ion channel-3 on

metaboreceptive afferents in muscle arterioles, it has been proposed that TRPV1 channels at this location may function as sensors for reflexes triggered by the acidic environment and elevated temperature of working muscles (Molliver et al., 2005). In those situations, when physical activity is especially strenuous (*e.g.*, when an animal is running for life from a predator), T_bs can reach extremely high values. In a study by Taylor and Lyman (1972), an abdominal temperature of > 47°C was recorded in a running gazelle. By the same token, high T_bs (whether shell or core) inhibit physical performance (Cheung & Sleivert, 2004; Nybo, Rasmussen, & Sawka, 2014; Schlader, Simmons, Stannard, & Mundel, 2011). Hence, a negative-feedback cycle is formed: an animal has to run as fast as it can to survive → T_bs increase → the capability to run decreases. Would it not be highly beneficial to counteract the development of hyperthermia by inhibiting cold-defense responses (thermoregulatory heat conservation and heat production), thus cancelling the performance-inhibiting feedback? We think it would, and the TRPV1-mediated acido-antithermogenic and acido-antivasoconstrictor reflexes discussed herein may do just that. When an animal runs, its internal environment acidifies, and the low pH, *via* TRPV1 channels (perhaps in the massive trunk muscles that are rich with slow-twitch, type-1 muscle fibers and are involved in breathing), inhibits cold defenses, thus preventing a further rise in T_b or bringing it down. This speculative line of thought has already found support in the studies showing that acute administration of CAP causes sympathetic activation and increases exercise endurance in rats and mice (Kim, Kawada, Ishihara, Inoue, & Fushiki, 1997; Oh, Oh, & Ohta, 2003). Furthermore, Luo et al. (2012) have shown that TRPV1 activation by chronic dietary CAP or transgenic TRPV1 overexpression also increases exercise endurance in mice, and that this effect of CAP does not occur in TRPV1-decifient mice. The concept presented here can be tested further by blocking TRPV1-mediated reflexes in exercising animals.

At the first glance, the proposed scenario is difficult to reconcile with the fact that lactic acid is a potent inhibitor (not activator) of TRPV1 (de la Roche et al., 2016), whereas acidosis during physical activity is accompanied by massive production of lactate (Bangsbo, Madsen, Kiens, & Richter, 1996) and, for a long time, was known as "lactic acidosis." It was believed that the increased production of lactic acid causes the release of protons and the formation of the acid salt sodium lactate, eventually exceeding the cellular buffering capacity and resulting in proton accumulation and a pH decrease. As explained by Robergs et al. (2004), the lactic acidosis concept has been disproved. While lactate production coincides with metabolic acidosis in strenuous exercise, it retards – not causes – it. In intense physical work, nonmitochondrial ATP from glycolysis is used heavily to fuel muscle contraction, thus releasing protons and causing acidosis. While extracellular lactate inhibits TRPV1, at least *in vitro* (de la Roche et al., 2016), the channel is gated open by extracellular protons (pH < 6), and milder acidosis (pH between 6 and 7) sensitizes it (reviewed by Holzer, 2009). The critical importance of TRPV1 in acid-sensing has been also demonstrated *in vivo* – using mice genetically deficient in TRPV1 (Caterina et al., 2000; Leffler, Monter, & Koltzenburg, 2006).

3. The hyperthermic effect of TRPV1 antagonists in human clinical trials

3.1. Phenomenology: effects of TRPV1 antagonists on T_b in humans

As of today, a relatively large number of TRPV1 antagonists has already been tested in humans (Table 3). Besides healthy adult volunteers, patients with a variety of conditions and symptoms were studied, often involving pain and inflammation (*e.g.*, dental or neuropathic pain, arthritis, or dermatitis), but also itching, coughing, and chronic pulmonary obstruction. As part of safety assessment, deep T_b was measured in several trials, and it is now well-known that many TRPV1 antagonists had adverse effects on T_b in humans (Gavva et al., 2008; Krarup et al., 2011; Lee et al., 2017; Manitpisitkul et al., 2015; Rowbotham et al., 2011).

As in laboratory animals (see 2.1), the thermoregulatory effects of TRPV1 antagonists in humans are diverse (Fig. 9). In one of the first human trials, AMG 517 caused marked hyperthermia with deep T_b exceeding 40°C (Gavva et al., 2008), which led to a premature termination of the trial. A dose-dependent (and, at higher doses, pronounced: 0.8-1.4°C) T_b rise was also observed in human trials with ABT-102 and V116517 (Fig. 9A and 9B, respectively), as well as with XEN-D0501 (Round, Priestley, & Robinson, 2011), while AZD1386 caused a milder (0.4°C) elevation in T_b (Fig. 9C). Another TRPV1 antagonist, SB-705498, had no thermal effect in humans, even at doses as high as 600 mg p.o. (Fig. 9D), whereas the mode-selective antagonist NEO6860 seemed to cause a small (0.2°C) decrease in deep T_b (Fig. 9E). As in laboratory animals, the thermal effects of TRPV1 antagonists in humans show no obvious association with the antagonist's chemical structure, thus suggesting an on-target action.

Because TRPV1 remains a promising therapeutic target in many human diseases and conditions (Brito, Sheth, Mukherjea, Rybak, & Ramkumar, 2014; Feketa & Marrelli, 2015; Gram, Holst, & Szallasi, 2017; Nilius & Appendino, 2013), it is important to understand what determines the thermoregulatory effects of TRPV1 antagonists in our own species. We already know that some TRPV1 antagonists, *e.g.*, CPZ and JYL1421, affect thermoregulation in a species-specific manner (see Section 2.1 above). We also know that at least some TRPV1 antagonists affect the TRPV1 channel in a species-specific manner as well and, hence, have different activation-mode pharmacological profiles against the TRPV1 channel in different species. A famous example is the TRPV1 antagonist AMG8562, which was the first one reported to cause analgesia without hyperthermia in rats (Lehto et al., 2008). It is thought to cause no hyperthermia in this species, because it does not inhibit proton activation of rTRPV1. However, if this assumption is true, the success of AMG8562 in causing hyperthermia-free analgesia in rats cannot be reproduced in humans – this antagonist is a potent inhibitor of all modes of activation of hTRPV1, including the proton mode (Lehto et al., 2008). Furthermore, the regulation of thermoregulatory responses in rats and humans is not likely to be identical; at the very least, rats do not sweat, whereas humans do not use tail-skin vasodilation or thermoregulatory salivation, and neither do humans rely on nonshivering thermogenesis in brown fat to the same extent as rats do (Romanovsky, 2018). Being several hundred times heavier than rats, humans take advantage of substantial thermal inertia and, accordingly, do not face the problem of readily losing the constancy of T_b (homeothermia) in view of environmental perturbations, at least not to the same extent as small rodents do. Consequently, peripheral thermal signals (including those mediated by TRPV1) are expected to play a smaller role in humans than in rodents (Romanovsky, 2014, 2018).

Last but not least, TRPV1 has different sensitivity to both temperature and chemical ligands in different species. For example, a TRPV1 isoform found in vampire bats is activated by temperatures as low as 30°C, which presumably makes TRPV1 a radiant-heat sensor for the detection of warm-blooded prey (Gracheva et al., 2011). On the other extreme, Bactrian camels express a TRPV1 ortholog that is not activated by temperature as high as 46°C (Laursen, Schneider, Merriman, Bagriantsev, & Gracheva, 2016), which, we think, is a genetic adaptation to high surface temperatures in camels' habitat during summer. Another dramatic example refers to TRPV1 sensitivity to CAP. Avian TRPV1 is not sensitive to CAP (Nagy & Rang, 2000), which allows birds to feed on spicy, pungent, CAP-rich fruits such as hot chili peppers. In contrast, most mammals, including mice (Garami et al., 2011), do not consume hot chili. In fact, CAP-containing repellants are used to protect crops from browsing by many species of mammalian pests (Romanovsky, 2015). In each of these examples, a pharmacological blockade of TRPV1 would have a distinct effect.

Based on the above, it is quite possible that TRPV1 antagonists cause their thermoregulatory effect somewhat differently in humans than in

Table 3
Characteristics of clinical studies of TRPV1 antagonists

Compound (company)	Patient or volunteer population	Thermometry site	Thermal effect	ClinicalTrials.gov identifier	Reference(s)
ABT-102 (Abbott)	Healthy male and female volunteers, 18-55 years	Oral	Increase in T_b	NCT00854659	Rowbotham et al., 2011
ABT-102 (Abbott)	Healthy volunteers	Oral or abdominal	Increase in T_b	Not reported	Othman et al., 2013
ABT-102 (Abbott)	Healthy male volunteers, 18-60 years	Aural	Dose-dependent increase in T_b	Not reported	Schaffler et al., 2013
AMG 517 (Amgen)	Patients with pain due to molar extraction	Oral and tympanic	Plasma concentration-dependent increase in T_b	Not reported	Gavva et al., 2008
AZD1386 (AstraZeneca)	Patients with pain due to lower "wisdom tooth" extraction	T_b not measured	T_b not measured	NCT00672646	ClinicalTrials.gov
AZD1386 (AstraZeneca)	Healthy volunteers	T_b not measured	T_b not measured	NCT00692146	ClinicalTrials.gov
AZD1386 (AstraZeneca)	Patients with post-traumatic or postherpetic neuralgia	T_b not measured	T_b not measured	NCT00976534	ClinicalTrials.gov
AZD1386 (AstraZeneca)	Patients with pain due to third molar extraction	Oral	Increase in T_b	Not reported	Quiding et al., 2013
AZD1386 (AstraZeneca)	Patients with knee osteoarthritis	T_b not measured	T_b not measured	NCT00878501	Miller, Bjornsson, Svensson, & Karlsten, 2014
AZD1386 (AstraZeneca)	Healthy male volunteers	Not reported	Dose-dependent increase in T_b	NCT00711048	Krarup et al., 2011
AZD1386 (AstraZeneca)	Patients with reflux disease responsive to proton-pump inhibitors	Not reported	Increase in maximum T_b	NCT01019928	Krarup et al., 2013
DWP05195 (Daewoong)	Patients with postherpetic neuralgia	T_b not measured	T_b not measured	NCT01557010	ClinicalTrials.gov
DWP05195 (Daewoong)	Healthy male volunteers	Not reported	Dose-dependent increase in T_b (tendency)	NCT00969787 (single dose) NCT01094834 (multiple doses)	Lee et al., 2017
GRC-6211 (Glenmark, Eli Lilly)	Patients with osteoarthritic pain, incontinence, or neuropathic pain	T_b not measured	T_b not measured	Not reported	Myers, 2008
JNJ-38893777 (Johnson & Johnson)	Healthy male volunteers	Oral	Small increase in T_b	Not reported	Manitpisitkul et al., 2015
JNJ-39439335 (Johnson & Johnson)	Healthy male volunteers	T_b not measured	T_b not measured	NCT01006304	ClinicalTrials.gov
JNJ-39439335 (Johnson & Johnson)	Healthy male volunteers	Oral	Small increase in T_b	Not reported	Manitpisitkul et al., 2016
JNJ-39439335 (Johnson & Johnson)	Healthy male volunteers	Oral	No clinically meaningful increase in T_b	Not reported	Manitpisitkul, Shalayda, Russel, Sanga, Solanki, et al., 2018
JNJ-39439335 (Johnson & Johnson)	Healthy male volunteers (Part 1); male and female patients with knee osteoarthritis (Part 2)	Oral	Plasma concentration-independent increase in T_b	Not reported	Manitpisitkul, Flores, et al., 2018
JNJ-39439335 (Johnson & Johnson)	Healthy male volunteers	Oral	Small increase in T_b	Not reported	Manitpisitkul, Shalayda, Russel, Sanga, Williams, et al., 2018
JNJ-39439335 (Johnson & Johnson)	Patients with knee osteoarthritis	Not reported	T_b increased in one patient (of 33 patients)	Not reported	Mayorga et al., 2017
MK-2295, or NGD-8243 (Merck)	Patients with postoperative dental pain	T_b not measured	T_b not measured	NCT00387140	ClinicalTrials.gov
MR-1817 (Mochida)	Healthy adult volunteers	T_b not measured	T_b not measured	NCT00960180	ClinicalTrials.gov
NEO6860 (NEOMED, Convance)	Healthy male volunteers	Gastrointestinal (ingestible transmitter)	No clinically significant change in T_b	NCT02337543	Brown et al., 2017
NEO6860 (NEOMED)	Adult patients with pain due to knee osteoarthritis	Oral	No increase of more than 1°C in T_b	NCT02712957	Arsenault et al., 2018
PAC-14028, or asivatrep (Amorepacific)	Patients with dermal pruritus	T_b not measured	T_b not measured	NCT02052531	ClinicalTrials.gov
PAC-14028, or asivatrep (Amorepacific)	Patients with dermal pruritus	T_b not measured	T_b not measured	NCT02565134	ClinicalTrials.gov
PAC-14028, or asivatrep (Amorepacific)	Patients with erythema-telangiectatic or papulopustular rosacea	T_b not measured	T_b not measured	NCT02052999	ClinicalTrials.gov
PAC-14028, or asivatrep (Amorepacific)	Patients with mild-to-moderate atopic dermatitis	T_b not measured	T_b not measured	NCT02757729 NCT02583022 NCT02965118	ClinicalTrials.gov
PAC-14028, or asivatrep (Amorepacific)	Patients with mild-to-moderate seborrheic dermatitis of the face	T_b not measured	T_b not measured	NCT02749383	ClinicalTrials.gov
PAC-14028, or asivatrep (Amorepacific, Seoul National University Hospital)	Healthy male volunteers	T_b not measured	T_b not measured	NCT02309008	ClinicalTrials.gov
PAC-14028, or asivatrep (Amorepacific)	Healthy male volunteers	T_b not measured	T_b not measured	NCT01264224	ClinicalTrials.gov

Table 3 (*continued*)

Compound (company)	Patient or volunteer population	Thermometry site	Thermal effect	ClinicalTrials.gov identifier	Reference(s)
PAC-14028, or asivatrep (Amorepacific)	Healthy adult volunteers	T_b not measured	T_b not measured	NCT01638117	ClinicalTrials.gov
PAC-14028, or asivatrep (Amorepacific)	Patients with rosacea	T_b not measured	T_b not measured	NCT02583009	ClinicalTrials.gov
PAC-14028, or asivatrep (Amorepacific)	Pediatric patients with atopic dermatitis	T_b not measured	T_b not measured	NCT02748993	ClinicalTrials.gov
PHE-377 (PharmEste)	Patients with neuropathic pain	T_b not measured	T_b not measured	Not reported	Evans, 2011
SB-705498 (GlaxoSmith-Kline)	Patients with migraine	T_b not measured	T_b not measured	NCT00269022	ClinicalTrials.gov
SB-705498 (GlaxoSmith-Kline)	Patients with third molar extraction	Tympanic	Maximal change in T_b from baseline was 0.8 ± 0.5°C at 1,000 mg (baseline not reported)	NCT00281684	ClinicalTrials.gov
SB-705498 (GlaxoSmith-Kline)	Healthy adult volunteers	Tympanic	Not reported	NCT01476098 (oral)	ClinicalTrials.gov
SB-705498 (GlaxoSmith-Kline)	Healthy adult volunteers	T_b not measured	T_b not measured	NCT01673529 (topical)	ClinicalTrials.gov
SB-705498 (GlaxoSmith-Kline)	Patients with rectal pain	T_b not measured	T_b not measured	NCT00461682	ClinicalTrials.gov
SB-705498 (GlaxoSmith-Kline)	Healthy adult volunteers	T_b not measured	T_b not measured	Not reported	Chizh et al., 2007
SB-705498 (GlaxoSmith-Kline)	Patients with refractory chronic cough	Tympanic	No change in T_b	Not reported	Khalid et al., 2014
SB-705498 (GlaxoSmith-Kline)	Patients with seasonal allergic rhinitis	Oral	No change in T_b	NCT01424397	Bareille et al., 2013
SYL-1001 (Sylentis)	Patients with ocular pain due to dry-eye syndrome	T_b not measured	T_b not measured	NCT01776658 NCT01438281 NCT02455999	Benitez-Del-Castillo et al., 2016
V116517 (Purdue)	Subjects with moderate-to-severe pain due to knee osteoarthritis	T_b not measured	T_b not measured	NCT01688934	ClinicalTrials.gov
V116517 (Purdue)	Patients with moderate-to-severe postherpetic neuralgia	T_b not measured	T_b not measured	NCT01688947	ClinicalTrials.gov
V116517 (Purdue)	Healthy male volunteers	Oral	No change in T_b	NCT02695745	Arendt-Nielsen et al., 2016
XEN-D0501 (Xention, Ario)	Patients with chronic idiopathic cough	T_b not measured	T_b not measured	NCT02233699	ClinicalTrials.gov
XEN-D0501 (Xention, Ario)	Patients with refractory chronic cough	Not reported	Not reported	Not reported	Belvisi et al., 2017
XEN-D0501 (Xention)	Healthy male (Part 1) or male and female (Part 2) volunteers	Aural	Dose-dependent increase in T_b	Not reported	Round et al., 2011
XEN-D0501 (Xention, Ario)	Patients with chronic obstructive pulmonary disease	T_b not measured	T_b not measured	NCT02233686	Smith et al., 2017

rats. Nothing is known about the contributions of different modes of hTRPV1 activation to the thermoregulatory effect of TRPV1 antagonists in humans. By conducting the mathematical analyses presented below, we intended to fill this void.

3.2. Mathematical modeling: which modes of hTRPV1 activation contribute to the hyperthermic response in humans?

For the present work, we identified 87 published reports on human clinical trials of TRPV1 antagonists, of which 18 trials involved recording some measure of deep (core) T_b (Fig. S1). Using the sets of inclusion and exclusion criteria (Tables S1 and S2), we were able to select five studies for a mathematical-modeling analysis, and also for meta-analysis (see Section 3.3. below). The full set of data used for modeling analysis is shown in Table S3. The model, which is based on the one we developed earlier (Garami et al., 2010), is described in detail in Supplementary Methods.

The modeling analysis has found that the hyperthermic effect of TRPV1 antagonists in humans is the most sensitive to the extent of hTRPV1 blockade in the proton activation mode. The sensitivity coefficient of the hyperthermic response to potency changes in the proton mode (mean ± SE) is 1.37 ± 0.00 ($P = 2.48 \times 10^{-39}$). In this respect, the hyperthermic response to TRPV1 antagonists in humans is similar to that in rats: in both species, potent blockers of proton activation cause an increase in T_b (Fig. 10). While preparing our recent study (Garami, Pakai, et al., 2018), we already had the information about the importance of the proton activation mode, based on preliminary results of our modeling analysis presented here, and we used these results to explain the phenomenon that was not well understood. It was reported that the TRPV1 antagonist V116517 potently blocked rTRPV1 activation by protons and caused hyperthermia in rats (Tafesse et al., 2014), but yet it did not cause hyperthermia in a human clinical trial, while potently blocking hTRPV1 activation by protons (Arendt-Nielsen et al., 2016). We proposed (Garami, Pakai, et al., 2018) that this discrepancy could be explained by the use of subthreshold (for causing hyperthermia) doses of V116517 in that particular trial, and that V116517 is an intrinsically hyperthermic (not neutral) compound. The data presented in the present work (Fig. 9B) show that our hypothesis is correct.

The present analysis has also found that, differently from the hyperthermic response in rats (which is insensitive to potency changes in the heat activation mode), the response in humans is highly sensitive to the antagonist's potency in the heat mode. For the human response, the sensitivity coefficient to potency changes in the heat mode is 1.09 ± 0.00 ($P = 1.92 \times 10^{-39}$). As for the CAP mode of activation, blocking it is not important for the hyperthermic response in either rats or humans. In humans, the response sensitivity to potency changes in the CAP mode is negative and six times lower in magnitude than the sensitivity in the proton activation mode. The sensitivity coefficient of the human response to potency changes in the CAP mode is -0.23 ± 0.00 ($P = 4.71 \times 10^{-38}$).

In addition to the set of mean sensitivity coefficients listed above and illustrated in Fig. 10, we have also found the best-fitted set of sensitivity

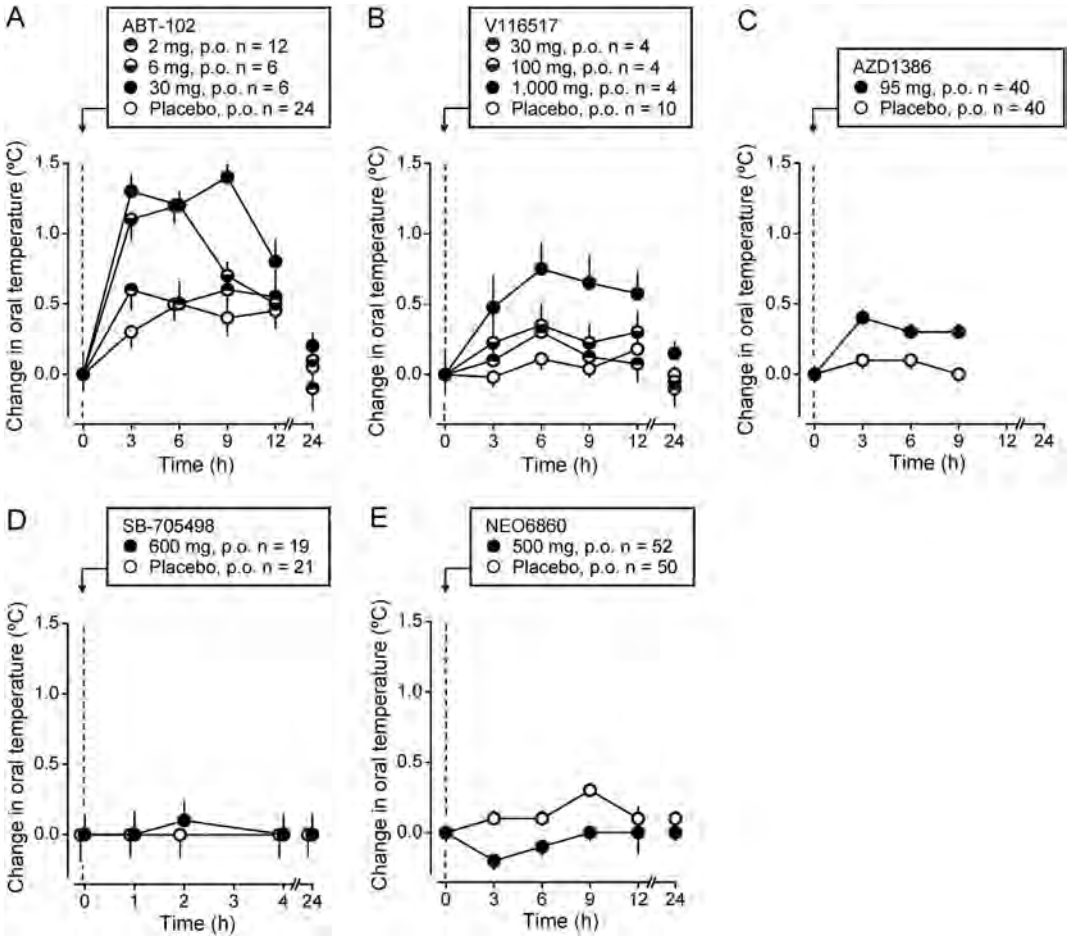

Fig. 9. Deep T_b responses of humans to TRPV1 antagonists vary from high hyperthermia to low hyperthermia, no effect, or hypothermia. A) Effect of ABT-102 (2, 6, and 30 mg/kg, p.o.) or placebo on oral temperature. B) Effect of V116517 (30, 100, and 1,000 mg/kg, p.o.) or placebo on oral temperature. C) Effect of AZD1386 (95 mg/kg, p.o.) or placebo on oral temperature. D) Effect of SB-705498 (600 mg/kg, p.o.) or placebo on oral temperature. E) Effect of NEO6860 (500 mg/kg, p.o.) or placebo on oral temperature. Plotted for this work based on data from Othman et al. (2013) (A), Arendt-Nielsen et al. (2016) (see also Table S6) (B), Quiding et al. (2013) (C), Khalid et al. (2014) (D), and Arsenault et al. (2018) (E).

coefficients (by using a Monte Carlo technique to run the model with randomly generated parameters; see Mathematical model description, Supplementary Information). The best-fitted set includes the following sensitivity coefficient values: -0.21 (CAP mode), 1.25 (proton mode), and 1.00 (heat mode). With this set, the model accounts for a maximal share of the hyperthermic response variation: 83% ($r^2 = 0.83$). The best-fitted set complements the mean values in characterizing how the potency in each activation mode affects the hyperthermic response to TRPV1 antagonists in humans.

In addition to the high value of r^2, the high quality of data fitting in our model is demonstrated by the low values of the so-called "mismatch errors", *i.e.*, the differences between the hyperthermic response values measured in clinical trials for the 16 antagonist doses used in this analysis (Table S3) and the corresponding response values predicted by our model. In our current analysis, the mismatch error varies from -0.3 to 0.4°C; the quadratic mean is 0.2°C.

The more complex nature of the hyperthermic response to TRPV1 antagonists in humans, as compared to that in rats (dependence on two activation modes *vs.* one), is also evident from how varying the antagonist potency in different activation modes contributes to the statistical variance of the human hyperthermic response (Fig. 10). In rats, > 81% of the statistical variance of the hyperthermic response is determined by the proton mode; the contributions of both the heat mode and the CAP mode are negligible (~1% each); and the contribution of factors unaccounted for by our model is 16%. In humans, these contributions are: 35% for the proton mode, 37% for the heat mode, 10% for the CAP mode, and 17% for unaccounted factors.

The main finding of our analysis is that the hyperthermic response to TRPV1 antagonists depends on the activation-mode TRPV1 pharmacology differently (and in a more complex fashion) in the human than in the rat. In rats, of the three modes studied (CAP, proton, and heat), only the proton mode matters: the hyperthermic response is highly sensitive to potency changes in this mode and is totally insensitive to potency changes in the CAP or heat mode. Accordingly, only potent blockers of rTRPV1 activation by protons cause hyperthermia in rats. In humans, the hyperthermic response is highly sensitive to potency changes in the proton mode of hTRPV1 activation as well. But it is also sensitive, almost to the same extent, to potency changes in the heat mode. Hence, perhaps the most interesting and somewhat unexpected conclusion from our analysis is that TRPV1 plays different roles in thermoregulation in humans, as compared to rats. In rats, it mediates the tonic suppression of thermogenesis and skin vasoconstriction by protons (or proton-like stimuli) in the abdomen, perhaps in trunk muscles; it does not serve as a thermosensor to the thermoregulation system in this species, or at least not in young male adults. In humans, TRPV1 also seems to mediate the effect of acidity on thermoregulation, but, in addition to this, it is likely to play a role of a true thermal sensor for the thermoregulation system – detecting T_bs to drive thermoeffector responses. Our analysis suggests that, in order to be thermally neutral in humans, a TRPV1 antagonist should have low potency in both temperature and proton activation modes of hTRPV1.

Our model also predicts that the strongest hyperthermic response in humans is induced by TRPV1 antagonists that are highly potent inhibitors of hTRPV1 activation by both protons and heat. This prediction is

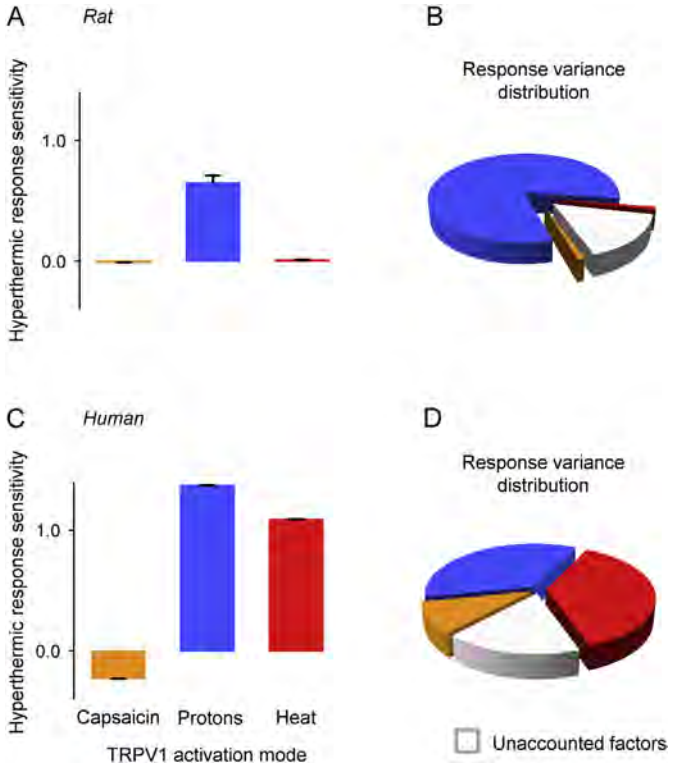

Fig. 10. Contribution of different modes of TRPV1 activation to the development of TRPV1 antagonist-induced hyperthermia in rats (A, B) and humans (C, D). TRPV1 antagonists differentially affect TRPV1 activation by CAP, protons, and heat; some TRPV1 antagonists cause the hyperthermic response. The sensitivity of the hyperthermic response to the antagonist potency in each of the three activation modes is presented as bars (mean ± standard error) for rats (A) and humans (C). While the hyperthermic response to TRPV1 antagonists in rats depends solely on the antagonist's potency to block TRPV1 activation by protons, the hyperthermic response in humans depends on the antagonist's potencies in both proton and heat modes. The pie charts depict the relative contributions of each mode (as well as of the factors unaccounted for by the model) to the statistical variance of the hyperthermic response in rats (B) and humans (D). Graphs are plotted from data reported by Garami et al. (2010) (A, B) or derived from the present mathematical analysis (C, D).

supported by the clinical trial of AMG 517 reported by Gavva et al. (2008). We could not use the Gavva et al. (2008) data in our analysis (Table S2), because the published report contained only the maximal values of T_b over a period of time (instead of the exact values at different time points). However, from the maximal T_b data presented, it is clear that AMG 517 causes marked hyperthermia in humans already at the very low dose of 2 mg (~5 μmol). AMG 517 is a highly potent blocker of hTRPV1 activation by both protons and heat; the corresponding IC_{50} values are 0.6 and 1.3 nM, respectively [Table S2; also see Gavva, Bannon, Surapaneni, et al. (2007)].

Furthermore, T_b data reported for most of the TRPV1 antagonists with a known pharmacological profile against hTRPV1 seem to be compatible with our model results (Arsenault et al., 2018; Gavva et al., 2008; Othman, Nothaft, Awni, & Dutta, 2013; Quiding et al., 2013; Rowbotham et al., 2011). SB-705498 may be an exception, as this compound blocks all three activation modes of hTRPV1 with high (3-6 nM) potency (Gunthorpe et al., 2007), and yet, in the trial reported by Khalid et al. (2014), a high dose of this compound (600 mg, p.o.) failed to increase deep T_b (Fig. 9D). However, the TRPV1 channel occupancy in that trial (based on the peak plasma SB-705498 concentration) was only 45%, thus suggesting that the dose used might have been insufficient for triggering on-target effects, including hyperthermia.

While the modeling analysis presented here enables us to make unique conclusions about the relationship between the activation-mode pharmacological profiles of TRPV1 antagonists and their thermal effects in humans, some caution should be exercised when these conclusions are interpreted or applied.

First, the model processed pharmacological profiles obtained against hTRPV1 *in vitro* and thermal responses recorded in humans *in vivo*, with measurements performed by different groups and by different methodologies. We do not know whether the channel behaves similarly *in vitro* and *in vivo*. We also ignore any pharmacokinetics-related differences between different compounds.

A less obvious, but perhaps very important, reason for caution is that the model assumes that there are only three modes of TRPV1 activation, and that they are independent of each other. In reality, however, the TRPV1 channel can be activated not only by CAP (and other vanilloids and some endocannabinoids), protons (acidic pH), and heat, but also by the myriad of other endogenous and exogenous ligands, including – just to give some examples – divalent (*e.g.*, Ni^{2+}) and trivalent (*e.g.*, Gd^{3+}) cations, polyamines, basic pH, eicosanoids, phospholipids, and terpenoids (for review, see Calixto, Kassuya, Andre, & Ferreira, 2005; Nilius & Szallasi, 2014). In fact, TRPV1 is known as the polymodal receptor *par excellence* (Holzer, 2009). Furthermore, there is certainly a crosstalk between different modes of TRPV1 activation (Blumberg et al., 2011), *e.g.*, mild acidosis sensitizes TRPV1 to both CAP and heat (reviewed by Holzer, 2009). The interdependence of different modes of TRPV1 activation clouds any interpretations of our modeling results.

Last but not least, the inordinate promiscuity of the TRPV1 channel makes it utterly sensitive to its environment. Accordingly, the channel is likely to play different physiological roles with changes in conditions, even in the same species. Consequently, effects of TRPV1 antagonists can also be expected to differ under different conditions. In the study by Gram et al. (2019) in Zucker obese rats, the TRPV1 antagonist BCTC improved insulin secretion at a young age of 6 months, but did not have this effect at a more mature age of 9 months. In our study (Wanner et al., 2012), the hyperthermic effect of AMG 517 in aged (44 weeks) mice was very similar to that in young (12 weeks) mice, but the effect of the antagonist on systemic inflammation changed with aging from anti-inflammatory to proinflammatory. Furthermore, the effect of AMG 517 on mortality of systemic inflammation in aged mice depended on whether the inflammation was septic (induced by colonic ligation and puncture) or aseptic (induced by a high dose of bacterial lipopolysaccharide).

A sex-dependent effect of the TRPV1 antagonist ABT-116 on locomotor activity was found in dogs; this compound produced a stronger effect in males than in females (Malek et al., 2012). Sex-dependent differences in the effects of CPZ on audiogenic seizures in rats (Cho, Vaca, Miranda, & N'Gouemo, 2018) and on the urethral response to noxious stimulation in mice (Yoshiyama, Araki, Kobayashi, Zakoji, & Takeda, 2010) have also been reported. These differences may be due to the demonstrated effects of ovariectomy, pregnancy, and sex hormones (*viz.*, progesterone, estradiol, and prolactin) on the expression and activity of TRPV1 in mice and rats (Diogenes et al., 2006; Ortiz-Renteria et al., 2018; Payrits et al., 2017; Wu et al., 2010). Estradiol has also been shown to upregulate TRPV1 at the mRNA level in human sensory neurons derived from embryonic stem cells (Greaves, Grieve, Horne, & Saunders, 2014).

Some thermoregulatory effects of TRPV1 antagonists may also be sex-dependent. Based on our experiments with AMG0347 in male rats (Steiner et al., 2007), we concluded that TRPV1 antagonists readily recruit autonomic thermoeffectors in the hyperthermic response, but do not use behavioral regulation (we are now revisiting the latter conclusion in a new study). Based on our experiments with multiple TRPV1 antagonists in male rats (Garami et al., 2010; Steiner et al., 2007), we concluded that blocking thermal signals does not affect thermoregulation. To our surprise, our recent experiments have shown that several TRPV1 antagonists affect behavioral thermoregulation in female rats, possibly by blocking thermal signals (Romanovsky, 2019). Clearly, more studies are needed, and any results obtained with TRPV1 antagonists should be generalized with great care. Without having a model of a

biological phenomenon in one's head, it is impossible to further study this phenomenon in a systematic way, but one should always be ready to change the model as new experimental data arrive.

3.3. Human clinical trials: meta-analysis

In addition to mathematical modeling, we also conducted a meta-analysis of the data reported in the identified articles, using standard meta-analysis tools (see Supplementary Methods), in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews (Table S4) and Meta-Analysis Protocols (Moher, Liberati, Tetzlaff, & Altman, 2009). This analysis was registered with PROSPERO (CRD 42018095220).

We included the studies that reported deep T_b values in both TRPV1 antagonist-treated and placebo groups at least at two time points: (i) at or shortly before the drug (or placebo) administration and (ii) at 3 h after the time of administration (Table S1). Studies for which all the necessary information could not be obtained were excluded from the analysis (Table S2). In certain cases, we had to make limited assumptions or simplifications, as explained in Table S5. The demographic profiles of the studies are outlined in Table S6. For each included study, we calculated the change in deep T_b as a difference between the T_b values at 3 h after the drug (placebo) administration *vs.* at the time of administration (0 h); this is the T_b change that was used in the modeling analysis as the value *H* of the hyperthermic response. We then calculated the difference between the T_b changes induced by a drug and those induced by placebo (difference in means); we considered the latter difference to represent the thermal effect of the drug in our meta-analysis. For all doses, the differences in means were standardized (based on variances) to obtain standardized differences in means (SDMs). The SDMs with 95% confidence intervals (CIs) were used as primary measures of effect size and are presented as a "forest plot" (Fig. 11). We considered each TRPV1 antagonist to be either mode-nonselective (ABT-102, AZD1386, and V116517) or mode-selective (NEO6860). To characterize the non-selective group, SDMs for the corresponding antagonists were weighted based on sample size and inversed variance.

All three antagonists in the mode-nonselective group caused hyperthermia, which was dose-dependent for those compounds that were administered at multiple doses, *viz.*, ABT-102 and V116517 (Fig. 11). The highest hyperthermic effect (SDM: 2.5; CI: 1.4-3.5) occurred in the group treated with the 86 μmol dose of ABT-102. The mean thermal effect of all doses of all mode-nonselective TRPV1 antagonists in the analyzed clinical trials was an SDM of 1.2 (CI: 0.9-1.6; $P < 0.001$). These results of meta-analysis agree with our mathematical-modeling results in that the blockade of the proton and heat activation modes of the hTRPV1 channel, as typically observed for polymodal TRPV1 antagonists, results in hyperthermia.

NEO6860, the only mode-selective TRPV1 antagonist that we were able to include in our analysis, did not cause hyperthermia at the dose used (1.2 mmol) but, instead, decreased the deep T_b (SDM: -0.7; CI: -0.3 to -1.1; $P < 0.001$). The lack of hyperthermia in response to NEO6860 agrees with our mathematical model, since this compound blocks neither proton nor heat activation of hTRPV1. The hypothermic effect of NEO6860 could be explained by the reported partial agonistic activity of this compound against hTRPV1 (Arsenault et al., 2018). TRPV1 agonists are well-known to cause hypothermia (Romanovsky et al., 2009; Szolcsanyi, 2015). TRPV1 antagonists with partial TRPV1 agonistic properties, such as 5'-iodo-RTX (Blumberg et al., 2011; Dogan et al., 2004; Shimizu, Iida, Horiuchi, & Caterina, 2005) or AbbVie's Compound 3 (Gomtsyan et al., 2015), also cause hypothermia. As a limitation of our meta-analysis, it should be mentioned that we analyzed only one dose of a single mode-selective drug; this analysis should be repeated as more data become available.

4. Tackling the thermal effects of TRPV1 antagonists: approaches to drug development

4.1. Designing thermally neutral antagonists based on mode-selective TRPV1 pharmacology

Perhaps the best solution would be to design a TRPV1 antagonist that possesses sufficient efficacy as an analgesic (or in respect to any other desired effects) but does not cause any of the on-target adverse effects on T_b, *i.e.*, hypo- or especially hyperthermia. Based on the current work, the thermal effects are likely to be minimal in humans for the compounds that do not block hTRPV1 activation by protons and temperature, even if they are still potent blockers of the channel activation by CAP. If the proton- and heat-blocking potencies are low enough,

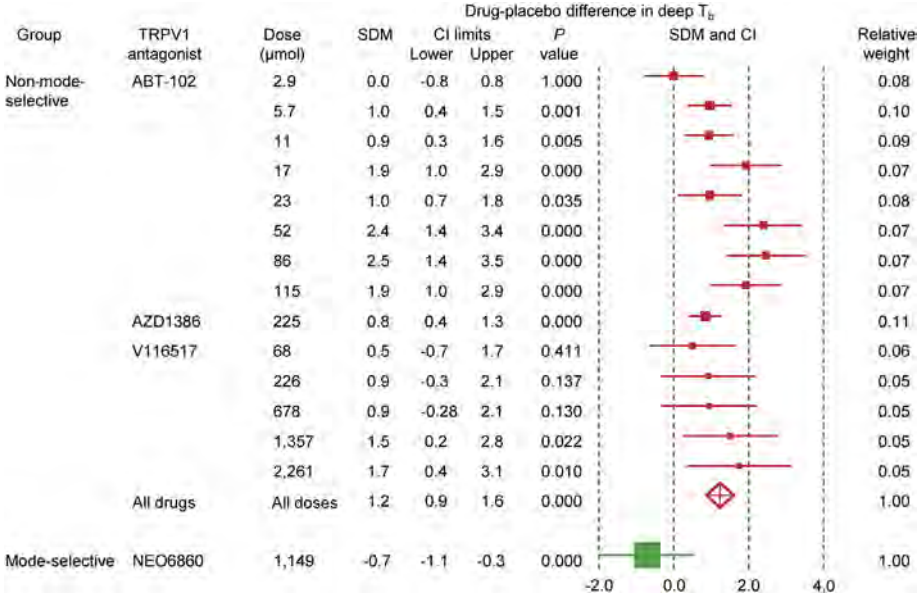

Fig. 11. Forest plot of the thermal effects of TRPV1 antagonists. For each antagonist dose, an SDM (a measure of the T_b response) and CI were calculated as described in the text (section 3.3) and are shown in the figure with a square and a horizontal bar, respectively. The area of the square is proportional to the sample size and inversed variance. The rhombus refers to the weighted mean SDM for the mode-nonselective group; the vertical diagonal of the rhombus points at the SDM value, whereas the horizontal diagonal represents the CI. Red symbols refer to mode-nonselective TRPV1 antagonists; the green square and bar refer to the selective antagonist NEO6860.

while the CAP-blocking potency is high enough, such a compound or biological has a chance of possessing sufficient efficacy (by potently blocking hTRPV1 activation by vanilloids) without affecting T_b. Rational design of therapeutics with this activation-mode profile clearly pushes the current boundaries of molecular pharmacology. Nonetheless, there are hints from TRPV1 mutational data that this may be possible. Early studies of TRPV1 orthologs showed that residues in the vanilloid-binding pocket could be mutated (Tyr511Ala and Ser512Tyr), which would selectively disrupt the CAP sensitivity, while retaining high sensitivity in the heat and proton activation modes (Jordt & Julius, 2002). Similarly, mutations in the pore turret of murine TRPV1 can selectively abrogate heat activation without impacting CAP activation (Cui et al., 2012), while distinct but proximal mutations have been shown to selectively disrupt proton activation (Jordt et al., 2000). More recently, random scanning mutagenesis of rTRPV1 has identified hundreds of specific mutations across the TRPV1 protein that retain either CAP- or heat-selective activation – although it is unclear how these mutations impact proton activation (Sosa-Pagan, Iversen, & Grandl, 2017). Taken together, these data prove that TRPV1 activation involves at least some stimuli-specific mechanismic differences. Leveraging these differences in gating-coupling modes of TRPV1 activation can potentially be instrumental for developing therapeutics. Indeed, multiple TRPV1 antagonists with various degrees of mode-selectivity (second-generation antagonists) have been synthesized, and some even tested in humans (*vide supra*).

There are two main obstacles for this approach. First, limited by the extent of the current knowledge, rational engineering of mode-selective TRPV1 compounds remains at an early stage. Second, there may be an intrinsic limit on how much the overall activation of TRPV1 can be blocked (for therapeutic purposes) without blocking channel activation by protons and temperature (to prevent thermoregulatory side effects). Reflecting this inherent limitation, the reasons for failure of TRPV1 antagonist clinical trials are now different than they were a decade ago. The early trials were conducted with first-generation (mode-nonselective) antagonists, and they typically failed due to the adverse hyperthermic effect; the trial with AMG 517 being a well-known example (Gavva et al., 2008). In contrast, several more recent trials were conducted with second-generation (mode-selective) TRPV1 antagonists, and some of these trials did not demonstrate the anticipated level of analgesia; trials of NEO6860 (Arsenault et al., 2018) being an example.

While rational engineering of TRPV1 antagonists with a desired profile is maturing, less efficient tools can also be used to achieve the same results, *e.g.*, "random" synthesis and *in-vitro* pharmacological screening. Based on publications, several thermally neutral, mode-selective TRPV1 antagonists have been (or were) considered by pharmaceutical companies for further development, including A-1165442 and A-1115760 by Abbott Laboratories and AbbVie (Reilly et al., 2012; Voight et al., 2014), AS1928370 (ASP8370) by Astellas Pharma (Oka et al., 2018; Watabiki, Kiso, Kuramochi, et al., 2011; Watabiki, Kiso, Tsukamoto, Aoki, & Matsuoka, 2011), and NEO6860 by NEOMED Institute (Arsenault et al., 2018; Brown et al., 2017).

4.2. Using therapeutic doses that are subthreshold for the hyperthermic response

In the section above, we discussed development of TRPV1 antagonists that are thermally neutral, meaning that they do not cause a thermal response in the entire dose range that can be reasonably expected to be used. However, the developers will always face conflicting demands: a thermally neutral antagonist, at least according to our model, should not block heat and proton activation of hTRPV1, whereas in order to be an efficacious analgesic (or whatever the desired therapeutic effect might be), it should potently block TRPV1 activation, sometimes in all modes. As a result, "compromise" antagonists are likely to emerge that exhibit some antagonism in non-CAP modes of activation at the price of causing hyperthermia at higher doses. Finding such a compromise, *i.e.*, using potentially hyperthermic antagonists at doses that are subthreshold for causing hyperthermia, can be viewed as a separate approach for dealing with the thermoregulatory side effects. This approach exploits differential sensitivity of the therapeutic *vs.* side effects to changes in exposure; it is also known as the therapeutic index-based approach. As evident from the literature, this approach might have been explored by Purdue Pharma in connection with the development of V116517, as discussed in section 3.2 (also see Fig. 9B). Since in an earlier trial in humans the TRPV1 antagonist XEN-D0501 caused dose-dependent hyperthermia (Round et al., 2011), one could speculate that the same approach may be used by the young pharmaceutical company Pila Pharma, which is developing XEN-D0501 for treating obesity and diabetes (Gram et al., 2017). Pila Pharma is also likely to be taking advantage of the approach described in the next section.

4.3. Taking advantage of the tachyphylaxis phenomenon

Another strategy to minimize the hyperthermia is by taking advantage of the fact that the hyperthermic effect of some TRPV1 antagonists fades away with repeated administration, whereas the desired effect (*e.g.*, analgesic) may not undergo such an attenuation (Gavva, Bannon, Hovland Jr., et al., 2007). The fading of the effect with repeated administration is called desensitization, or tachyphylaxis; we will use the latter term – to avoid confusion with CAP- or RTX-induced desensitization discussed in previous sections. In different animal models it has been shown that repeated dosing of AMG 517, AMG8163 (Gavva, Bannon, Hovland Jr., et al., 2007) or ABT-102 (Honore et al., 2009) can favorably shift the ratio of the desired effect (analgesia) to the adverse effect (hyperthermia). It is speculated that the selective modulation of some (but not other) effects of TRPV1 antagonists with repeated dosing can be due to the antagonist-sensitive regulation of the subcellular distribution of TRPV1, which can lead to relative strengthening or weakening of TRPV1-mediated responses generated from different locations within a cell (Johansen, Reilly, & Yost, 2006). While the exact molecular mechanisms of the effect-specific tachyphylaxis seen at the whole-body level are unknown, it is interesting that rTRPV1 desensitization *in vitro* (revealed as reduced ion conductance) has different mechanisms when caused by different stimuli. Whereas CAP-induced desensitization is Ca^{2+}- and calmodulin-dependent (Rosenbaum, Gordon-Shaag, Munari, & Gordon, 2004) and at least partially reversible (Numazaki et al., 2003), heat-induced desensitization is independent of either Ca^{2+} or calmodulin and, on the experimental time scale, is irreversible (Sanchez-Moreno et al., 2018).

Repeated administration of TRPV1 antagonists was also tested in human clinical trials. In the trial by Amgen (Gavva et al., 2008), the hyperthermic response to the highest dose (10 mg) of AMG 517 (but not to a lower dose of 2 or 5 mg) was attenuated with repeated antagonist administration (Fig. 12A). In the trials conducted by Abbott Laboratories, the hyperthermic response to any dose of ABT-102 used (1, 2, or 4 mg, twice a day) attenuated gradually with repeated dosing and faded by day 7 [Fig. 12B; also see Rowbotham et al. (2011)]. In the trial reported by Round et al. (2011), XEN-D0501 caused a dose-dependent core T_b increase, which was greatest on the first day of dosing but rapidly attenuated thereafter for all doses tested (1, 2.5, and 5 mg, twice a day) and, by day 14, was 0.3°C or less (as compared to the placebo group) for all doses studied.

A priori, any pharmaceutical company that deals with a chronic condition, thus requiring repeated drug administration, and works on TRPV1 antagonists with an inherent hyperthermic activity may try to use a tachyphylaxis protocol, whether by desire or as an added benefit of repeated dosing. Purdue Pharma conducted clinical trials of their TRPV1 antagonist V116517 (NCT01688934, 2012; NCT01688947, 2012). According to R. Kapil and D. J. Kyle (unpublished observations), the company saw a rapid abatement of hyperthermia with repeated dosing of V116517 in laboratory animals and humans alike, thus

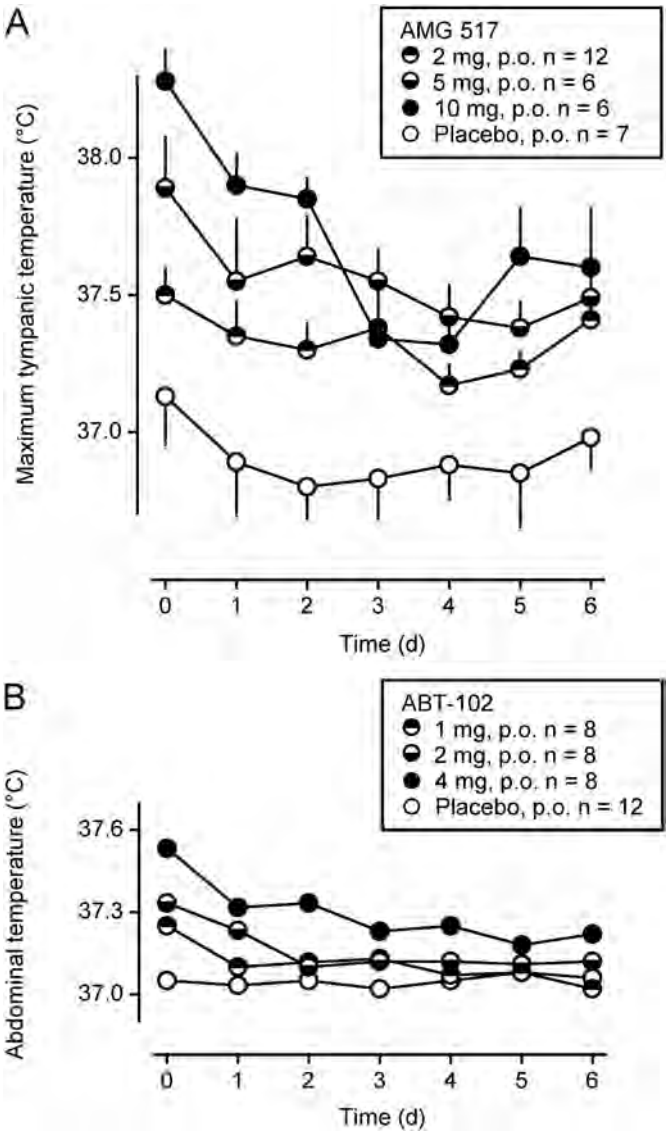

Fig. 12. The magnitude of the hyperthermic response to TRPV1 antagonists decreases with subsequent administration in humans. A) The effect of AMG 517 (2, 5, and 10 mg/kg, p.o., twice a day) or placebo on maximum tympanic temperature is shown for the first 7 days of treatment (days 0-6). B) The effect of ABT-102 (1, 2, and 4 mg/kg, p.o., twice a day) or placebo on abdominal temperature is shown for the first 7 days of treatment. Plotted for this work based on data from Gavva et al. (2008) (A) and Rowbotham et al. (2011) (B).

reducing the possible safety concerns for the drug. In addition to Purdue Pharma, several other companies, including Amgen, Daewoong Pharmaceutical, Pila Pharma, and Abbott Laboratories exercised (or at least considered) this approach, as is evident from the published reports (Gavva et al., 2008; Lee et al., 2017; Round et al., 2011; Rowbotham et al., 2011).

4.4. Combining a TRPV1 antagonist with an antihyperthermic drug

The low-hanging fruit was picked first. Several TRP programs at pharmaceutical companies considered combining a hyperthermic TRPV1 antagonist with some of the most obvious choices for drugs decreasing T_b – conventional antipyretics. Amgen published some research on this topic. Amgen's first report (Gavva, Bannon, Hovland Jr., et al., 2007) described an attempt of combining the TRPV1 antagonist AMG8163 with acetaminophen, an active constituent of Tylenol (and of more than 500 other over-the-counter and prescription medicines used to treat fever and pain). It appeared that acetaminophen did attenuate the hyperthermia induced by AMG8163 but only at a super high, hypothermia-inducing dose of 300 mg/kg, which is analogous to a 21-g bolus dose for a 70-kg human. Lower doses of acetaminophen that are normally used to treat fever (and that do not affect T_b in afebrile – without fever – conditions) do not diminish the hyperthermic response to AMG8163 in rats (Gavva, Bannon, Hovland Jr., et al., 2007). Nor do they affect the hyperthermic response to AMG 517 in human patients undergoing molar extraction (Gavva et al., 2008).

These negative results should not come as a surprise, because acetaminophen is thought to block fever by inhibiting cyclooxygenase (Engstrom Ruud et al., 2013), the key enzyme in the synthesizing cascade of prostaglandin E_2 (Ivanov & Romanovsky, 2004). While prostaglandin E_2 is the principal mediator of fever (Garami, Steiner, & Romanovsky, 2018), there are no data on prostaglandin involvement in the genesis of the hyperthermic response to TRPV1 antagonists. The noninvolvement of prostaglandin E_2 would explain the resistance of AMG8163- or AMG 517-induced hyperthermia to acetaminophen. On the other hand, the attenuation of AMG8163-induced hyperthermia in rats by the super high dose of acetaminophen (Gavva, Bannon, Hovland Jr., et al., 2007) is likely to be independent of TRPV1 and has been proposed to involve TRPA1 (Mirrasekhian et al., 2018), even though TRPA1 does not seem to play a role in T_b regulation in rats (de Oliveira et al., 2014). Alternatively, the hypothermic action of very high doses of acetaminophen can be, at least partially, due to the accumulation of acetaminophen metabolites, some of which exert a TRPV1-agonistic action (Eberhardt et al., 2017; Ohashi et al., 2017; Stueber et al., 2018).

A more fruitful approach can be combining a hyperthermic TRPV1 antagonist with a drug that blocks the sympathetic activation. Indeed, TRPV1 antagonists increase T_b by mounting the autonomic cold-defense responses: thermogenesis and skin vasoconstriction (Gavva et al., 2008; Steiner et al., 2007). These responses are sympathetically-driven not only when they are triggered by cold exposure or pyrogens (Morrison, 2011), but also when they are induced by TRPV1 antagonists, *e.g.*, AMG9810 (Alawi et al., 2015). The sympathetic mediation of TRPV1 antagonist-induced hyperthermia paves a way for combining a TRPV1 antagonist with a sympatholytic (sympathoplegic) drug, *i.e.*, a drug that opposes the downstream propagation of the neural signal in the sympathetic nervous system. The broad class of sympatholytics exploits a plethora of mechanisms and includes postsynaptic α- and β-adrenoreceptor antagonists, presynaptic α_2-adrenoreceptor agonists, catecholamine synthesis inhibitors, vesicular monoamine transporter inhibitors, and drugs with some other mechanisms of action (Stowe & Ebert, 2013). Based on the public information (Gomtsian, 2019), the pharmaceutical startup Synventa may be using the strategy described in this section. Synventa proposes that the α_2-adrenoceptor agonist lofexidine can prevent the hyperthermic effect of a TRPV1 antagonist and that using the two together will result in a thermally neutral analgesic combination.

4.5. Turning the adverse hyperthermia into a desired effect

Yet another approach to dealing with the adverse effect of TRPV1 antagonists on T_b would be to exploit their hyperthermic potential for a therapeutic purpose, *e.g.*, for counteracting hypothermia associated with general anesthesia in surgery (Schmidt, 2017) and possibly other types of hypothermia, especially when accompanied by pain or hyperalgesia. Anesthesia-associated hypothermia can be dramatic in magnitude (several degrees Celsius) and carries a significant risk of serious adverse effects, including coagulopathy and blood loss, as well as an increased propensity for wound infection (Ruetzler & Kurz, 2018). In animal models (Fig. 13), TRPV1 antagonists readily prevent or reverse anesthesia-associated hypothermia, while reducing the need for opioids for coping with postsurgical pain (Garami et al., 2017). As evident from the literature (Schmidt, 2017) and a published patent application

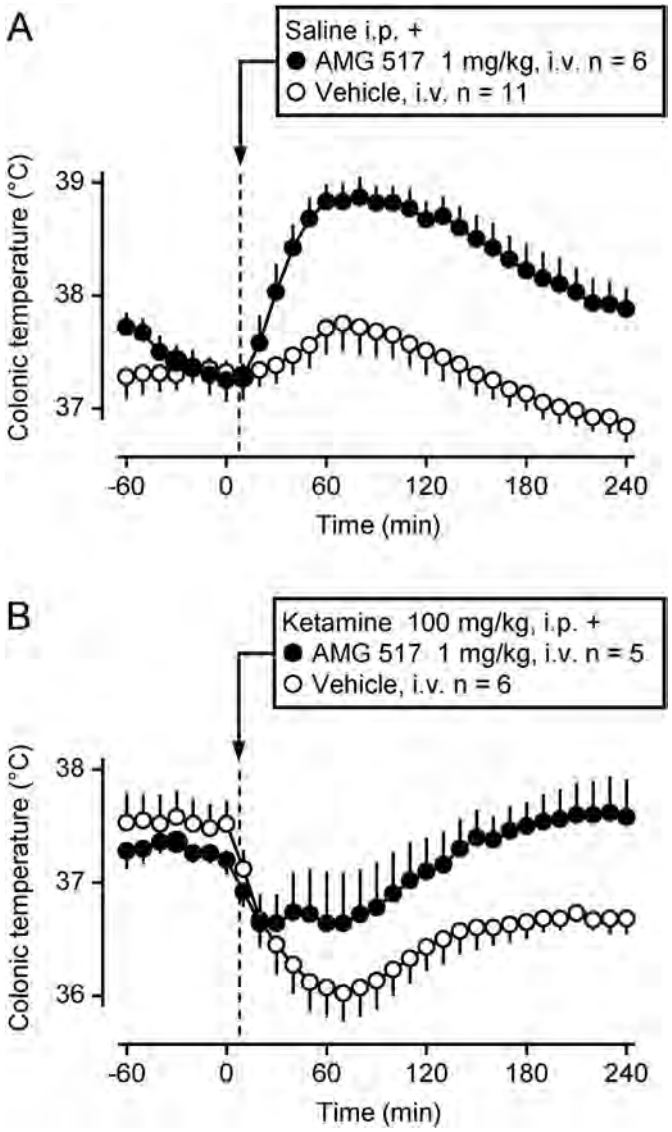

Fig. 13. AMG 517 causes hyperthermia in conscious rats but prevents hypothermia during anesthesia – without causing hyperthermia. A) The effect of AMG 517 (1 mg/kg, i.v.) or its vehicle on colonic temperature of rats pretreated with saline (i.p.) at a subneutral T_a of 23°C. B) The effect of AMG 517 (1 mg/kg, i.v.) or its vehicle on colonic temperature of rats pretreated with ketamine (100 mg/kg, i.p.) at a subneutral T_a of 23°C. Modified with permission from Garami et al. (2017).

(Patwardhan, Porreca, & Romanovsky, 2017), the strategy described in this section is used by the pharmaceutical startup Catalina Pharma.

5. Summary and conclusions

1. In rats and other laboratory animals, TRPV1 antagonists alter the level of T_b: most cause hyperthermia, whereas some produce hypothermia, and yet others exert no effect on thermoregulation. In general, the thermoregulatory effects of TRPV1 antagonists are dose-dependent. For some compounds, the effects are species-specific. The most common effect – hyperthermia – often fades with repeated dosing. Despite their diversity, all these thermoregulatory responses are likely to reflect an on-target (TRPV1-mediated) action of TRPV1 antagonists.
2. The TRPV1 protein is a species-specific channel that can be activated by a variety of stimuli, including (but not limited to) CAP and other vanilloids, protons (low pH), and heat. Activation of TRPV1 by different stimuli involves at least some mechanismic differences related to distinct portions on the channel structure and different loci of the molecule. These activation-mode-specific differences pave the way for developing mode-selective (mode-specific) TRPV1 antagonists. TRPV1 antagonists that potently block all activation modes are called mode-nonselective (or mode-nonspecific); they represent the first generation of TRPV1 antagonists. Second-generation (mode-selective) TRPV1 antagonists potently block the channel activation by CAP, but exert different effects (*e.g.*, potentiation, no effect, or low-potency inhibition) in either the proton mode or the heat mode, or both.
3. In young male rats, TRPV1 channels are not used as thermosensors for the thermoregulation system: thermal (T_b) signals that are mediated by TRPV1 do not normally drive thermoeffector responses in this species. Hence, blocking thermal activation by TRPV1 antagonists has no thermoregulatory effect in rats. Contrary to the widespread assumption, the mechanism of thermoregulatory effects of TRPV1 antagonists in this species does not involve blocking TRPV1-mediated thermosensation. Instead, TRPV1 antagonists affect thermoregulation by blocking the tonic TRPV1 activation by protons (or other ligands that share the proton activation mechanism) on sensory afferents somewhere in the trunk (abdomen), perhaps in the muscles. The hyper- and hypothermic responses share the same mechanism, which utilizes the acido-antithermogenic and acido-antivasoconstrictor reflexes. When the tonic activation of truncal TRPV1 channels is blocked (by hyperthermic antagonists) or potentiated (by hypothermic antagonists), the autonomic cold defenses (thermogenesis and cutaneous vasoconstriction) become either disinhibited or further inhibited, respectively, and either hyper- or hypothermia occurs. This mechanism may play a vital role during strenuous exercise by attenuating the inhibitory effect of T_b on physical performance.
4. If one were to design an "ideal" TRPV1 antagonist to treat pain in rats, such a compound would belong to the second generation and be a potent blocker of rTRPV1 activation by CAP and heat (to be an efficacious analgesic) but would not affect rTRPV1 activation by protons (to be free of the adverse effects on T_b).
5. Similar to their effects in laboratory rodents, TRPV1 antagonists alter the level of T_b in humans: most cause dose-dependent hyperthermia, whereas some may produce hypothermia. The hyperthermic effect often fades with repeated dosing.
6. The mathematical modeling used in the present work shows that the hyperthermic effect of a TRPV1 antagonist in humans depends on the compound's potency to block channel activation not only by protons, but also by heat. The connection between the hyperthermic response to TRPV1 antagonists and the heat mode of TRPV1 activation is present only in humans, not in rats. Similar to the hyperthermia in rats, the thermoregulatory response to a TRPV1 antagonist in humans does not depend on the compound's potency to block TRPV1 activation by CAP.
7. Based on the model analysis, we speculate that the role of TRPV1 in thermoregulation differs drastically between rats and humans. Whereas in rats TRPV1 channels modulate T_b *via*, most likely, pH signals from the trunk but are not used as thermosensors by the thermoregulation system, TRPV1 channels in humans may play both roles. The location of TRPV1 channels that sense T_bs (whether shell or core) to drive thermoeffector responses in humans is unknown and can be different from the location of the channels that are tonically activated by protons. Knowing that the skin plays a prominent thermosensory role in all species, at least some TRPV1 channels that mediate thermal signals to drive thermoeffectors in humans can be speculated to be located in the skin.

8. An “ideal” TRPV1 antagonist to treat pain in humans would belong to the second generation and be a potent blocker of hTRPV1 activation by CAP (to be an efficacious analgesic) but would not affect hTRPV1 activation by protons or heat (to be free of the adverse effects on T_b). The main concern about such an antagonist would be potentially a reduction in the level of efficacy. Such a drug would be most suited for treating pain caused exclusively through the CAP mode of TRPV1 activation – without any involvement of the heat and proton activation modes of activation; it is unclear whether such pain exists under natural conditions.
9. Our meta-analysis of the human-trial data has confirmed that the first-generation TRPV1 antagonists cause hyperthermia in humans, whereas the second-generation compounds may lack this effect.
10. The strategies of harnessing the thermoregulatory effects of TRPV1 antagonists in humans include: (i) creating inherently thermally neutral compounds based on mode-selective TRPV1 pharmacology, either by rational design or by more traditional approaches; (ii) creating potentially hyperthermic TRPV1 antagonists but with such profiles that would allow balancing the therapeutic effect with the adverse hyperthermic effect by using therapeutic doses that are subthreshold for the hyperthermia; (iii) taking advantage of the tachyphylaxis phenomenon (fading of the hyperthermic effect with repeated dosing) – in those cases when the desired therapeutic effect does not fade; (iv) combining a hyperthermic TRPV1 antagonist with an antihyperthermic drug (*e.g.*, a sympatholytic); and (v) turning the adverse hyperthermia into a desired effect, *e.g.*, by using TRPV1 antagonists to prevent anesthesia-associated hypothermia.

Declaration of Competing Interest

DAC is employed by NEOMED Institute. RK and DJK are employed by Purdue Pharma LP. AAR is an officer and director of Catalina Pharma Inc. and Zharko Pharma Inc.; he has consulted for TRPV1 programs at several pharmaceutical companies, and his laboratory conducted paid research on TRPV1 for Amgen Inc., Abbott Laboratories, and AbbVie Inc.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pharmthera.2020.107474.

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PT# 23524

SUPPLEMENTARY INFORMATION

Supplementary Methods

Mathematical model description

The hyperthermic effect H of a transient receptor potential vanilloid-1 (TRPV1) channel antagonist was defined as a body temperature (T_b) difference at 3 h *vs.* 0 h post-administration (Table S3). We assumed that the hyperthermic effect occurs due to a blockade of three independent modes of TRPV1 activation: by heat (M_1), protons (M_2), and capsaicin (CAP) (M_3). Thus,

$$H = H(\Delta M_1, \Delta M_2, \Delta M_3),$$

where ΔM_i is the extent to which a TRPV1 antagonist blocks the i-th mode of activation (i is either 1, 2, or 3). For the j-th antagonist (j is an integer between 1 and 4), the extent of blockade in each activation mode was considered to be a function of antagonist dose D:

$$\Delta M_i = \Delta M_i(D).$$

Hence, the effect H was also a function of a dose:

$$H = H(D) = H(\Delta M_1(D), \Delta M_2(D), \Delta M_3(D)).$$

The $H(D)$ function was tabulated for each antagonist based on the data reported in Table S3. It was required to determine how sensitive H is to the extent of blockade of each mode of TRPV1 activation, *i.e.*, to find the sensitivity coefficients k_i. A high value of k_i would indicate that

the potency of an antagonist in the *i*-th activation mode strongly and positively affects the hyperthermic response; a 0 value would indicate the absence of such an effect; a negative value would mean that the potency in the *i*-th mode has a negative effect on *H*. To find k_i, we approximated the tabular *H*(*D*) data for each antagonist with a sigmoid function commonly used for pharmacological dose-effect relationships (see, *e.g.*, Di Veroli et al., 2015; Meddings, Scott, & Fick, 1989):

$$H(D) = v_0 + \frac{v_1}{e^{-(w_0 + w_1 D)} + 1}.$$

In the above formula, v_0, v_1, and w_0 are constants, and the coefficient w_1 is defined as follows:

$$w_1 = \frac{k_1}{d_1} + \frac{k_2}{d_2} + \frac{k_3}{d_3},$$

where d_i is the 50% inhibitory concentration of an antagonist (IC_{50}) for the *i*-th mode of TRPV1 activation (Table S3).

Hence, *H*(*D*) was a function that contained six unknown parameters: the sensitivities k_1, k_2, and k_3 and the constants v_0, v_1, and w_0, all of which were identified through best-fitting the mathematical model described above to the tabulated data. Each row of that table corresponded to an equation, thus resulting in 15 equations.

To minimize a systematic approximation error in this nonlinear model, and also to estimate the statistical significance of the results of model fitting, we accounted for the statistical variance of *H*. Using a Monte Carlo simulation technique (Metropolis & Ulam, 1949), 10 datasets (replicates) were generated randomly, according to the Gaussian probability distribution, with the mean and standard deviation (SD) values taken from Table S3. The datasets generated were used to find the unknown parameters k_1, k_2, k_3, v_0, v_1, and w_0 by applying the standard least squares technique (Wolberg, 2006). The set of parameter values that corresponded to the minimal fitting

error was taken as an initial approximation. This step was needed to maximize the consistency of model fitting. Next, the initial approximation was used for model fitting into each of additional 20 replicates. Thus, 20 sets of the six unknown parameters were obtained, from which the mean value and the variance estimates were calculated for each sensitivity. We assumed that the T_b measurements at 0 h *vs.* 3 h were uncorrelated. Then, the statistical variance *H* (the difference between these two values) should be equal to the sum of the respective variances, which were calculated as squares of the respective SD values (Table S3).

Meta-analysis description

Search strategy

Our meta-analysis was conducted in accordance with the guidelines of the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) protocols (Moher, Liberati, Tetzlaff, Altman, & Group, 2009) (Table S4). The analysis was based on the so-called PICO [Patients, Intervention (or Indicator), Comparison, Outcome] model: in adult human subjects, we aimed at assessing the effect of TRPV1 antagonists (as compared to placebo) on deep T_b. This meta-analysis has been registered with PROSPERO (CRD42018095220).

A search in the PubMed, EMBASE, and Cochrane Controlled Trials Registry databases was performed for the period from database inception to February 2019 using the following terms: "(TRPV1 OR TRPV-1 OR VR1 OR VR-1 OR vanilloid OR capsaicin) AND (antagonist OR blocker OR inhibitor) AND (temperature OR hyperthermia OR fever OR hypothermia)". We restricted our search to original human studies published in English. A manual search through the

reference lists of relevant full-text articles was also conducted to identify additional articles. The search was conducted independently by two authors (AG, ZR), who also selected studies for analysis and extracted data from the selected studies (see below). Any disagreements were resolved by consensus, with the help of a third author (AAR).

Study selection, data extraction, and risk of bias assessment

The titles and abstracts of the publications identified by the literature search were screened, and the full text of potentially eligible articles was obtained. We included studies in which deep T_b was reported in both TRPV1 antagonist-treated and placebo groups at (at least) two time points: (i) at or before and (ii) 3 h after the drug (or placebo) administration. Furthermore, an *in-vitro* activation-mode pharmacological profile of the TRPV1 antagonist used had to be available (Table S1). From all included articles, we extracted the mean deep T_b with the corresponding SD and the sample size (or other statistical information, which allowed us to calculate or estimate these parameters).

Two authors (AG and ZR) evaluated the quality of the trials according to the Cochrane risk-of-bias tool (Higgins et al., 2011), *i.e.*, by assessing independently the methodology of random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, completeness of outcome data, and selective outcome reporting (Table S7).

Statistical analysis

We used a standardized difference in means (SDM) as a measure of the effect on T_b response (see main text, section 3.3). For standardization, the means were divided by their corresponding SD values, which was required because the different T_b-measuring methods and

dose ranges could result in different variances among the study groups and influence the results. SDM values were calculated by using the random effect model by DerSimonian and Laird (1986), and then compared using standard meta-analysis tools, such as "forest plot" (Fig. 11). The presence of publication bias was determined by visual inspection of a "funnel plot" for the lack of asymmetry and evaluated quantitatively by Egger's test ($P < 0.1$ indicating publication bias). Both the visual inspection (Fig. S2) and the results of Egger's test revealed no asymmetry ($P = 0.128$). The meta-analyses were performed with Comprehensive Meta-Analysis (version 3.3; Biostat, Engelwood, MJ, USA) software.

Recording thermal effects of V116517 in trial NCT02695745

Thirty-six healthy male volunteers (age: 18-45 years; body mass: 50-100 kg) were randomized in a double-blind, controlled clinical trial. Participants received 30, 100, 300, 600, or 1,000 mg of V116517 in suspension or placebo *per os*. Oral temperature was recorded at different time points in each treatment group from 24 h before to 72 h after drug or placebo administration. The results (for 0-24 h) are shown in Table S8.

TABLE S1

Human studies that used a TRPV1 antagonist and measured T_b: included in analyses

TRPV1 antagonist	ABT-102	AZD1386	NEO6860	V116517
For trial details, refer to the following lines in Table 3	1, 2	8	25	48
Molecular mass (g/mol), as reported in the literature	349	423	Not reported*	442
IC_{50} against CAP (nM)	7.0	7.9	32	35
IC_{50} against protons (nM)	6.0	79	> 10,000	40
IC_{50} against heat (nM)	Unknown	100	> 10,000	Unknown
Dose range used (mg) [dose range used (µmol)]	1.0-40 [2.9-115]	95 [225]	500 [1,150]	30-1,000 [68-2,261]
Did the study have a placebo group?	Yes	Yes	Yes	Yes
Was a patient-level or group mean T_b before or at the time of drug (placebo) administration reported?	Yes	Yes	Yes	No*
Was a patient-level or group mean T_b at 3 h after drug (placebo) administration reported?	Yes	Yes	Yes	No*

*For explanation on how the missing information was obtained, see Table S5.

TABLE S2

Human studies that used a TRPV1 antagonist and measured T_b: excluded from analyses

TRPV1 antagonist	ABT-102	AMG 517	AZD 1386	DWP 05195	JNJ-38893777	JNJ-39439335	JNJ-39439335	NEO 6860	SB-705498	SB-705498	XEN-D0501	XEN-D0501
For trial details, refer to the following lines in Table 3	3	4	10, 11	13	15	17-19, 21	20	24	43	44	50	51
Molecular mass (g/mol), as reported in the literature	348	430	423	Not reported	537	423	423	435	429	429	Not reported	Not reported
IC_{50} against CAP (nM)	7.0	0.8	7.9	Not reported	8.3	4.6-23	4.6-23	32	3.0	3.0	Not reported	Not reported
IC_{50} against protons (nM)	6.0	0.6	79	Not reported	7.4	6.8	6.8	> 10,000	5.3	5.3	Not reported	Not reported
IC_{50} against heat (nM)	Not reported	1.3	100	Not reported	Not reported	Not reported	Not reported	> 10,000	6.0	6.0	Not reported	Not reported
Did the study have a placebo group?	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
Was a patient-level or group mean T_b before or at the time of drug (placebo) administration reported?	No	No*	No	Yes	No	No	No	No	Yes	No	No	Yes
Was a patient-level or group mean T_b at 3 h after drug (placebo) administration reported?	No	No*	No	No	No	No	No	No	No	No	No	Yes
Additional reasons for exclusion									Low target occupancy	Intranasal administration		

Reasons for exclusion are highlighted in pink. *Only maximal T_b values for different periods of time were reported.

TABLE S3

Data on T_b and IC_{50} values of TRPV1 antagonists: used in the modeling analysis

TRPV1 antagonist	Dose (μmol)	T_b at 0 h (°C)		T_b at 3 h (°C)		3 h – 0 h T_b difference (°C)*		Number of subjects	Reference(s)	IC_{50} values against human TRPV1 in different activation modes (nM)			
		Mean	SD	Mean	SD	Mean	SD			Heat	pH (5.0-5.5)	CAP (50-100 nM)	Reference
ABT-102	2.9	37.0	0.3	37.3	0.2	0.3	0.4	8	Othman, Nothaft, Awni, & Dutta, 2013; Rowbotham, et al., 2011		6.0	7.0	Surowy, et al., 2008
	5.7	36.5	0.3	37.2	0.3	0.7	0.4	20					
	11	36.7	0.3	37.4	0.3	0.7	0.5	14					
	17	36.3	0.2	37.4	0.3	1.1	0.4	6					
	23	36.5	0.3	37.2	0.2	0.7	0.4	6					
	52	36.3	0.2	37.6	0.3	1.3	0.4	6					
	86	36.2	0.2	37.5	0.2	1.3	0.3	6					
	115	36.5	0.3	37.6	0.2	1.1	0.4	6					
AZD1386	225	36.8	0.3	37.2	0.3	0.4	0.4	40	Quiding, et al., 2013	100	79	7.9	Chiche, Brown, & Walker, 2016**
NEO6860	1,149	36.6	0.3	36.4	0.3	-0.2	0.4	52	Arsenault, et al., 2018	> 10,000	> 10,000	31.62	Chiche, et al., 2016**
V116517	68	37.0	0.2	37.1	0.3	0.1	0.3	4	Unpublished***		40	35	Tafesse, et al., 2014
	226	36.9	0.3	37.1	0.3	0.2	0.4	4					
	678	36.8	0.1	37.0	0.2	0.2	0.2	4					
	1,357	36.8	0.2	37.2	0.4	0.4	0.4	4					
	2,261	36.9	0.2	37.4	0.4	0.5	0.5	4					

*These values are used as the hyperthermic index *H* values (see *Mathematical model description* in *Supplementary Information)*. **The applied temperature, pH, and concentration of CAP to activate human TRPV1 were not reported. ***Patient-level T_b data were provided by Purdue Pharma (see Table S8).

TABLE S4

PRISMA checklist

Section/topic	#	Checklist item	Reported
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	Page 1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	Page 1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	Section 1
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	Page 3 in Supplementary Information
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (*e.g.*, Web address), and, if available, provide registration information, including registration number.	Section 3.3
Eligibility criteria	6	Specify study characteristics (*e.g.*, PICOS, length of follow-up) and report characteristics (*e.g.*, years considered, language, publication status) used as criteria for eligibility, giving rationale.	Section 3.3
Information sources	7	Describe all information sources (*e.g.*, databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	Pages 3-4 in Supplementary Information
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Page 3 in Supplementary Information
Study selection	9	State the process for selecting studies (*i.e.*, screening) and list the inclusion and exclusion criteria.	Page 4 in Supplementary Information
Data collection process	10	Describe method of data extraction from reports (*e.g.*, piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	Page 4 in Supplementary Information
Data items	11	List and define all variables for which data were sought (*e.g.*, PICOS, funding sources) and any assumptions and simplifications made.	Page 4 in Supplementary Information and Table S5
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	Page 4 in Supplementary Information
Summary measures	13	State the principal summary measures (*e.g.*, risk ratio, difference in means).	Section 3.3; Pages 4-5 in Supplementary Information
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency for each meta-analysis.	Section 3.3; Pages 4-5 in Supplementary Information
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (*e.g.*, publication bias, selective reporting within studies).	Page 5 in Supplementary Information

Additional analyses	16	Describe methods of additional analyses (*e.g.*, sensitivity or subgroup analyses, meta-regression), if done, indicating which were prespecified.	Section 3.3
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusion at each stage, ideally with a flow diagram.	Section 3.2; Fig. S1
Study characteristics	18	For each study, present characteristics for which data were extracted (*e.g.*, study size, PICOS, follow-up period) and provide the citations.	Tables S3 and S6
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Table S7
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group and (b) effect estimates and confidence intervals, ideally with a "forest plot".	Section 3.3; Fig. 11
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	Section 3.3; Fig. 11
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	Fig. S2
Additional analysis	23	Give results of additional analyses, if done [(*e.g.*, sensitivity or subgroup analyses, meta-regression (see Item 16)].	Section 3.3; Fig. 11
DISCUSSION			
Summary of evidence	24	Summarize the main findings, including the strength of evidence for each main outcome; consider their relevance to key groups (*e.g.*, healthcare providers, users, and policy makers).	Section 5
Limitations	25	Discuss limitations at study and outcome level (*e.g.*, risk of bias), and at review-level (*e.g.*, incomplete retrieval of identified research, reporting bias).	Sections 3.2 and 3.3
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	Section 5
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (*e.g.*, supply of data); role of funders for the systematic review.	Acknowledgements

TABLE S5

Assumptions and simplifications used in the modeling analysis

TRPV1 antagonist	Problem	Solution
ABT-102	Of the three trials reported by Othman et al. (2013) and Rowbotham et al. (2011), oral T_b was measured in two and gastrointestinal T_b (with an ingested transmitter) in the third one.	Oral and gastrointestinal T_bs were considered equivalent.
	In Othman et al. (2013), T_b was reported as a median with percentiles.	SD values were calculated from percentiles; when only the median was reported, it was considered to equal the mean.
	Two treatments, *viz.*, 2 mg of ABT-102 and placebo were studied in three trials; 4 mg of ABT-102 was studied in two trials (Othman et al., 2013; Rowbotham et al., 2011).	For each treatment, the data were pooled.
AZD1386	Neither standard error (SE) nor SD values for the mean T_b were reported by Quiding et al. (2013).	SD for T_b was calculated based on the SD for the pain intensity difference, as estimated by Quiding et al. (2013).
V116517	Neither the molecular mass of V116517 nor T_b data were reported by Arendt-Nielsen et al. (2016).	The molecular mass (442 g/mol) and the T_b data (Table S8) were provided by Purdue Pharma.
NEO6860	The molecular mass was reported by neither Arsenault et al. (2018) nor Brown et al. (2017).	Molecular mass values for small-molecule TRPV1 antagonists fall in a narrow range. For this analysis, the molecular mass of NEO6860 was assumed to be 435 g/mol, which is the mean value for all antagonists presented in Tables S1 and S2.
	For both the proton and heat mode, IC_{50} > 10 μM.	For the analysis, both values were considered to be 10 μM.

TABLE S6

Demographic characteristics of the analyzed studies

Study report	TRPV1 antagonist	Subject population	Age (year)		Gender		Body mass (kg)		Body mass index (kg/m^2)	
			Mean	SD	Male	Female	Mean	SD	Mean	SD
Arsenault, et al., 2018	NEO6860	With knee osteoarthritis	61	9	20	34	79	14	29	4
Arendt-Nielsen, et al., 2016	V116517	Healthy	22	3	36	0	80	10	24	2
	Placebo				35	0				
Othman, et al., 2013*	ABT-102	Healthy	38	10	35	10	76	11	25	2
Othman, et al., 2013*	ABT-102	Healthy	43	10	23	4	79	11	25	2
Rowbotham, et al., 2011*	ABT-102	Healthy	30	9	18	18	70	11	24	3
Quiding, et al., 2013	AZD1386	Following molar extraction	22	4	40	0	76	11	24	4
	Placebo		22	5	40	0	77	12	25	3

*The demographic data for this study are reported by Othman, Nothaft, Awni, & Dutta (2012).

TABLE S7

Risk of bias assessment

TABLE S8

The thermal effect of V116517 in healthy male volunteers (trial NCT02695745)

Treatment	Dose (mg)	Oral T_b at different times post-dosing (°C)												Number of subjects
		0 h		3 h		6 h		8 h		12 h		24 h		
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
V116517	30	37.0	0.1	37.1	0.1	37.3	0.2	37.1	0.1	37.0	0.1	36.9	0.1	4
	100	36.9	0.1	37.1	0.2	37.2	0.1	37.1	0.1	37.2	0.1	36.9	0.1	4
	300	36.8	0.1	37.0	0.1	37.4	0.2	37.1	0.2	37.3	0.2	36.8	0.1	4
	600	36.8	0.1	37.2	0.2	37.4	0.2	37.7	0.2	37.5	0.3	36.9	0.1	4
	1,000	36.9	0.1	37.4	0.2	37.6	0.2	37.5	0.1	37.5	0.1	37.0	0.0	4
Placebo		36.8	0.0	36.8	0.1	36.9	0.1	36.9	0.1	37.0	0.1	36.8	0.0	10

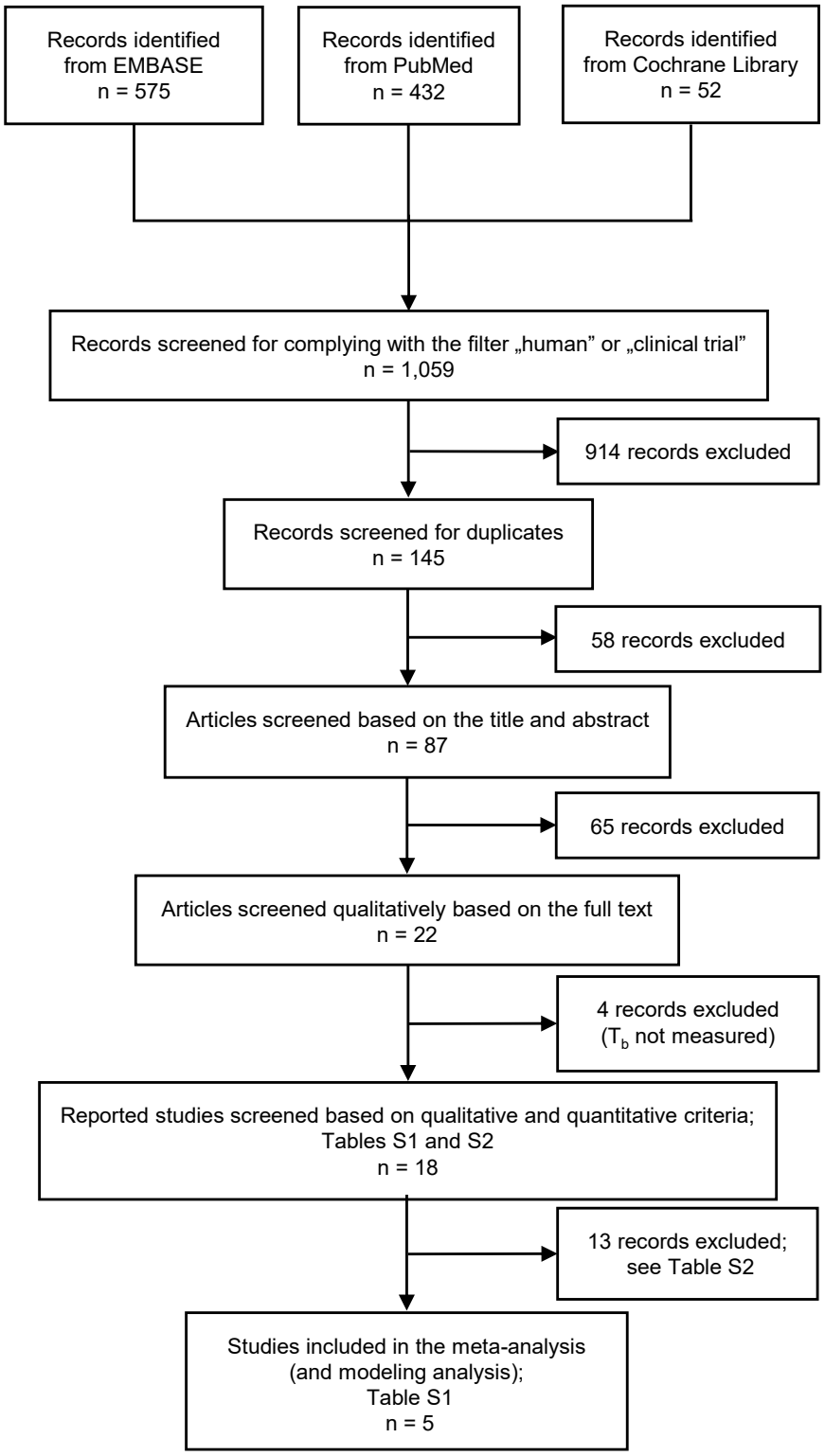

FIGURE S1. PRISMA Flow Diagram

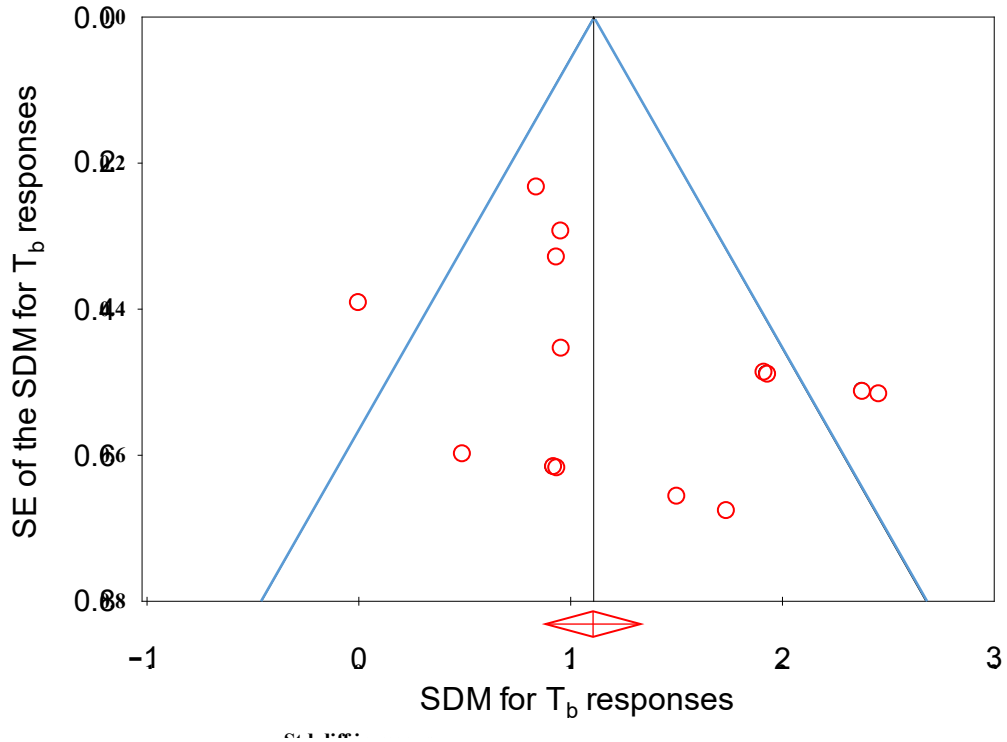

FIGURE S2. "Funnel plot" of the studies with mode-nonselective antagonists. Each circle represents a group treated with a single dose a TRPV1 antagonist; the same groups are shown in Figure 11. The "funnel sides" (slotted blue lines) form a triangular area that is expected, in the absence of publication bias, to harbor 95% of data. The average SDM for all doses of all antagonists corresponds to the "funnel axis" (vertical black line) and also to the vertical diagonal of the rhombus; the horizontal diagonal of the rhombus represents the 95% confidence interval. A high degree of symmetry in the distribution of data relative to the funnel axis (as in this plot) indicates that the results are unlikely to be affected by a bias.

Supplementary References

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Ammonium chloride-induced hypothermia is attenuated by transient receptor potential channel vanilloid-1, but augmented by ankyrin-1 in rodents

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ABSTRACT

Aims: Systemic administration of ammonium chloride (NH_4Cl), an acidifying agent used in human patients and experimental conditions, causes hypothermia in mice, however, the mechanisms of the thermoregulatory response to NH_4Cl and whether it develops in other species remained unknown.
Main methods: We studied body temperature (T_b) changes in rats and mice induced by intraperitoneal administration of NH_4Cl after blockade of transient receptor potential vanilloid-1 (TRPV1) or ankyrin-1 (TRPA1) channels.
Key findings: In rats, NH_4Cl decreased T_b by 0.4–0.8°C ($p < 0.05$). The NH_4Cl-induced hypothermia also developed in *Trpv1* knockout (*Trpv1*$^{-/-}$) and wild-type (*Trpv1*$^{+/+}$) mice, however, the T_b drop was exaggerated in *Trpv1*$^{-/-}$ mice compared to *Trpv1*$^{+/+}$ controls with maximal decreases of 4.0 vs. 2.1°C, respectively ($p < 0.05$). Pharmacological blockade of TRPV1 channels with AMG 517 augmented the hypothermic response to NH_4Cl in genetically unmodified mice and rats ($p < 0.05$ for both). In contrast, when NH_4Cl was infused to mice genetically lacking the TRPA1 channel, the hypothermic response was significantly attenuated compared to wild-type controls with maximal mean T_b difference of 1.0°C between the genotypes ($p = 0.008$). Pretreatment of rats with a TRPA1 antagonist (A967079) also attenuated the NH_4Cl-induced T_b drop with a maximal difference of 0.7°C between the pretreatment groups ($p = 0.003$).
Significance: TRPV1 channels limit, whereas TRPA1 channels exaggerate the development of NH_4Cl-induced hypothermia in rats and mice, but other mechanisms are also involved. Our results warrant for regular T_b control and careful consideration of NH_4Cl treatment in patients with TRPA1 and TRPV1 channel dysfunctions.

1. Introduction

Ammonium chloride (NH_4Cl) is a systemic and urinary acidifying agent that can be used in the treatment of metabolic alkalosis [1–3]. As an expectorant, it is a common ingredient of many cough mixtures, which explains why the excessive consumption of such over-the-counter medications can lead to metabolic acidosis [4,5] that was also observed in subjects receiving NH_4Cl in other types of medicines [6–8]. Extending its application areas, more recently, the use of NH_4Cl was implicated in COVID-19 [9,10].

The oral and/or parenteral administration of NH_4Cl is often used to induce systemic (extracellular) acidosis in animal models [11–15] and in human studies [16–19]. Although interspecies differences, for example, between rats and mice, were demonstrated in the response to the same NH_4Cl-loading protocol [13,20], NH_4Cl administration has been widely used to study the effect of acidosis associated with different conditions, such as physical exercise [16,21], kidney functions [22,23], iron metabolism [24], or parathyroid functions [25].

In 1988, Gordon showed that the systemic administration of NH_4Cl leads to hypothermia in mice [26]. This finding can explain why its overconsumption was also associated with hypothermia in case of a human patient [5]. However, the molecular mediators of the NH_4Cl-induced hypothermia have remained largely unknown. Our literature search did not reveal any studies that aimed to clarify the underlying

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mechanisms of this thermoregulatory phenomenon.

Transient receptor potential (TRP) vanilloid-1 (V1) and ankyrin-1 (A1) channels are temperature-sensitive members of the TRP channel family, for the reason that *in vitro* they can be activated by noxious heat and cold, respectively [27,28]. In addition to thermal signals, they can be both activated by ligand agonists and by changes in pH, hence they function as polymodal receptors [29,30], especially on primary afferent neurons where they are often co-expressed [31,32]. Their thermosensor function has been well established in pain sensation [29,33,34], however, neither of them was found to serve as a thermosensor for the thermoregulation system in rodents [35,36]. It was proposed that their activation by agonists other than temperature (by protons for TRPV1 and by sulfides for TRPA1) contributes to the regulation of deep body temperature (T_b) [37,38]. Interestingly, both channels can be activated by NH_4Cl [39,40], and also by low pH [41–44]. However, to our best knowledge, the contribution of the TRPV1 or TRPA1 channel to the thermal response to NH_4Cl has not been investigated yet.

In the present work, we studied whether NH_4Cl induces hypothermia also in rats in addition to mice to exclude the possibility of a mouse-specific effect, and whether the blockade of TRPV1 and TRPA1 channels influences the NH_4Cl-induced hypothermia by using genetic deletion of the channels. To avoid chronic compensatory mechanisms, which may develop for the function of a knocked out gene, we also used pharmacological inhibition of TRPV1 and TRPA1 in genetically unaltered animals.

2. Methods

2.1. Animals

Experiments were conducted in adult male rats and mice under protocols approved by the Institutional Animal Use and Care Committee of the University of Pecs (registration no.: BA02/2000–13/2021) and in accordance with the directives of the National Ethical Council for Animal Research and those of the European Communities Council (86/609/EEC). One hundred and ten Wistar rats and thirty-seven C57BL/6 mice were obtained from the Laboratory Animal Centre of the University of Pecs. In addition, mice with ($^{-/-}$ aka knockout, KO) or without ($^{+/+}$ aka wild type, WT) a homozygous targeted mutation in the *Trpv1* gene (*Trpv1* KO: n = 21; *Trpv1* WT: n = 14) or in the *Trpa1* gene (*Trpa1* KO: n = 24; *Trpa1* WT: n = 16) were also obtained from the Laboratory Animal Centre of the University of Pecs, where they were bred and kept as described in details elsewhere [35,45]. Mice from these strains were also used in previous studies aiming to investigate the thermoregulatory function of TRPA1 [35,37] and TRPV1 [38,46]. The animals were housed in polycarbonate cages kept in temperature-controlled rooms on a 12 h light/dark cycle (lights on at 5:00 A.M.). The ambient temperature was maintained at 24–25°C and humidity at 30–40%. Standard rodent chow and tap water were available *ad libitum*. Animals were extensively habituated to the experimental setup, as described elsewhere [47,48]. At the time of the experiments, the mice and rats weighed 25 ± 1 and 328 ± 3 g, respectively.

2.2. Surgeries

2.2.1. Anesthesia and perioperative care

Animals were anesthetized with intraperitoneal (i.p.) administration of a ketamine–xylazine cocktail (81.7 and 9.3 mg/kg for mice, 55.6 and 5.5 mg/kg for rats, respectively), and they received antibiotic protection intramuscularly (gentamycin, 6 mg/kg). To prevent intra- and postoperative hypothermia, during the surgery mice were kept on a heating pad (PECO Services Ltd., Brough, United Kingdom), and then they were allowed to recover from anesthesia in a temperature-controlled chamber (model MIDI F230S; PL Maschine Ltd., Tarnok, Hungary) set to 31°C. Each rat and mouse was implanted with an i.p. catheter, additionally rats assigned to experiments with pharmacological antagonists were also implanted with an intravenous (i.v.) catheter during the same surgery. The i.p. and i.v. catheter implantations have been widely used in thermoregulation experiments, and the animals tolerated these interventions well [37,38,46,49]. These preimplanted catheters were used for the non-stressful administration of substances to conscious animals in our experiments. In case of a bolus injection (i.p. or i.v.), the handling of the animal and the puncture of the abdominal wall with the needle would have resulted in pain and stress-induced hyperthermia (for review, see [50]), which could interfere with our results. All experiments were performed 3–5 days after the surgery.

2.2.2. I.p. catheter implantation

For the non-stressful i.p. administration of the substances during the experiment, a polyethylene (PE)-50 catheter filled with pyrogen-free saline was implanted into the peritoneal cavity of each mouse and rat, similarly as in previous studies [38,46]. In brief, through a small midline incision on the abdomen, the internal end of the catheter was fixed to the left side of the abdominal wall with a suture, while the external end of the catheter was tunneled under the skin to the nape, where it was exteriorized and heat-sealed. The surgical wound was sutured in layers. The catheter was flushed with 0.1 ml of saline on the day after the surgery and every other day thereafter.

2.2.3. I.v. catheter implantation

This procedure was performed in rats assigned to treatment with a TRPV1 or a TRPA1 antagonist. As described before [46], the i.v. catheter was implanted during the same surgery as the i.p. catheter. A small longitudinal incision was made on the ventral surface of the neck, left of the trachea. The left jugular vein was exposed, freed from its surrounding connective tissue, and ligated. A silicone catheter (with inner and outer diameter of 0.5 and 0.9 mm, respectively) was filled with heparinized (10 U/ml) saline, then it was inserted into the left jugular vein, passed into the superior vena cava, and secured in place with ligatures. The free end of the catheter was knotted and exteriorized at the nape. The skin wound was sutured. The catheter was flushed with heparinized saline on the day following the surgery and every other day thereafter.

2.3. Thermocouple thermometry

The mice and the rats were placed in cylindrical confiners and equipped with copper-constantan thermocouples (Omega Engineering, Stamford, CT, USA) to measure colonic temperature (a form of deep T_b). The colonic thermocouple was inserted beyond the anal sphincter (10 and 3 cm deep for rats and mice, respectively); fixed to the base of the tail with adhesive tape; and plugged into a data logger device (Cole-Palmer, Vernon Hills, IL, USA) connected to a computer. Animals in their confiners were then placed into a biochemistry incubator (model BJPX-Newark; Biobase; Jinan, China). As the expected T_b change was hypothermia, the ambient temperature was set to 25°C, which is slightly subneutral for rats and mice in this setup. The preimplanted i.p. and i.v. catheter (when present) was connected to a PE-50 extension, which was prefilled with the substance of interest and connected to a syringe placed in an infusion pump (model 975; Harvard Apparatus Inc., Holliston, MA, USA).

2.4. Drugs and drug administration

2.4.1. I.p. NH_4Cl to mice and rats

NH_4Cl was purchased from VWR Chemicals (Leuven, Belgium). On the day of an experiment, NH_4Cl was freshly dissolved in sterile water to achieve final concentrations of 32.1, 220 or 280 mg/ml. For the i.p. administration of NH_4Cl to mice, the working solution (32.1 mg/ml) was infused (10 ml/kg) over 16 min to deliver NH_4Cl at 321 mg/kg (~6 mmol/kg). In rats, the working solutions (220 and 280 mg/ml) were infused (1 ml/kg) over 5 min to deliver NH_4Cl at 220 and 280 mg/kg (ca.

4 and 5 mmol/kg), respectively. The decreased dose of NH_4Cl in the rats compared to mice was necessary, because rats were more sensitive to the effect of the same NH_4Cl-loading protocol on protein expression than mice [13]. Control animals were infused with sterile water. All infusion rates and volumes were selected with the goal to minimize the stress and discomfort of the animals that may originate from the substance administration procedure itself. Similar i.p. infusion rates as in the present experiments were successfully applied in our previous studies for non-stressful administration of substances to mice [51] and rats [46].

2.4.2. I.v. AMG 517 and A967079 to rats

AMG 517, a highly potent TRPV1 antagonist [52], and A967079, a highly potent TRPA1 antagonist [53], were purchased from Tocris (Bristol, UK). A stock solution of AMG 517 (1 mg/ml) was prepared with 10% dimethyl sulfoxide (DMSO) and 10% Tween-80 in saline, aliquoted, and stored at −80°C. On the day of the experiment, the stock was diluted to give a working solution of AMG 517 at 210 μg/ml in 10% DMSO and 10% Tween-80 in saline. A stock solution of A967079 (10 mg/ml) was prepared with polyethylene glycol 400 (PEG 400), aliquoted, and stored at −80°C. On the day of the experiment, the stock was diluted with PEG 400 and saline to give a working solution of A967079 at 5 mg/ml in 80% PEG 400 in saline. For the i.v. administration, the working solution of AMG 517 or A967079 (210 μg/ml or 5 mg/ml, respectively) was infused (1 ml/kg) over 10 min to deliver AMG 517 and A967079 at 210 μg/kg and 5 mg/kg, respectively. Both antagonists were infused i.v. to the rats 20 min before the i.p. infusion of NH_4Cl. Control rats were infused with the vehicle of the antagonist of interest. The efficacies of AMG 517 and A967079 administered i.v. at similar doses and rates as in the present experiments were shown in previous studies [54,55].

2.4.3. Subcutaneous (s.c.) AMG 517 to mice

To study the effect of AMG 517 on NH_4Cl-induced hypothermia in mice, we used a drug dose and administration model that was successfully applied earlier [56]. The stock and working solutions of AMG 517 were prepared as described above. The working solution (or its vehicle) was injected s.c. as a bolus to deliver AMG 517 at a dose of 210 μg/kg just before setting up the mice for the experiment (i.e., ~120 min before the administration of NH_4Cl). Then, the mice were allowed to accommodate to the experimental conditions for ~2 h before they received the non-stressful, i.p. infusion of NH_4Cl (321 mg/kg) through a pre-implanted catheter. The 2-h latency until the NH_4Cl infusion was needed to reduce the stress-induced hyperthermia resulting from the needle prick associated with the s.c. injection of AMG 517 (or its vehicle). Because of the long (31h) half-life of AMG 517 in rodents [57], the effect of the drug could be reasonably expected at the time of NH_4Cl infusion even with this latency.

The drug administration procedures in the different experiments are summarized in Table 1.

2.5. Blood pH measurements

One hour after the i.p. administration of NH_4Cl, the animals were anesthetized with a ketamine-xylazine cocktail, and then blood samples were collected by cardiac puncture with a heparinized syringe. The pH of the blood samples was measured by a pH meter (model OP-211/2; Radelkis Ltd., Budapest, Hungary) within 1 min after collection. After the blood withdrawal, the animals were euthanized with sodium pentobarbital (100 mg/kg, i.p.).

2.6. Data processing and analysis

Data on deep T_b and on blood pH were compared by ANOVA, as appropriate. ANOVA was followed by the Student-Newman-Keuls *post hoc* test as in our earlier study [49]. Sigmaplot 11.0 (Systat Software, San Jose, CA, USA) software was used for statistical analyses. Differences were considered significant when $p < 0.05$. Data are presented as mean ± SE.

3. Results

3.1. Systemic administration of NH_4Cl causes hypothermia in rats

First, we studied the thermal effect of NH_4Cl administered systemically (i.p.) to rats in order to exclude the possibility that the NH_4Cl-induced hypothermic response is specific only for mice. We found that the i.p. injection of NH_4Cl to the rats caused hypothermia, which was more pronounced at the higher dose (Fig. 1). The colonic temperature of the rats started to drop promptly, already at 10 min after the injection of NH_4Cl at both doses. Compared to the baseline, the NH_4Cl-induced maximal mean (± SE) decrease in deep T_b was −0.4 ± 0.1°C at 20 min in case of 220 mg/kg and −0.8 ± 0.2°C at 30 min in case of 280 mg/kg. From that timepoint, the T_b of the NH_4Cl-treated rats gradually increased and reached the T_b level of the control (sterile water-treated) group by the end of the experiment. The T_b of the sterile water-treated rats tended to increase, and then to decrease during the experiment after the i.p. infusion, but it did not differ significantly from the baseline at any timepoints. The initial increase in the T_b could be caused by an unwanted stress response to sterile water infusion despite all of our efforts to minimize the discomfort associated with substance administration, while the gradual fall might have reflected ultradian body temperature rhythms in rats maintained at an ambient temperature of ~25°C, which is below the thermoneutral zone. Statistically, both doses of NH_4Cl had a significant effect as compared to controls ($p < 0.001$ for both), and a statistical difference was also present between the lower and the higher dose groups ($p < 0.001$). At 220 mg/kg, the NH_4Cl-induced decrease in deep T_b was significant compared to the control group between 20 and 50 min, while at 280 mg/kg, the T_b was significantly lower than in controls between 20 and 90 min. The development of hypothermia in response to NH_4Cl in rats is a novel finding of our study, especially considering that the effect occurred already at an i.p. dose of 220 mg/kg, which is almost 50% smaller than the threshold dose predicted earlier in mice [26].

Table 1
Summary of drug administration procedures in the experiments.

Species (strain)	Pretreatment			NH_4Cl	Figure number
	Drug	Dose (mg/kg) and route	Time prior NH_4Cl (min)	I.p. dose (mg/kg)	
Rat (Wistar)	N/A			220; 280	1
Mouse (*Trpv1*$^{-/-}$ and $^{+/+}$)	N/A			321	2
Mouse (C57BL/6)	AMG 517	0.21 s.c.	−120	321	3
Rat (Wistar)	AMG 517	0.21 i.v.	−20	280	4
Mouse (*Trpa1*$^{-/-}$ and $^{+/+}$)	N/A			321	5
Rat (Wistar)	A967079	5 i.v.	−20	280	6

N/A, not applicable.

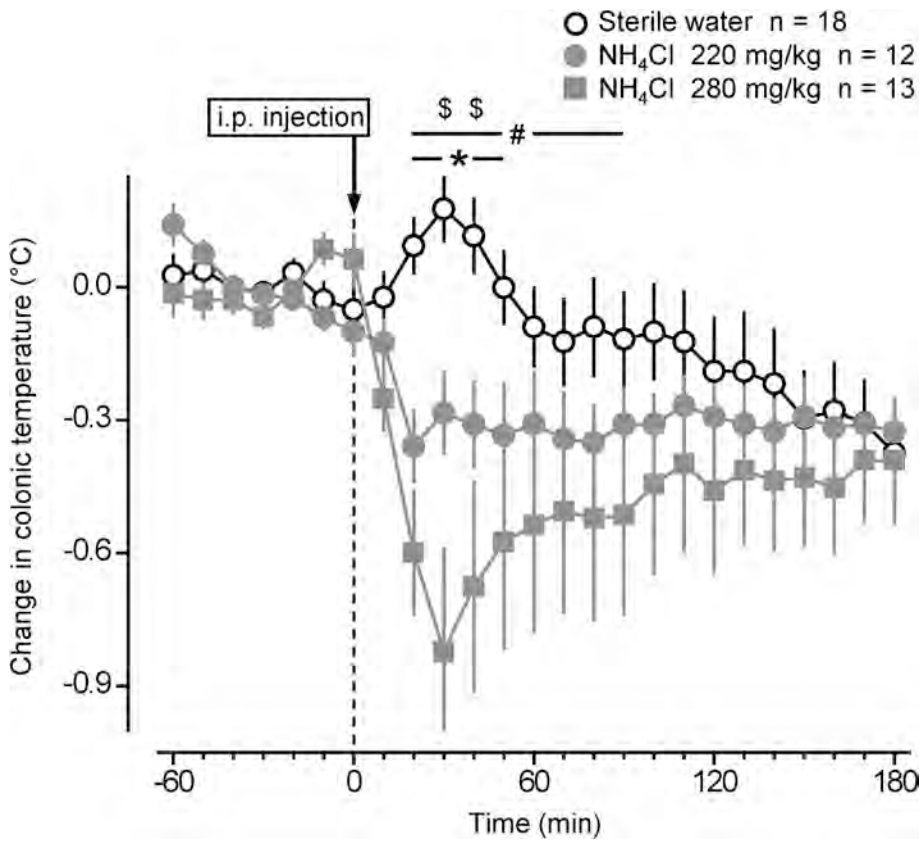

Fig. 1. Changes in deep (colonic) T_b in response to i.p. administration of NH_4Cl (doses indicated) or sterile water in rats. Here and in Figs. 2–6, n is the number of animals in each experimental group. $^{*}p < 0.05$, 220 mg/kg NH_4Cl vs. sterile water difference; $^{\#}p < 0.05$, 280 mg/kg NH_4Cl vs. sterile water difference; $^{\$}p < 0.05$, 220 vs. 280 mg/kg NH_4Cl difference.

3.2. NH_4Cl-induced hypothermia is augmented in mice genetically lacking the TRPV1 channel

After we showed that the hypothermic response to NH_4Cl is not specific to mice only, we wanted to know if two of the most studied thermo-TRP channels, TRPV1 or TRPA1, are involved in this thermoregulatory response. In our first approach, we compared the hypothermic response to NH_4Cl between *Trpv1*$^{-/-}$ and *Trpv1*$^{+/+}$ mice. Since the threshold i.p. dose of NH_4Cl to trigger hypothermia in mice was above 300 mg/kg according to Gordon [26], in our experiments we infused the mice i.p. with 321 mg/kg of NH_4Cl. As expected, NH_4Cl at this dose caused a sudden drop in the colonic temperature of *Trpv1*$^{+/+}$ mice, which reached the biggest mean decrease of -2.1 ± 0.5°C at 30 min post-injection (Fig. 2). Compared to the vehicle (sterile water), the deep T_b of NH_4Cl-treated *Trpv1*$^{+/+}$ mice was markedly lower between 20 and 50 min post-injection ($p < 0.05$). In *Trpv1*$^{-/-}$ mice, NH_4Cl also caused hypothermia compared to sterile water, which was significant between 20 and 70 min ($p < 0.05$) with a maximal mean decrease of -4.0 ± 0.4°C ($p < 0.001$). Interestingly, however, the hypothermic response to NH_4Cl was much more pronounced in the *Trpv1*$^{-/-}$ mice than in their *Trpv1*$^{+/+}$ littermates (Fig. 2). The intergenotype difference was significant ($p < 0.05$) between 20 and 60 min post-NH_4Cl administration with a maximum of ~2.0°C difference between the groups at 40 min ($p < 0.001$).

Nevertheless, it should be noted that the NH_4Cl-induced hypothermia developed in both genotypes of the mice (though to a different extent), which suggests that it also involves TRPV1-independent mechanisms.

3.3. Pharmacological blockade of the TRPV1 channel exaggerates NH_4Cl-induced hypothermia in mice and rats

Our findings in the genetically modified mouse model clearly indicated a bigger hypothermic response in *Trpv1*$^{-/-}$ mice. However, it could not be excluded that the *Trpv1*$^{-/-}$ mice had developed chronic compensatory mechanisms for the absence of TRPV1, which could have influenced the results. To avoid the potential development of chronic compensation, we decided to use genetically unaltered animals (*viz.*, C57BL/6 mice and Wistar rats) and block their TRPV1 channels acutely by a pharmacological antagonist, AMG 517.

AMG 517 is a highly potent and selective, in itself hyperthermia-inducing TRPV1 antagonist [52], which has been tested in hypothermic conditions associated with severe systemic inflammation and with general anesthesia [46,56]. Similarly as in our earlier study [56], in the present experiments the efficacy of AMG 517 could be confirmed by the higher deep T_b of the AMG 517-treated mice compared to vehicle-treated controls before NH_4Cl administration (Fig. 3a). As it could be expected based on our results obtained in *Trpv1*$^{+/+}$ mice, in the vehicle-pretreated C57BL/6 mice the hypothermic response to NH_4Cl developed rapidly and it reached the biggest mean decrease of -2.4 ± 0.4°C at 30 min ($p < 0.001$ compared to sterile water) (Fig. 3b). In the vehicle-pretreated mice, the NH_4Cl-induced decrease in deep T_b was significant ($p < 0.05$) compared to the sterile water-treated group between 20 and 70 min post-injection. Pretreatment with AMG 517 exaggerated the hypothermic effect of NH_4Cl to a maximal mean T_b decrease of -3.5 ± 0.6°C at 40 min ($p < 0.001$ compared to vehicle pretreatment) (Fig. 3b). The biggest difference between the mean T_b of the pretreatment groups was 1.6°C at 50 min post-NH_4Cl injection ($p < 0.001$). In response to NH_4Cl, the deep T_b of the AMG 517-pretreated mice was significantly

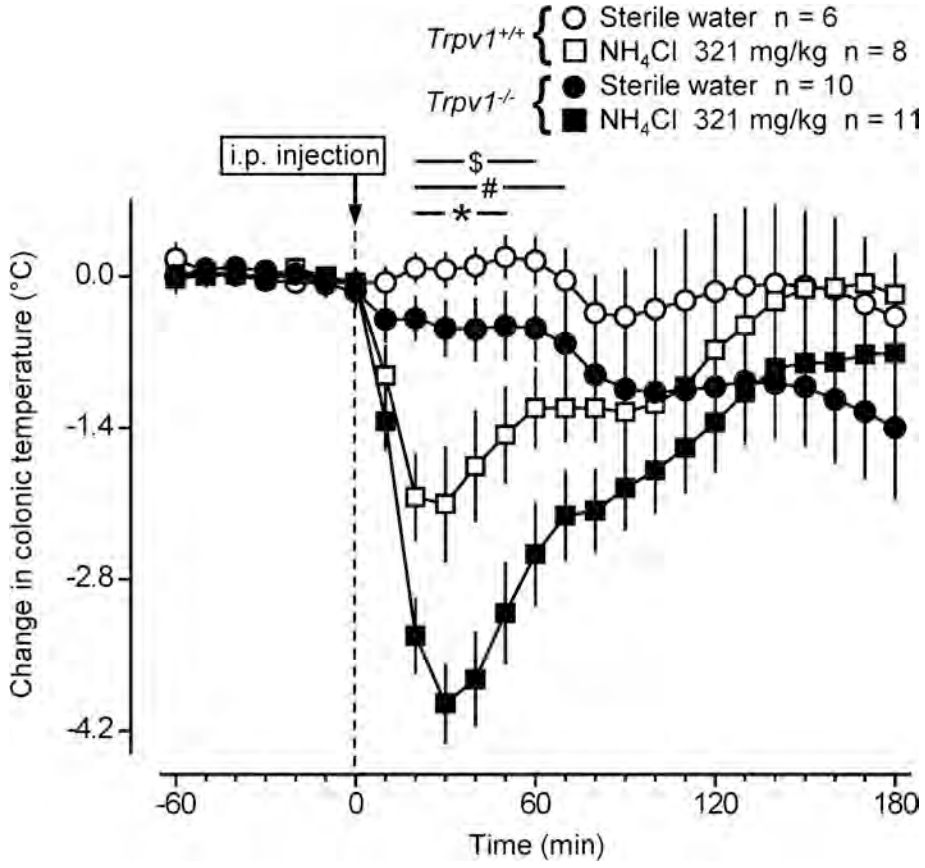

Fig. 2. Deep T_b responses of *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$ mice to i.p. administration of NH_4Cl (dose indicated) or sterile water. $^{*}p < 0.05$, NH_4Cl vs. sterile water difference in *Trpv1*$^{+/+}$ mice; $^{\#}p < 0.05$, NH_4Cl vs. sterile water difference in *Trpv1*$^{-/-}$ mice; $^{\$}p < 0.05$, intergenotype difference in response to NH_4Cl.

lower than that of the vehicle-pretreated mice between 30 and 70 min ($p < 0.05$).

We also wanted to confirm that the blockade of TRPV1 leads to the augmentation of NH_4Cl-induced hypothermia not only in mice, but also in rats. We administered the same dose (210 μg/kg) of AMG 517 to rats i.v. 20 min before the i.p. injection of NH_4Cl. As expected, AMG 517 caused prompt hyperthermia, which was present also at the time of the i.p NH_4Cl injection (Fig. 4a). After the injection of NH_4Cl, the deep T_b of vehicle-pretreated rats started to decrease immediately and reached a mean maximal decrease of -1.2 ± 0.2°C at 30 min ($p < 0.001$ compared to sterile water injection); it was significantly ($p < 0.05$) different between the two i.p. treatment groups (NH_4Cl vs. sterile water) between 20 and 100 min (Fig. 4b). Importantly, in AMG 517-pretreated rats both the magnitude and the duration of the NH_4Cl-induced hypothermia was markedly exaggerated. The maximal mean T_b fall of -1.7 ± 0.2°C developed at 40 min, and the T_b of the NH_4Cl-treated rats remained lower than the T_b of the sterile water-treated rats between 20 and 130 min ($p < 0.05$). Accordingly, the effect of NH_4Cl on T_b was significantly different between the i.v. pretreatment groups (AMG 517 vs. vehicle) from 40 to 70 and at 110 min post-NH_4Cl injection (Fig. 4b).

Similarly to our results with the genetic blockade of TRPV1, the NH_4Cl-induced hypothermia developed both with and without the pharmacological blockade of the TRPV1 channel (though to a different extent), which suggests the involvement of TRPV1-independent mechanisms.

3.4. The hypothermic response to NH_4Cl is attenuated in the absence of the TRPA1 channel in mice

Because TRPV1 and TRPA1 channels are often co-expressed [31,32,58] and crosstalk between them was also reported [59,60], after discovering the exaggeration of NH_4Cl-induced hypothermia in different models of TRPV1 blockade, we studied whether the TRPA1 channel also plays a role in this thermal response. Similarly as in the case of TRPV1, in our first approach we used mice genetically lacking the channel (*Trpa1*$^{-/-}$) and their WT littermates (*Trpa1*$^{+/+}$). As expected, the injection of NH_4Cl (321 mg/kg, i.p.) caused marked hypothermia in *Trpa1*$^{+/+}$ mice as compared to sterile water (Fig. 5). In *Trpa1*$^{+/+}$ mice, the biggest NH_4Cl-induced drop in T_b (-2.8 ± 0.3°C) developed 30 min after the injection ($p < 0.001$), and the T_b of NH_4Cl-tretaed mice was significantly lower than the T_b of the sterile water-treated group from 20 to 110 min post-injection ($p < 0.05$). In the *Trpa1*$^{-/-}$ mice, however, the hypothermic response to NH_4Cl was greatly attenuated: the biggest mean T_b decrease was -2.5 ± 0.2°C at 20 min, and it was significantly ($p < 0.05$) lower than that of sterile water-treated mice only between 20 and 50 min (Fig. 5). With regards to intergenotype difference, *Trpa1*$^{+/+}$ mice had significantly lower deep T_b than *Trpa1*$^{-/-}$ mice between 40 and 100 min after the administration of NH_4Cl with a maximal mean T_b difference of 1.0°C at 70 min ($p = 0.008$).

It should be noted that although the NH_4Cl-induced hypothermia was markedly attenuated in the genetic absence of the TRPA1 channel, it was still present in the KO mice suggesting the contribution of TRPA1-independent mechanisms to the response.

3.5. The hypothermic response to NH_4Cl is attenuated by the pharmacological blockade of the TRPA1 channel in rats

Similarly as in the case of TRPV1, it was important to exclude the potential presence of chronic compensatory mechanisms that may have developed in the absence of the TRPA1 channel in the *Trpa1*$^{-/-}$ mice. For that, we used pharmacological blockade of the channel with a highly potent and selective TRPA1 antagonist, A967079 [53]. However, we

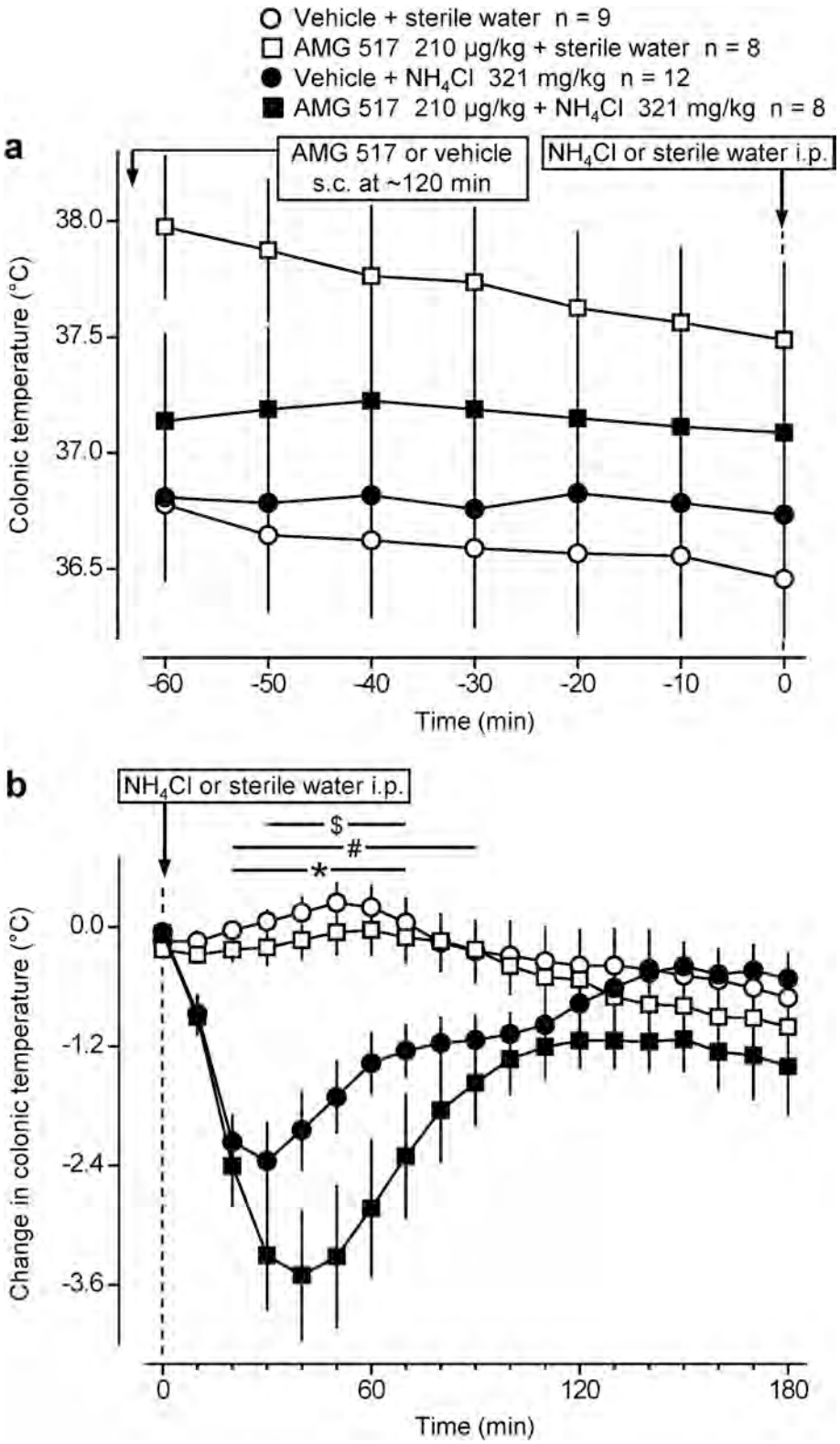

Fig. 3. (a) Colonic temperature responses to s.c. administration of the TRPV1 antagonist AMG 517 (dose indicated) or vehicle in C57BL/6 mice. (b) Changes in deep T_b of the mice in response to i.p. administration of NH_4Cl (dose indicated) or sterile water after s.c. pretreatment at −120 min with AMG 517 (dose indicated) or vehicle. $^{*}p < 0.05$, NH_4Cl vs. sterile water difference in vehicle-pretreated mice; $^{\#}p < 0.05$, NH_4Cl vs. sterile water difference in AMG 517-pretreated mice; $^{\$}p < 0.05$, AMG 517 vs. vehicle pretreatment difference in response to NH_4Cl.

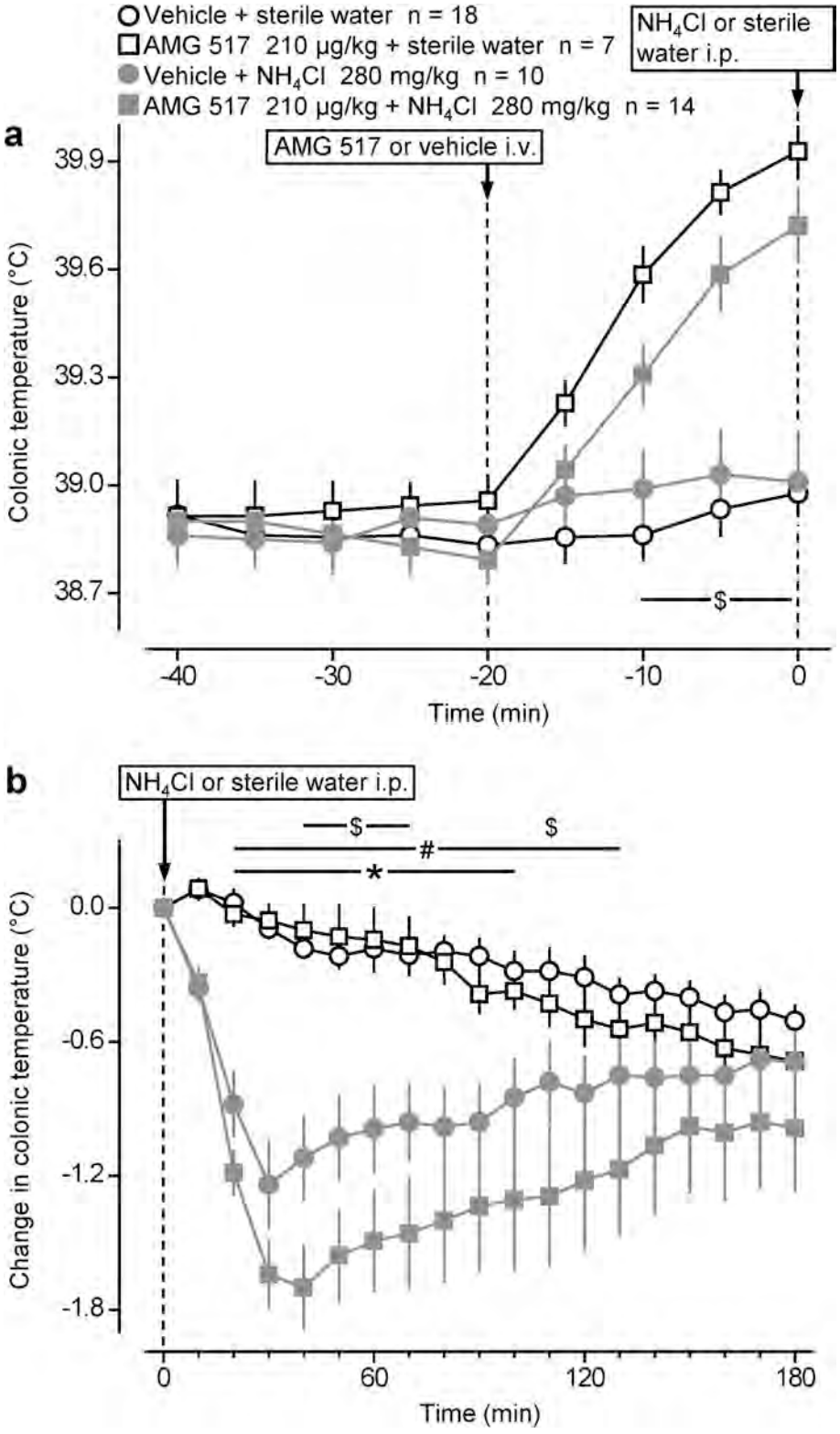

Fig. 4. (a) Colonic temperature responses to i.v. administration of the TRPV1 antagonist AMG517 (dose indicated) or vehicle in rats. $^{\$}p < 0.05$, AMG 517 vs. vehicle pretreatment difference. (b) Changes in deep T_b of the mice in response to i.p. administration of NH_4Cl (dose indicated) or sterile water after i.v. pretreatment at −20 min with AMG 517 (dose indicated) or vehicle. $^{*}p < 0.05$, NH_4Cl vs. sterile water difference in vehicle-pretreated rats; $^{\#}p < 0.05$, NH_4Cl vs. sterile water difference in AMG 517-pretreated rats; $^{\$}p < 0.05$, AMG 517 vs. vehicle pretreatment difference in response to NH_4Cl.

could not use the mouse experimental model that was applied in case of AMG 517, because the half-life of A967079 is relatively short, ca. 49 min [53], thus the efficacy of the drug could have been questionable 2 h after its s.c. administration, when NH_4Cl could be injected to mice (for details, see Table 1). In addition, it was of crucial importance to test the contribution of the TRPA1 channel in another species than mice, because interspecies differences were demonstrated in the temperature-sensitivity of the channel as well as in its response to pharmacological

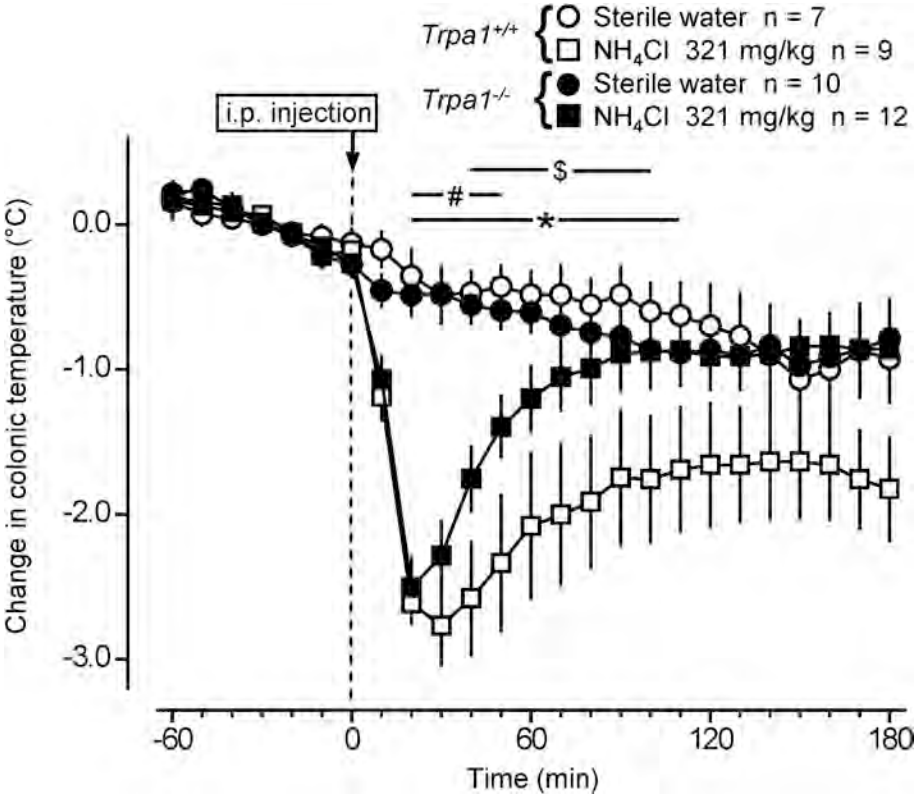

Fig. 5. Deep T_b responses of *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice to i.p. administration of NH_4Cl (dose indicated) or sterile water. $^{*}p < 0.05$, NH_4Cl vs. sterile water difference in *Trpa1*$^{+/+}$ mice; $^{\#}p < 0.05$, NH_4Cl vs. sterile water difference in *Trpa1*$^{-/-}$ mice; $^{\$}p < 0.05$, intergenotype difference in response to NH_4Cl.

agents (for review, see [61]). Therefore, we studied the effects of i.v. administered A967079 on NH_4Cl-induced hypothermia in rats with a similar experimental design as in the case of AMG 517 (for details, Table 1). In accordance with previous studies [35,53], A967079 did not have any meaningful effect on the colonic temperature of the rats before the i.p injection of NH_4Cl or sterile water (Fig. 6). On the contrary, when the same dose (5 mg/kg) of A967079 was infused 20 min before the i.p. injection of NH_4Cl, it markedly attenuated the hypothermic response. While the T_b of NH_4Cl-treated rats was significantly ($p < 0.05$) lower at 20–150 min compared to sterile water injection after pretreatment with vehicle, in the A967079-pretreated rats the NH_4Cl-induced hypothermia was significant only between 10 and 90 min (Fig. 6). In accordance, a statistically significant difference was also present between the two pretreatments in the NH_4Cl-treated rats between 40 and 120 min with a maximal difference of 0.7°C at 50 min ($p = 0.003$).

In line with our results with the genetic blockade of TRPA1, the NH_4Cl-induced hypothermia was substantially attenuated, but still present after the pharmacological inhibition of the TRPA1 channel suggesting the contribution of TRPA1-independent mediators to the response.

3.6. I.p. administration of NH_4Cl decreases the blood pH in rats and mice

Last, we wanted to know how the applied doses of NH_4Cl affected the blood pH of the rats and the mice. In rats, the blood pH after i.p. administration of NH_4Cl was decreased at both doses (to 7.47 and 7.24) compared to sterile water treatment (pH = 7.51), however, the difference was significant only at the higher (280 mg/kg) dose ($p < 0.001$) (Fig. 7). The blood pH was also statistically different between two doses of NH_4Cl ($p < 0.001$). In mice, the i.p. injection of NH_4Cl (321 mg/kg) resulted in substantial drop in blood pH in all genotypes compared to sterile water injection (Fig. 7). The fall in the blood pH of the mice reached a similar extent in all genotypes ranging between 7.23 and 7.30 after the i.p. injection of NH_4Cl.

4. Discussion

In the present study, we show that the i.p. administration of NH_4Cl decreases deep T_b in rats and mice. The genetic and pharmacological blockade of the TRPV1 channel exaggerates the hypothermic effect of NH_4Cl. On the contrary, the hypothermic response to NH_4Cl is attenuated by the genetic ablation and pharmacological inhibition of the TRPA1 channel. Nevertheless, the hypothermic response occurred (to a different extent) in all of our experimental models, indicating that *per se* neither TRPA1 nor TRPV1 is essential for NH_4Cl-induced hypothermia. These findings suggest that TRPV1 channels are limiting regulators, whereas TRPA1 channels are potentiating signaling molecules of NH_4Cl-induced hypothermia.

The hypothermic effect of i.p. administered NH_4Cl has been shown in mice long time ago [26]. However, it has remained unknown whether it also develops in other species and which receptors are involved in the NH_4Cl-induced hypothermic response. NH_4Cl administration is often used to induce metabolic acidosis in rodents [12–15]. The mechanism of NH_4Cl-induced metabolic acidosis is consumption of bicarbonate during conversion of ammonia to urea nitrogen via the urea cycle [62]. In severe cases of the NH_4Cl-induced metabolic acidosis, a fall in intracellular pH also developed [19], although the intra- and extracellular pH levels did not always correlate in milder cases of acidosis (extracellular pH drop of 0.11–0.19) [14,19,63]. The different effects on pH can originate from the used species (rat vs. mouse), experimental model (*in vitro* vs. *in vivo*), and acid loading protocol (acute vs. chronic). In our experiments, NH_4Cl decreased the blood pH to ~7.25 in rats and mice, which was markedly lower than in the control animals, however, in this *in vivo* experimental design the intracellular pH could not be measured. The decreased blood pH could potentially serve as a direct mechanism for the development of the hypothermia, for example, through the

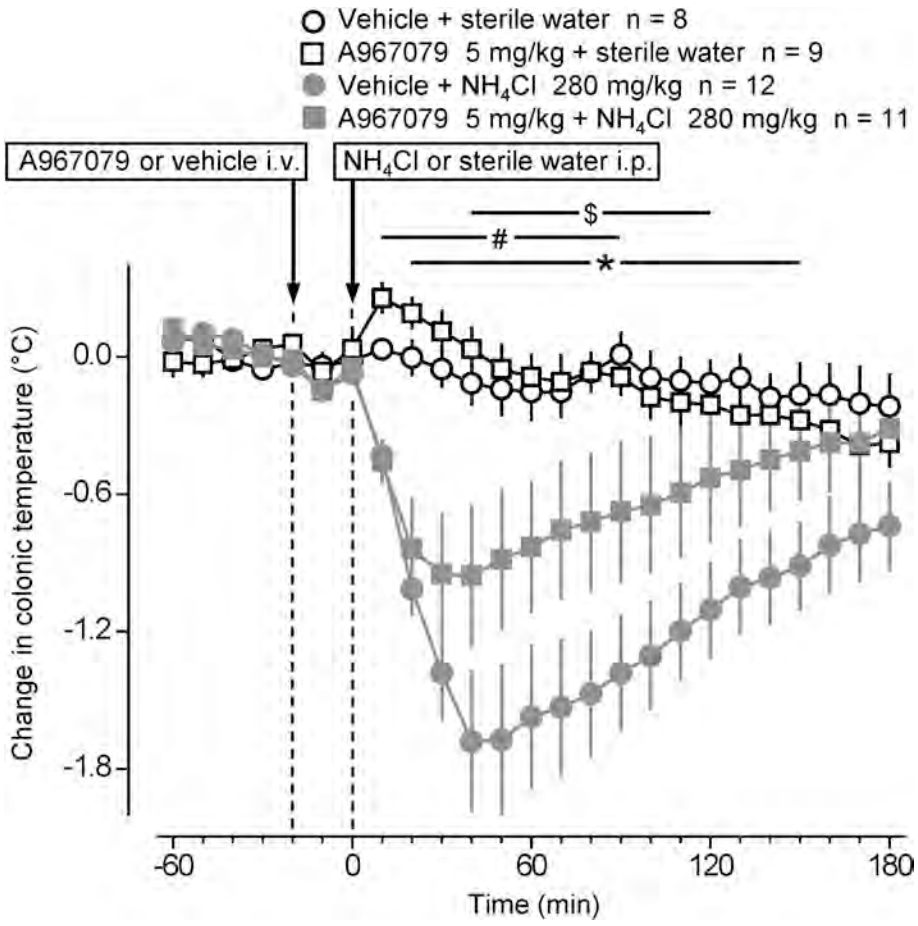

Fig. 6. Changes in deep T_b of rats in response to i.p. administration of NH_4Cl (dose indicated) or sterile water after i.v. pretreatment at −20 min with A967079 (dose indicated) or vehicle. $^{*}p < 0.05$, NH_4Cl vs. sterile water difference in vehicle-pretreated rats; $^{\#}p < 0.05$, NH_4Cl vs. sterile water difference in A967079-pretreated rats; $^{\$}p < 0.05$, A967079 vs. vehicle pretreatment difference in response to NH_4Cl.

stimulation of the TRPV1-mediated acido-antithermogenic and acido-antivasoconstrictor reflexes that originate from trunk muscles and limit the increase in deep T_b during physical exercise (for review, see [64]). However, since the NH_4Cl-induced hypothermia was still detectable after genetic and pharmacological blockade of both the TRPV1 and the TRPA1 channels, it cannot be excluded that the hypothermic response to the decreased blood pH involved TRPV1- and TRPA1-independent mechanisms. Several acid-sensitive ion channels, other than TRPV1 and TRPA1, are expressed on primary sensory neurons, which are involved in a number of physiological and pathophysiological reactions to acidosis (for review, see [65]). Whether any of these acid sensors contribute to the development of NH_4Cl-induced hypothermia remains subject for future studies.

It is also possible, however, that the hypothermic effect of NH_4Cl was triggered by mechanisms that are not related solely to the decreased blood pH, because the lower dose of NH_4Cl caused hypothermia in rats, but it did not influence the blood pH significantly, which argues against a direct acid-induced effect. Unfortunately, the pH of the mice was not reported in the study by Gordon [26]. It should be also noted that although the acidosis clearly developed in the rats (at the higher dose) as well as in the mice of all genotypes in our experiments, the extent of decrease in blood pH was not very severe. The drop in blood pH was probably not sufficient to reach the proton activation threshold of the TRPV1 channel, because the half-maximal response of the rat TRPV1 channel to protons occurs at a pH of ~5.78 *in vitro* [66]. However, the elevated proton concentrations could potentiate responses evoked by other agonists, resulting in increased activity of the channel [43].

With regards to the TRPA1 channel, the pH activation is complex. It was demonstrated that weak acids activate rodent TRPA1, but this was due to intracellular acidosis and direct proton activation of the channel from the cytosolic side [44,67]. Although protons can rapidly permeate through the membrane to induce an intracellular acidosis when the extracellular H^+ concentration is increased [68], rodent TRPA1 failed to respond to extracellular acidosis, and protons even inhibited the channel in a later study [41]. Nevertheless, exclusively in humans, TRPA1 is an important (nociceptive) proton sensor [41], which complicates the translation of results from animal experiments to humans. Moreover, in case of NH_4Cl administration the intracellular pH level does not always correlate with the extracellular pH and an increased pH within the cell was also described instead of acidosis (for review, see [69]). Ammonia and intracellular alkalization were shown to activate both the TRPV1 and the TRPA1 channels [39,40]. Hence, the rapid inward diffusion of gaseous ammonia and the resulting intracellular alkalization could have also contributed to our results. It is known that systemic (i.v.) administration of NH_4Cl leads to formation of ammonia, which can readily cross the blood-brain barrier [70]. This raises the possibility that activation of TRPA1 channels in thermoregulatory neurons in the brain triggered the hypothermic response, as shown earlier in case of another gasotransmitter, hydrogen sulfide [37], but the investigation of the exact molecular interaction between NH_4Cl and TRP channels, as well as finding the site of the thermoregulatory action of NH_4Cl were beyond the scope of the present study and remain subjects for future research.

The exaggeration of NH_4Cl-induced hypothermia by the blockade of TRPV1 channels was an unexpected novel finding. However, it should be mentioned that it was shown earlier that inhibition of TRPV1 channels potentiated the neuronal responses to NH_4^+ [71]. We can only speculate

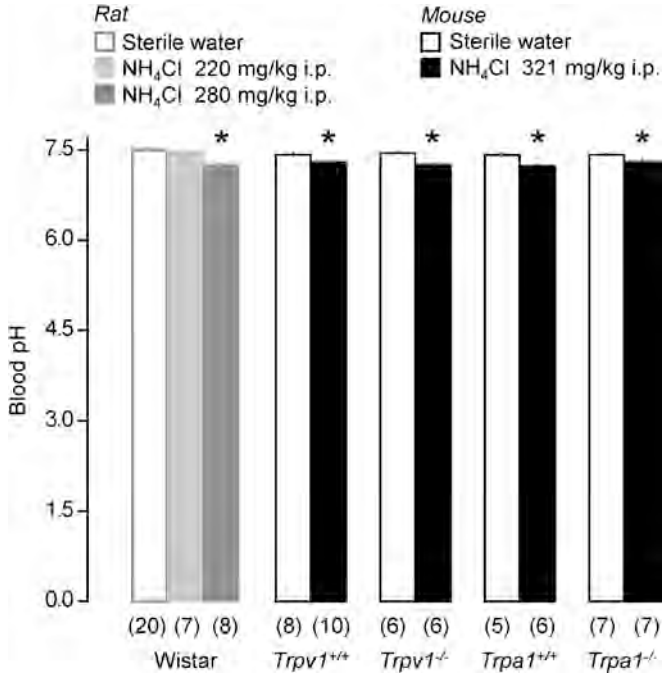

Fig. 7. Blood pH of rats and mice of different genotypes (*Trpv1*$^{+/+}$, *Trpv1*$^{-/-}$, *Trpa1*$^{+/+}$, and *Trpa1*$^{-/-}$) one hour after the i.p. administration of NH_4Cl (doses indicated) or sterile water. Numbers in parentheses indicate the number of animals in the corresponding groups. *$p < 0.05$, NH_4Cl vs. sterile water difference within the same genotype.

that NH_4^+ ions could inhibit TRPV1 channels located in the abdominal wall, which were shown to tonically suppress skin vasoconstriction and thermogenesis [72]. When these TRPV1 channels are blocked with genetic or pharmacological tools the hypothermia-counteracting effect is absent, hence the thermal response to NH_4Cl becomes augmented. In support of this assumption, it was found that different quaternary ammonium ions blocked the TRPV1 channel from the intracellular surface with the bigger molecules becoming slower blockers [73]. As an alternative theory, we showed earlier that acidosis-induced vasodilation of rat and mouse tail arteries are limited by non-neuronal TRPV1 channels in the vascular wall [74], thus it can be assumed that the blockade of these vasodilation-limiting TRPV1 channels could result in higher heat loss and exaggerated hypothermic response to NH_4Cl. It has to be noted, however, that the abovementioned action mechanisms of NH_4Cl on TRPV1 channels remain hypothetical until directly tested in future experiments. As another limitation of our study, it should be mentioned that we used only male animals in our experiments, hence sex differences in the thermal response to NH_4Cl could not be investigated. Nevertheless, our results can also serve as an encouraging basis for designing future studies, which are required to determine the potential presence of sex differences in the development of NH_4Cl-induced hypothermia and its relation to TRPA1 and TRPV1 channels.

In conclusion, TRPA1 channels contribute to the development of NH_4Cl-induced hypothermia in mice and rats, whereas TRPV1 channels play a limiting function, although in itself neither of them is essential for the occurrence of the response. It can be hypothesized that activation of TRPA1 channels in thermoregulation-related brain nuclei are responsible for the induction of NH_4Cl-induced hypothermia, while TRPV1 channels on the periphery, possibly in abdominal muscles or vascular smooth muscle exert a limiting effect on the response. Our findings highlight the importance of the nonthermal activation of TRPV1 and TRPA1 channels in thermoregulation. Since NH_4Cl is still used in clinical practice (*e.g.*, as a diuretic, expectorant, and perhaps COVID-19 treatment), the present results warrant for regular T_b control and careful consideration of the treatment, especially in patients with TRPA1 and TRPV1 channel function disorders.

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CRediT authorship contribution statement

Zoltan Rumbus: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kata Fekete:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Leonardo Kelava:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Bibor Gardos:** Writing – review & editing, Investigation, Formal analysis. **Krisztian Klonfar:** Formal analysis, Investigation, Writing – review & editing. **Patrik Keringer:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Erika Pinter:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Writing – review & editing. **Eszter Pakai:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Andras Garami:** Investigation, Funding acquisition, Formal analysis, Conceptualization, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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.

Data availability

All data generated or analyzed during this study are included in this published article.

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FRONT MATTER: DISCOVERY

Fever, hypothermia, and mortality in sepsis*

Comment on: Rumbus Z, Matics R, Hegyi P, Zsiboras C, Szabo I, Illes A, Petervari E, Balasko M, Marta K, Miko A, Parniczky A, Tenk J, Rostas I, Solymar M, Garami A. Fever is associated with reduced, hypothermia with increased mortality in septic patients: a meta-analysis of clinical trials. PLoS One. 2017;12(1):e0170152. DOI: 10.1371/journal.pone.0170152

Sepsis presents a major challenge for critical care and the society worldwide. Despite the ample research interest in understanding the underlying mechanisms, mortality rate remains considerably high in sepsis even nowadays. In order to assess the prognosis and the severity of the disease, thus to initiate the most optimal treatment, it is necessary to identify vital signs and biomarkers, which can predict the outcome. As a manifestation of systemic inflammation, sepsis is often accompanied by changes in body temperature (T_b): fever or hypothermia. Our understanding of the thermoregulatory manifestations of systemic inflammation has advanced in the past decades, but it has remained unanswered whether fever and hypothermia can serve as predictors of the outcome in sepsis. In the highlighted study, we investigated the association between the alterations of T_b and the rate of mortality in septic patients [1].

By conducting a meta-analysis of clinical trials, we studied the association between changes in T_b and mortality in a big number (> 10,000) of septic patients [1]. We found that in septic patients with fever the estimated mortality rate was ~ 22%, which was higher (~ 31%) in normothermic patients, while it was the highest (~ 47%) in hypothermic patients [1]. When we compared the T_b data of all septic patients divided into mortality quartiles, we found that T_b was by 1°C higher in patients with the lowest (< 25%) mortality than in patients with the highest rate of death (> 75%). The results of our meta-analyses clearly demonstrate a negative correlation between T_b and mortality in sepsis: fever is associated with decreased, whereas hypothermia with increased rate of death. However, this association does not automatically imply that fever is always beneficial and hypothermia is harmful in sepsis (Table 1). The causative relationship between the thermoregulatory manifestations and the outcome in systemic inflammation could not be assessed in our study and it deserves discussion.

The beneficial versus harmful effect of fever has been debated since the time of Hippocrates. Despite the advancements in our understanding of the molecular, cellular, and physiological mechanisms of the fever response, the question of whether it is a friend or foe is asked even in recent days. The controversies between the findings on beneficial versus harmful effects of fever can be mitigated by focusing on the question of when instead of whether fever is a friend or a foe as suggested by Romanovsky and Szekely [2]. It was proposed that the thermoregulatory manifestations of systemic inflammation can be regarded as adaptations in constellation of the sickness syndrome. Two sickness patterns can be distinguished as part of the syndrome, which represent sequential stages of the body's response to systemic inflammation and constitute two different adaptive strategies to infection [2]. The two distinguished patterns of the syndrome, the early and the late phase syndrome, correspond to mild and severe forms of systemic inflammation. Romanovsky and Szekely [2] proposed that fever, as part of the early phase syndrome, should be regarded as an adaptive strategy of the organism, which occurs at the onset of the infection and constitutes a response of the healthy organism to the forthcoming disease. In this regard, the biological purpose of the early phase syndrome is to engage active defense mechanisms (fever), notify the host about the pathogenic insult (hyperalgesia), and secure the means (vigilance, hyperactivity, hypertension, and anorexia), which can empower the active search for optimal environment (warmth seeking, adequate water supply, protection from external stressors) for fighting the disease. This type of adaptation to

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Table 1. Disease coping strategies in different premorbid conditions, thermoregulatory manifestations, and potential outcomes in different severities of sepsis.

Infection severity	Premorbid condition	Coping strategy	Deep body temperature	Predicted outcome	Effect on mortality
Mild	Healthy	Disease fighting	Fever	Pathogen clearance	Not applicable
Moderate	Healthy	Disease fighting	Fever	Pathogen clearance	↓
			Extreme fever	Organ failure, energy depletion	↑
	Comorbidities* or exhaustion†	Disease fighting	Fever	Organ failure, energy depletion	↑
		Energy saving	Hypothermia	Disease tolerance††	↓
Severe (e.g., septic shock)	Healthy	Energy saving	Hypothermia	Disease tolerance	↓
	Comorbidities or exhaustion		Extreme hypothermia	Organ failure	↑

Effects on mortality are marked as: ↓, decrease; ↑, increase. *E.g., pre-existing cardiovascular, pulmonary, neurological disease. †E.g., because of old age, starvation, prolonged systemic inflammation. ††The host's ability to tolerate the presence of the pathogen; for details, see Garami et al. [3].

infections develops at a high energy cost. A short-lasting, mild infectious challenge (common cold or influenza) is often characterized by the early phase syndrome only. In a previously fit and healthy patient, fever will be likely beneficial (Table 1). On the contrary, the beneficial value of fever can be compromised by an already existing or forthcoming energy deficiency, for example, in moderate-to-severe infections of patients with different (e.g., nutritional, cardiovascular, and respiratory) comorbidities, who lack adequate protection from external stressors. The energy resources are depleted more rapidly in extremely high fevers, resulting in the worsening of the outcome of the disease. In such situations, the beneficial value of fever diminishes and its harmful consequences emerge (Table 1). It has to be noted that because of data unavailability, extreme fever responses (T_b above 39.9°C), which could have detrimental consequences, were not included in the meta-analysis in focus [1].

In contrast to fever, the biological significance of hypothermia has been almost completely ignored in systemic inflammation. The possible reasons for this inattention include the lower incidence rate of hypothermia compared to fever, improper T_b-measuring techniques that result in cases that go undetected, and the low priority of decreased T_b in critically ill patients who are often in extremely poor conditions when presented to the physician. From a physiological perspective, hypothermia can be regarded as a part of the late phase syndrome, which represents the systemic response to infection when the disease has already progressed, thus damaged and weakened the organism [2]. The pain has lost its warning function, resulting in hypoalgesia. High energy-consuming responses are not affordable; consequently, somnolence, motor depression, and normo- or hypotension are characteristic. In severe clinical cases (e.g., septic shock), the late phase syndrome becomes predominant and it can completely replace the early phase syndrome. As a general rule, hypothermia is a beneficial response when the damage is severe enough to cause or facilitate energy depletion (Table 1). In severe forms of inflammation, the energy resources can be reduced by the overwhelming inflammatory response, involving pathological energy expenditure and increased oxygen demand of damaged tissues. At the same time, the energy supply is commonly decreased or completely absent due to the compromised ability to get food and the development of adaptive anorexia. Because of the dependence of biochemical rates on temperature (van't Hoff's rule), tissue metabolic requirements decrease by more than 10% for every 1°C drop in T_b. Direct experiments have shown that spontaneous hypothermia is more advantageous than fever in rats with severe forms of aseptic (lipopolysaccharide-induced) or septic (Escherichia coli-induced) systemic inflammation [reviewed in ref. 3]. In these studies, the development of hypothermia (vs fever) exerted a pronounced influence on survival rates of the rats with systemic inflammation: the survival rate of hypothermic rats was markedly higher than that of the febrile rats in both aseptic and

septic models of severe systemic inflammation. Further advantages of hypothermia over fever included the suppression of endotoxemia and reduced lung infiltration by neutrophil leukocytes. The more severe the pathogenic challenge, premorbid pathology, and actual conditions, the more likely that hypothermia and its energy-saving actions will be advantageous for the host (Table 1). If the patient is sleepy, depressed, hypoactive, hypotensive, and hypoalgesia occurs, the energy-saving strategy of hypothermia is likely to be protective [2]. It should be also noted that in severe cases of sepsis, particularly in patients with pre-existing energy exhaustion or comorbidities, T_b may fall to critical levels and the adverse consequences of the extreme hypothermia (e.g., cardiac arrhythmias, neurological dysfunctions) can overcome its adaptive value (Table 1).

Despite the growing body of evidence for the adaptive value of hypothermia in systemic inflammation, hypothermia is generally perceived in clinical settings as something dysregulated and accidental, and prompt rewarming is regularly considered for those septic patients who display a fall in T_b. The first effort to reconcile experimental and clinical evidence in septic hypothermia was made only recently; it was revealed that hypothermia is predominantly transient, self-limiting, and nonterminal response, which naturally occurs in human sepsis [4]. Consequently, it can be questioned whether rewarming is at all necessary in the subset of septic patients who naturally develop hypothermia. To answer the question, well-designed, interventional clinical studies are warranted, in which spontaneous hypothermia is allowed or prevented within the hypothermic subset of septic patients.

As a perspectival approach, the utilization of controlled, targeted modulation of T_b by pharmacological tools could also be studied in systemic inflammation; new hypo- and hyperthermia-inducing drugs may be used one day, like the antagonists of the transient receptor potential vanilloid-1 channel [5]. Such and similar studies with targeted modulation of T_b in systemic inflammation could unequivocally determine the cause-effect relationship between the thermoregulatory manifestations and the outcome in sepsis, thus further advance our understanding of the association between T_b and mortality observed in the highlighted article [1].

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