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Mechanisms of cardiac ethanol toxicity and novel treatment options

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ABSTRACT

Ethanol can acutely and chronically alter cardiomyocyte and whole-organ function in the heart. Importantly, ethanol acutely and chronically predisposes to arrhythmias, while chronic abuse can induce heart failure. However, the molecular mechanisms of ethanol toxicity in the heart are incompletely understood.
In this review, we summarize the current mechanistic knowledge on cardiac ethanol toxicity, with a focus on druggable pathways. Ethanol effects on excitation-contraction coupling, oxidative stress, apoptosis, and cardiac metabolism, as well as effects of ethanol metabolites will be discussed. Important recent findings have been gained by investigation of acute ethanol effects. These include a renewed focus on reactive oxygen species (ROS) and induction of SR Ca^{2+} leak by CaMKII-mediated pathways downstream of ROS. Furthermore, a clinical outlook into potential novel treatment options is provided.

Contents

1. Introduction & clinical relevance

The relevance of ethanol abuse for public health systems, as well as patient morbidity and mortality is well established (GBD, 2016; Hasin, Stinson, Ogburn, & Grant, 2007). Indeed, alcohol consumption was the seventh leading risk factor for death and loss of disability-adjusted life-years in 2016 (GBD, 2016). While abstinence would of course be the most effective preventative measure (Pavan et al., 1987), the overwhelming prevalence of chronic or regular ethanol consumption in most societies (GBD, 2016; Hasin et al., 2007; Voskoboinik, Prabhu, Ling, Kalman, & Kistler, 2016; Whitman et al., 2017) in our opinion results in a need for novel pharmacological approaches for ethanol-induced pathologies.

Chronically, alcohol can induce contractile dysfunction and arrhythmias, leading to heart failure, increased risk of sudden cardiac death, myocardial infarction, and stroke (Whitman et al., 2017). The supposed beneficial cardiac effects of small amounts of alcohol consumed (Piano, 2002) turn into detrimental consequences, when the daily number of drinks increases (Charakida et al., 2018; Li et al., 2016; Walker et al., 2013) and heavy drinkers have a much higher risk of severe heart failure compared to non-drinkers (Li et al., 2016). Indeed, up to 40% of patients classified as idiopathic dilated cardiomyopathy (DCM) are excessive drinkers (Gavazzi, De Maria, Parolini, & Porcu, 2000; McKenna, Codd, McCann, & Sugrue, 1998), suggesting underdiagnosis of alcoholic

Abbreviations: ACA, acetaldehyde; ACM, alcoholic cardiomyopathy; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; APD, action potential duration; ATP, adenosine triphosphate; Ca^{2+}, calcium; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; DAD, delayed afterdepolarization; DCM, dilated cardiomyopathy; JNK, c-Jun N-terminal kinases; mTORC1, mammalian target of rapamycin complex 1; NCX, sodium calcium exchanger; NOX2, NADPH-Oxidase II; QTc, frequency-corrected QT interval; ROS, reactive oxygen species; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; ULK1, Unc51-like kinase.

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cardiomyopathy (ACM) and/or overlap between these entities. Furthermore, there are studies that have observed negative effects on cardiac function, like reduced left-ventricular ejection-fraction, already in regular moderate drinkers (Li et al., 2016). Besides reduced cardiac contractility and symptoms of heart failure, chronic drinkers also have an increased risk for both atrial and ventricular arrhythmias (Buckingham et al., 1985; Corović, Duraković, & Misigoj-Duraković, 2006; Ettinger et al., 1978; Frost & Vestergaard, 2004; George & Figueredo, 2010; Guzzo-Merello et al., 2015; Regan, 1984; Voskoboinik et al., 2016; Wannamethee & Shaper, 1992; Whitman et al., 2017). Additionally, heavy drinkers have a higher risk for sudden cardiac death, independent from pre-existing ischemic heart disease (Wannamethee & Shaper, 1992).

Even *acutely*, ethanol can induce atrial fibrillation, the so-called holiday heart syndrome (Buckingham et al., 1985; Ettinger et al., 1978; Whitman et al., 2017), and further worsen preexisting morbidities (such as further impairing reduced cardiac contractility in heart failure (Gould, Zahir, Demartino, & Gomprecht, 1971) or acutely increasing the propensity for lethal arrhythmias in patients with myocardial infarction (Jabbari et al., 2015)). Interestingly, as much as two thirds of patients admitted with new-onset atrial fibrillation had consumed alcohol prior to admission (Voskoboinik et al., 2016).

While these detrimental effects of increased alcohol intake on the heart are well documented, the pathophysiological mechanisms are less well understood. Especially in chronic ethanol abuse, alcoholic cardiomyopathy and contractile dysfunction are the end-stage of complex remodeling processes, which are often difficult to attribute to ethanol toxicity alone rather than unspecific heart failure remodeling, and might be influenced by other cardiac co-morbidities such as cardiac ischemia, making it difficult to discern specific pathologic effects of ethanol on the heart. More so, research into the effects of acute versus chronic ethanol intake is often conflicting or at least mechanistically different, possibly due to such chronic (mal-) adaptive processes. Thus, it has proven difficult to discern specific pathologic effects of ethanol on the heart and to develop specific therapeutic strategies for alcoholic cardiac damage from these findings. As such, alcohol-induced cardiac diseases are commonly treated according to the standards of the general cardiac condition, without specifically addressing the underlying effects of ethanol on the heart. More so, strategies to prevent or ameliorate cardiac damage due to alcohol (besides abstinence) are lacking.

However, in the absence of chronic confounders (such as cardiac remodeling or systemic effects of ethanol), recent research into acute ethanol effects has provided important insights into cardiac ethanol toxicity, where disordered cardiomyocyte Ca^{2+}-handling (Mustroph et al., 2018; Yan et al., 2018) seems to mediate arrhythmias and contractile dysfunction.

The aim of this article is to review the current knowledge of cardiac toxicity of ethanol, with a focus on, where applicable, *direct* cardiac effects rather than systemic processes, featuring both in vivo and in vitro research. It will focus on mechanistic insights into the pathophysiology of ethanol toxicity and provide a clinical outlook from recent findings. Key aspects of this review are also illustrated in Fig. 1 and Fig. 2. With respect to clinical features, clinical reviews are available to the reader (Tonelo et al., 2013; Voskoboinik et al., 2016; Whitman et al., 2017).

2. Pathophysiology of cardiac ethanol toxicity & therapeutic strategies

While chronic ethanol abuse may result in alcoholic cardiomyopathy (ACM) and while this is considered as a specific disease, there is no clear definition of ACM and it is a diagnosis of exclusion (Guzzo-Merello, Cobo-Marcos, Gallego-Delgado, & Garcia-Pavia, 2014). This lack of distinction adds to the fact that as of yet, no specific differences have been found between ACM and DCM. Interestingly, Askanas et al. found echocardiographic abnormalities already in about 50% of asymptomatic alcoholics, who had still preserved left ventricular contractility, but increased left-ventricular mass (Askanas, Udoshi, & Sadjadi, 1980). Both acutely and chronically, ethanol consumption predisposes to arrhythmias. Excellent and extensive reviews on ACM with a more clinical focus are available (e.g. (Piano, 2002; Piano, 2017; Voskoboinik et al., 2016)).

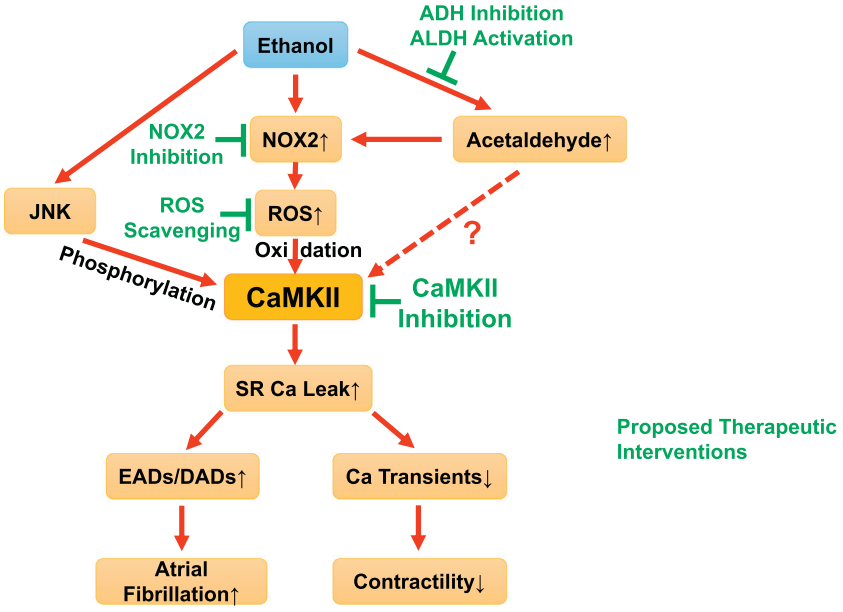

Fig. 1. Mechanisms of ethanol-induced CaMKII activation, downstream detrimental effects, and novel therapeutic options. Ethanol acutely activates NADPH oxidase II (NOX2) directly and indirectly (via induction of acetaldehyde, ACA). NOX2 activation (and effects of ethanol on the mitochondria) induces ROS production, which can activate Ca/calmodulin-dependent kinase II (CaMKII) by oxidation. CaMKII also is activated by phosphorylation via JNK. Activated CaMKII induces sarcoplasmic reticulum (SR) Ca leak, which leads to cellular arrhythmias and reduced contraction. Novel therapeutic options could be inhibition of alcohol dehydrogenase (ADH) to decrease metabolism of ethanol into ACA or activation of acetaldehyde dehydrogenase (ALDH) to increase acetaldehyde degradation. Furthermore, inhibition of NOX2 could prevent ROS production. Alternatively, ROS scavenging could also be a viable therapeutic option. Importantly, direct CaMKII inhibition utilizing novel orally applicable CaMKII inhibitors could prevent CaMKII-induced arrhythmias and contractile dysfunction.

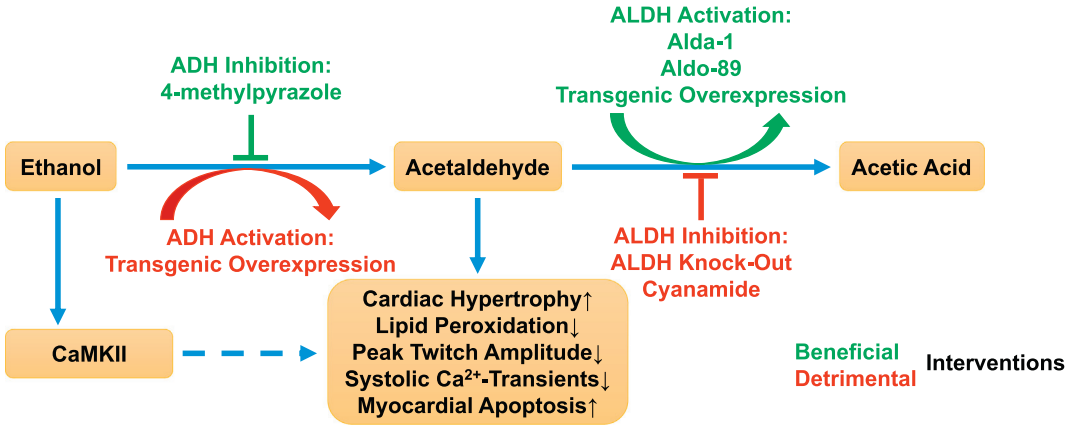

Fig. 2. Interventions in the degradation of ethanol can result in benefical or detrimental effects. Ethanol is metabolized in the liver (but also other organs, such as the heart) into acetaldehyde (ACA) by alcohol dehydrogenase (ADH). ACA is further metabolized into acetic acid by acetaldehyde dehydrogenase (ALDH). Interventions, which have been shown to result in beneficial downstream effects are shown in green. Interventions that have been shown to aggravate ethanol toxicity are shown in red. Interestingly, most ACA effects could also be explained by direct activation of Ca/calmodulin-dependent kinase II (CaMKII). However, while in vitro data have demonstrated beneficial potential of CaMKII-inhibition, in vivo assessment is lacking.

2.1. Excitation-contraction coupling and cellular electrophysiology

Ethanol exposure has long been known to be proarrhythmogenic, perhaps most prominently in the form of the "holiday heart syndrome", acute atrial fibrillation induced by one – or more – episode(s) of binge drinking (Ettinger et al., 1978). A large body of both clinical (Guzzo-Merello et al., n.d.; Brunner et al., 2017; Ettinger et al., 1978; Perkiömäki et al., 2016; Voskoboinik et al., 2016) and experimental (Anadon et al., 1996; Gao et al., 2012; Mustroph, Wagemann, Lebek, et al., 2018; Voskoboinik et al., 2016; Yan, Thomson, et al., 2018) evidence shows that even acute alcohol consumption increases the propensity for both atrial and ventricular arrhythmias (while a few experimental studies using animals suggested antiarrhythmic effects of small amounts of ethanol (Gao et al., 2012; Yang et al., 2017)). The holiday heart syndrome, specifically, can affect people who do not regularly consume ethanol (Tonelo et al., 2013), but also affects patients with chronic ethanol abuse (2-fold increase in AF incidence) (Voskoboinik et al., 2016; Whitman et al., 2017). Indeed, atrial fibrillation occurs in 6 of 10 binge drinkers, independent from underlying myocardial diseases (Ettinger et al., 1978; Frost & Vestergaard, 2004; Mukamal, Tolstrup, Friberg, Jensen, & Grønbaek, 2005).

Additionally, there are in vivo and in vitro data showing a negative inotropic effect after acute ethanol exposure in humans and animals (Cheng, Shihabi, & Little, 1990; Delgado, Gortuin, & Ross, 1975; Gould et al., 1971; Ibrahim, Fan, & Abdel-Rahman, 2014; Kelbaek et al., 1988; Kelbaek, Gjørup, Brynjolf, Christensen, & Godtfredsen, 1985; Nakano & Moore, 1972). While multiple investigations have shown decreased cell shortening and systolic Ca^{2+} release or detrimental effects on cardiac contractile function overall after acute ethanol exposure (Blomqvist, Saltin, Mitchell, & Vastagh, 1970; Child, Kovick, Levisman, & Pearce, 1979; Ge, Guo, & Ren, 2011; Guo & Ren, 2012; Guo, Scott, Ren, & Involvement of AMPK in Alcohol Dehydrogenase Accentuated Myocardial Dysfunction Following Acute Ethanol Challenge in mice, 2010; Mustroph, Wagemann, Lebek, et al., 2018), the precise mechanisms were thus far poorly understood.

Excitation-contraction coupling is the highly coordinated process linking electrical excitation of the cardiomyocyte to its mechanical contraction. Upon electrical excitation of the cell, voltage sensitive Na^+ channels ($Na_V1.5$) open, leading to depolarization of the cell membrane. This in turn causes the voltage-gated L-Type sarcolemmal Ca^{2+} channels to open, allowing Ca^{2+} influx into the dyadic cleft. The RyR2 is Ca^{2+} sensitive at the luminal side, so that this local increase of cytosolic Ca^{2+} leads to opening of the RyR2 with much larger amounts of Ca^{2+} being released from the SR ("Ca^{2+}-induced Ca^{2+} release"). The resulting rise of intracellular Ca^{2+} causes Ca^{2+} binding of troponin and triggers contraction of the myofilaments. Cytosolic Ca^{2+} is the eliminated back into the SR by reuptake via the SR Ca^{2+}-ATPase (SERCA2a, accounting for about 70% of Ca^{2+} elimination in healthy hearts) or extruded back into the extracellular space via the Na^+/Ca^{2+}-exchanger (NCX, accounting for about 28% in healthy hearts). Please note that the latter process is electrogenic, as 3 Na^+ ions enter the cell in exchange for 1 Ca^{2+} ion being eliminated, thus potentially transiently depolarizing the cell membrane to a certain extent. Also note, that even after deactivation of the $Na_V1.5$, a persistent Na^+ current can remain, the so called late Na^+ current. Although this current is small, it can lead to significant Na^+ influx into the cell due to its long persistence. (For more detailed reviews of excitation-contraction coupling and its pathologic remodeling in heart failure, refer to e.g. (Bers, 2002; Eisner, Caldwell, Kistamás, & Trafford, 2017; Neef & Maier, 2007; Neef & Maier, 2013; Voigt, Nattel, & Dobrev, 2012).

Ca^{2+}/calmodulin-dependent kinase II (CaMKII) has emerged as a key player in cardiac arrhythmogenesis and heart failure development (Anderson, Brown, & Bers, 2011a; Fischer, Neef, & Maier, 2013; Mustroph, Neef, & Maier, 2017; Neef et al., 2010; Neef et al., 2013; Neef, Mann, Zwenger, Dybkova, & Maier, 2017; Pellicena & Schulman, 2014). Evidence by independent groups has recently emerged that CaMKII is also centrally involved in atrial and ventricular arrhythmogenesis and contractile dysfunction upon *acute* ethanol exposure (Mustroph, Wagemann, Lebek, et al., 2018; Yan, Thomson, et al., 2018):

Our group could recently show increased CaMKII activation in isolated ventricular cardiomyocytes upon 3‰ and 6‰ ethanol, as assessed by CaMKII autophosphorylation at T286/287 and CaMKII-dependent phosphorylation of phospholamban (Mustroph, Wagemann, Lebek, et al., 2018), a canonical CaMKII target (Ai, Curran, Shannon, Bers, & Pogwizd, 2005; Koss & Kranias, 1996). CaMKII activation following acute ethanol was independently validated in HEK293 cells by Yan, Thomson, et al. (2018).

Yan et al. showed an increased incidence of atrial fibrillation in ethanol-exposed Langendorff-perfused isolated human atrial preparations subjected to electrical burst pacing (Yan, Thomson, et al., 2018), which they could also replicate in vivo in rabbits and mice. This suggests that there are direct cardiac effects of ethanol on cardiac tissue that induce arrhythmogenesis. In accordance, our group recently showed that ethanol induces arrhythmogenic Ca^{2+}-waves in isolated cardiomyocytes. Downstream of CaMKII, we found increased sarcoplasmic reticulum (SR) Ca^{2+}-leak in isolated human atrial cardiomyocytes, which has been associated with Ca^{2+} waves and can cause arrhythmias

via activation of the electrogenic sodium-calcium-exchanger (NCX) (Mustroph, Wagemann, Lebek, et al., 2018). Dose-dependency of ethanol-induced SR Ca^{2+} leak was demonstrated by us in murine atrial and ventricular cardiomyocytes (Mustroph, Wagemann, Lebek, et al., 2018). This SR leak led to significantly more delayed afterdepolarizations (DADs) in murine atrial cardiomyocytes (Mustroph, Wagemann, Lebek, et al., 2018). DADs are known as important triggers of arrhythmias in vitro and in vivo (Chen et al., 2001; Neef et al., 2010; van Oort et al., 2010). Thus, CaMKII-mediated SR Ca^{2+} leak could be an important mechanism for ethanol-induced arrhythmias. Importantly, pharmacological inhibition of CaMKII using the specific autocamtide-related inhibitory peptide (AIP) could significantly reduce SR Ca^{2+} leak in ventricular cardiomyocytes (Mustroph, Wagemann, Lebek, et al., 2018). Yan et al. could also show increased SR Ca^{2+} leak upon acute ethanol exposure in isolated rabbit and HL-1 atrial cardiomyocytes, which they could prevent using the allosteric CaMKII-inhibitor KN-93 (validated versus its inactive analogue KN-92 because of off-target effects of KN-93) (Yan, Thomson, et al., 2018). Of note, this important finding of ethanol-induced SR Ca^{2+} leak was verified by both groups using two different methodologies (Ca^{2+} sparks and tetracaine-sensitive Ca^{2+} shift) (Mustroph, Wagemann, Lebek, et al., 2018; Yan, Thomson, et al., 2018).

Moreover, we could show that acute ethanol-exposure reduces systolic Ca^{2+} release ("Ca^{2+} transients"). During systole, free cytosolic Ca^{2+} leads to contraction of the myofilaments. In line with these data, cardiomyocyte contractility was dose-dependently reduced by ethanol and also the developed force of intact human atrial trabeculae was severely impaired (while our investigation of myofilament Ca^{2+} sensitivity indicated that myofilament desensitization contributed to this, as discussed below). (Mustroph, Wagemann, Lebek, et al., 2018)

Besides Ca^{2+} dependent activation, CaMKII can be activated non-canonically by oxidation at the methionine residues 281/282 (Anderson, 2015; Purohit et al., 2013). Induction of reactive oxygen species (ROS) by ethanol has been repeatedly shown, importantly in both atrial and ventricular cardiomyocytes (Brandt et al., 2016; Mustroph, Wagemann, Lebek, et al., 2018). Thus, it appears reasonable that oxidative activation of CaMKII might be involved in ethanol-induced pathologic CaMKII signaling. Indeed, in our research, transgenic knock-out of the main ROS-producing cardiac enzyme, NADPH-oxidase II (NOX2 KOs), prevented CaMKII activation and CaMKII-dependent phospholamban phosphorylation in isolated ventricular cardiomyocytes exposed to ethanol (Mustroph, Wagemann, Lebek, et al., 2018). Furthermore, NOX2 KO consistently prevented the induction of SR Ca^{2+} leak by acute ethanol exposure (Mustroph, Wagemann, Lebek, et al., 2018). Also, Ca^{2+} transients were not decreased by ethanol concentrations even up to 6‰ in atrial and ventricular cardiomyocytes from NOX2 KO mice, in contrast to murine wildtype cells. Interestingly, Brandt et al. found that NOX2 expression is increased in patients with DCM and a history of ethanol abuse (compared to DCM without ethanol abuse). Similarly, they could show increased expression of several NOX2 subunits and NOX2 activators in isolated murine cardiomyocytes cultured with ethanol or the ethanol metabolite acetaldehyde (ACA). (Brandt et al., 2016) This suggests that increased NOX2 activity and consequently increased ROS production may also be important in chronic ethanol toxicity and the development of heart failure.

Mutation of CaMKII residues M281/282 (see above) to valine renders the kinase oxidation resistant to oxidative activation (CaMKII-MMVV mutation (Anderson, 2015; Erickson et al., 2008; Purohit et al., 2013)). We could show that in cardiomyocytes from mice with this oxidation-resistant CaMKII, induction of SR Ca^{2+} leak by acute ethanol-exposure (1–6‰) is completely prevented. Furthermore, systolic Ca^{2+} transients in both atrial and ventricular CaMKII-MMVV cardiomyocytes were not diminished by acute ethanol exposure even at 6‰ ethanol. (Mustroph, Wagemann, Lebek, et al., 2018) Thus, the oxidative activation of CaMKII by ROS generated via NOX2 appears to be a central mechanism for the acute detrimental alterations of Ca^{2+} homeostasis induced by ethanol.

Similar to us, Yan et al. found that acute ethanol exposure leads to SR Ca^{2+} leak in atrial cardiomyocytes (HL-1 cells), which was CaMKII-dependent (i.e. prevented by inhibition with the experimental CaMKII-inhibitor KN-93) (Yan, Thomson, et al., 2018). However, they propose another mechanism by which CaMKII becomes activated upon ethanol:

Li et al. found already in 2009 that the expression and activation of c-Jun N-terminal kinases (JNK) increased after chronic ethanol exposure (Li, Gilbert, Li, & Ren, 2009). Recent research found activation of CaMKII downstream of JNK2 (Yan et al., 2018). Yan et al. expressed human CaMKII in HEK293 cells and found increased CaMKII activity (T287 autophosphorylation) upon 24 h of ethanol. When they expressed oxidation resistant CaMKII (CaMKII MMVV mutation), ethanol could still induce CaMKII-T287 autophosphorylation, although to a lesser extent than in cells expressing WT-CaMKII. (Yan, Thomson, et al., 2018) Thus, there appear to be at least two different pathways by which CaMKII is activated downstream of ethanol. However, the oxidative activation of CaMKII seems to be necessary for the pathological effects of ethanol on disturbed excitation-contraction coupling and arrhythmogenesis, as knock-out of the ROS-producing enzyme NOX2, the transgenic mutation of the CaMKII oxidation site Met281/282, or ROS-scavenging with *N*-acetylcysteine consistently prevented SR Ca^{2+}-leak and contractile dysfunction in our murine and human experiments respectively (Mustroph, Wagemann, Lebek, et al., 2018).

Importantly, and unanimously, the data by Yan et al. (Yan, Thomson, et al., 2018) and our group (Mustroph, Wagemann, Lebek, et al., 2018) (at least on the cellular level) suggest that increased CaMKII activation and subsequent CaMKII-dependent SR Ca^{2+} leak could to be *the* major factor in atrial and ventricular arrhythmogenesis upon acute ethanol exposure (Mustroph, Wagemann, Lebek, et al., 2018; Yan, Thomson, et al., 2018). Noteworthy, this relevance of CaMKII seems to extend beyond the *acute* setting, as two other groups could demonstrate increased cardiac CaMKII activation in mice (Guo, Hu, Kandadi, & Ren, 2012) and an increased cardiac CaMKII expression in rats (Heshmati, Shirpoor, Kheradmand, Alizadeh, & Gharalari, 2018) also upon *chronic* ethanol consumption.

Ca^{2+} release from the SR during systole leads to contraction of the myofilaments (by Ca^{2+} binding to troponin). Myofilament regulation and myofibrillar structure are likely involved in cardiac ethanol toxicity. In chronic ethanol exposure in rodents, myofibrillar structure has been shown to be disturbed, with reductions in myosin and actin expression (Fogle et al., 2010). Furthermore, recent studies have identified several genetic abnormalities that predispose for ACM. Ware et al. showed in 141 patients with ACM that titin truncating variants were increased about 4.6-fold compared to control, but similar to patients with DCM (Ware et al., 2018). Moreover, patients with DCM and a titin truncating variant had a nearly 10% reduction in ejection fraction when they additionally consumed high levels of alcohol (Ware et al., 2018).

We could recently show that acute ethanol exposure decreases cardiomyocyte myofilament Ca^{2+} sensitivity (Mustroph, Wagemann, Lebek, et al., 2018). This is likely an effect of acute CaMKII activation and CaMKII-dependent myofilament regulation (speculatively e.g. by titin phosphorylation (Hamdani et al., 2013)), as myofilament Ca^{2+} response was normal, when either ROS production was genetically inhibited (NOX2 KO) or when oxidation of CaMKII was prevented by transgenic modification of CaMKII (CaMKII-MMVV mouse model) (Mustroph, Wagemann, Lebek, et al., 2018).

Altered action potentials and also early and delayed afterdepolarizations (EADs and DADs) are involved in the development of arrhythmias (Fischer et al., 2013; Mustroph et al., 2017). As already discussed, DADs were observed in isolated cardiomyocytes acutely exposed to ethanol and were attributed to SR Ca^{2+} leak (Mustroph, Wagemann, Lebek, et al., 2018).

Prolongation of cardiac repolarization (i.e. longer corrected QT interval (QTc) in EKG) is associated an increased propensity for torsade de pointes tachycardia. While chronic effects of binge drinking episodes

on QTc are marginal (i.e. 3–4 ms (Zhang et al., 2011)), acute intoxication leads to clinically relevant prolongation of QTc (13–23 ms (Rossinen, Sinisalo, Partanen, Nieminen, & Viitasalo, 1999)). However, action potential duration (APD) is reported to be even shorter upon ethanol (Carryl, Gallardo-Carpentier, & Carpentier, 1992; Williams, Mirro, & Bailey, 1980). Underlying this might be reduced sodium (I_{Na}) and calcium (I_{Ca}) currents, which have been reported following ethanol infusion over 5 days in rabbit atria (Laszlo et al., 2009). The discrepancy between prolonged QTc and shorter APD has been attempted to be resolved by simulation studies (Bébarová, Hořáková, & Kula, 2017; Pásek, Bébarová, Christé, Šimurdová, & Šimurda, 2016), which suggested that the dose-dependent effect of ethanol on APD (with lengthening of APD at low, but shortening at high ethanol concentrations) might be due to modulation of the repolarizing potassium current I_{Kr}. But conclusive experimental results resolving this issue are lacking. Cardiac effective refractory period, however, has been reported to be shortened by ethanol in clinical studies (Gould, Ramana Reddy, Becker, Oh, & Kim, 1978).

2.2. The role of acetaldehyde in the cardiotoxicity of ethanol

Acetaldehyde (ACA), a key product of ethanol metabolism, is also critically involved in the cardiotoxic effects of ethanol. Ethanol is first oxidized by alcohol dehydrogenase (ADH) to ACA and then further oxidized to acetic acid by aldehyde dehydrogenase (ALDH) (Leibing & Meyer, 2016; Liu et al., 2018; Ren, Davidoff, & Brown, 1997; Zhang & Ren, 2011). This process takes place predominantly in the liver, but not exclusively so, as ADH has also been found in cardiomyocytes (Brandt et al., 2016).

There are multiple investigations showing exacerbated cardiotoxic effects of ethanol due to increased ACA concentration. ACA, similar to ethanol, has been shown to reduce peak twitch amplitude, systolic Ca^{2+}-transients, but also caffeine-induced Ca^{2+}-transients (SR Ca^{2+} load) in rat ventricular cardiomyocytes (Ren et al., 1997). Duan et al. used a transgenic mouse overexpressing ADH specifically in the heart, which induced increased cardiac concentrations of ACA after acute and chronic ethanol ingestion (Duan et al., 2002; Hintz et al., 2003). They demonstrated a pronounced cardiac reduction of cell shortening and intracellular Ca^{2+} transients after both acute and chronic ethanol exposure in these mice (Duan et al., 2002; Hintz et al., 2003). Accordingly, in presence of either the ADH-inhibitor 4-methylpyrazole or the ALDH inhibitor cyanamide, the ethanol-induced damage was reduced or increased, respectively (Duan et al., 2002). ADH overexpressing mice exhibited further increased cardiac hypertrophy, lipid peroxidation, and protein damage after chronic ethanol exposure (Hintz et al., 2003; Li & Ren, 2008).

Increased concentrations of ACA occur, when either ADH is highly active or ALDH is inhibited (Duan et al., 2002). Consequently, increasing the activity of ALDH might be a promising possibility to counteract the detrimental effects of ethanol exposure (Ge et al., 2011; Ma, Li, Gao, & Ren, 2009). Indeed, mice overexpressing mitochondrial ALDH2, that consequently showed reduced levels of ACA after ethanol exposure (Ma et al., 2009), were protected against severe disturbance of cardiac contractile function, myocardial apoptosis, and enlargement of left ventricular end-systolic and end-diastolic parameters after acute ethanol exposure, compared to control mice, while the ethanol effects on Ca^{2+} transients and SERCA function were also diminished (Ge et al., 2011; Ma et al., 2009).

These findings suggest that ACA, the product of ADH and the substrate of ALDH, might play an important role in the cardiotoxicity of ethanol. Overall, ALDH2-activation seems to be cardioprotective in the context of increased ethanol levels, possibly due to decreased accumulation of ACA. This suggests that a decreased activity of ALDH2 may exacerbate the negative effects of ethanol. Consistently, ALDH2 knock-out mice showed increased levels of ACA and thus an even more pronounced depression of cardiac contractile function upon ethanol compared to wildtype mice, while similar results were obtained by ALDH2-inhibition with cyanamide (Ma et al., 2010).

Importantly, chronic ethanol-induced activation of CaMKII was even more pronounced in ADH overexpressing mice compared to wildtype mice (Guo et al., 2012), while ALDH2 activation reduced levels of ACA and resulted in decreased CaMKII activation (Woods et al., 2016). Thus ACA appears to be involved in CaMKII activation, while the exact mechanisms remain unclear. We deem this of key importance, since most acetaldehyde mediated cardiotoxic effects of ethanol could also be explained by CaMKII activation and consequently downstream CaMKII-dependent effects (Anderson, Brown, & Bers, 2011b; Mohler & Hund, 2011; Mustroph et al., 2017). Importantly, CaMKII – besides (dis-)regulating excitation-contraction coupling – has also been linked to several cardiac transcriptional pathways ("excitation-transcription coupling") by phosphorylation of cardiac gene regulators (especially in the HDAC/MEF2-axis), as recently reviewed in detail in (Dewenter, von der Lieth, Katus, & Backs, 2017). However, whether this occurs in alcoholic cardiomyopathy, remains to be investigated.

2.3. Ethanol induces oxidative stress

Recent investigations have suggested that increased oxidative stress, e. g. ROS, plays a key role in the development of alcoholic cardiomyopathy. Alcohol is known to disturb mitochondrial function, leading to decreased mitochondrial membrane potential and increased production of mitochondrial superoxide levels (Steiner & Lang, 2017). Research by Brandt et al. using their ALDH2-deficient mouse model showed that the most prominent increase in mitochondrial superoxide levels is not due to ethanol, but due to ACA (Brandt et al., 2016). This suggests that slowed degradation of ACA or high levels of ethanol leading to increased degradation into ACA should aggravate ethanol toxicity.

Brandt et al. found that the ethanol- and acetaldehyde (ACA)-induced increase in superoxide (O_2^-) levels was abolished by inhibition of NOX2 (with diphenyleneiodonium) (Brandt et al., 2016), which is commonly regarded as the most important source of ROS in cardiomyocytes (Thannickal & Fanburg, 2000). Cardiomyocytes cultured with ethanol or ACA showed an increased mitochondrial ROS production (by trend for ethanol and strongly for ACA), that could be lowered by NOX2 inhibition with aponycin. Consistently, crossbreeding of their ALDH2-deficient mouse line with NOX2-knock out mice led to a clear protection from chronic ethanol induced heart failure that they had previously observed in the ALDH2-deficient model (Brandt et al., 2016). Increased cardiac NOX activity and ROS production was also observed in rats after acute ethanol exposure, that had developed reduced left ventricular function (Yao & Abdel-Rahman, 2017).

Noteworthy, ROS are not confined to the mitochondria, as experiments by Brandt et al. assessing ROS concentrations in murine cardiomyocytes using the mitochondrial ROS dye MitoSox had shown a pronounced staining also of non-mitochondrial compartments (Brandt et al., 2016). Indeed, while in line with previous findings, they observed upregulation of NOX2 upon ethanol exposure, the only other isoform of NOX expressed in cardiomyocytes, NOX4 (which is expressed primarily in the mitochondria) appeared not to be involved (Brandt et al., 2016) (this is in contrast to the liver, where NOX4 seems to be upregulated upon ethanol (Sun, Zhang, Zhong, Sun, & Zhou, 2017)). Yet, other mitochondrial sources exist besides NOX4, such as monoamin oxidases (Nina et al., 2010). Thus, the exact compartment that mainly contributes to the ethanol-induced ROS generation is not yet clear. However, our group was able to confirm increased cytosolic [ROS] in isolated cardiomyocytes upon ethanol using the cytosolic ROS dye CMH_2DCFDA (Mustroph, Wagemann, Lebek, et al., 2018). Interestingly we could show that knockout of NOX2 protects from ethanol-induced SR Ca^{2+}-leak and contractile dysfunction, as discussed above, while we could also show that ROS-scavenging with *N*-acetyl-cysteine prevents the ethanol-induced impairment of human cardiac muscle strip contractility (Mustroph, Wagemann, Lebek, et al., 2018).

As mentioned above, ROS have been shown to activate CaMKII through oxidation at M281/282 and transgenic mutation of CaMKII at M281/M282 (CaMKII MMVV) renders CaMKII oxidation resistant (Erickson et al., 2008). Our group could show that murine CaMKII MMVV cardiomyocytes are completely protected from ethanol-induced proarrhythmic SR Ca^{2+} leak and contractile dysfunction. Overall, increased cardiotoxicity due to increased ROS production upon ethanol or its metabolites appears to strongly induce the NOX2 – CaMKII axis, highlighting the potential therapeutic applications of either NOX2 inhibition, ROS scavenging or CaMKII inhibition.

The potential of therapeutic intervention in this axis was demonstrated in catalase overexpressing mice. Catalase is an enzyme that is involved in the degradation of ROS. In these mice, the acute ethanol-induced reduction of intracellular Ca^{2+}, myocyte fractional shortening, and prolongated intracellular diastolic Ca^{2+} reuptake were attenuated (Zhang et al., 2003).

2.4. Cardiac energy homeostasis and cardiac metabolism

Interestingly, chronic ethanol decreases the expression of a large number of proteins involved in energy metabolism, such as fatty acid metabolism proteins, glycolytic proteins and mitochondrial proteins involved in the Krebs cycle and ATP-production (Fogle et al., 2010). However, the physiological significance of these results from a proteomic study is still mostly unclear. Hu et al. observed increased levels of cardiac triglycerides and cardiac fatty acid ethyl esters in mice following chronic ethanol consumption, mainly because of an increased expression of long chain fatty acid transporters (Hu et al., 2013). This may also be the reason for the clinically observed accumulation of cardiac triglyceride in patients with alcoholic cardiomyopathy (Tsiplenkova, Vikhert, & Cherpachenko, 1986). Noteworthy, in the animal model investigated by Hu et al., cardiac triglycerides were negatively correlated with cardiac contractile function. Also in this model, downregulation of PGC-1α and downstream targets of the oxidative phosphorylation pathway was reported, which included important members from the electron transport chain and mitochondrial ATP synthase. This was associated with decreased ATP-level. Thus, downregulation of PGC-1α and its target genes results in reduced ATP generation, which in turn was found to correlate with impaired cardiac ejection fraction. (Hu et al., 2013)

As discussed below, the AMP-to-ATP ratio is increased (Guo et al., 2010) upon ethanol. Several proteins of the oxidative phosphorylation, including the electron transport and ATP synthase complexes of the inner mitochondrial membrane are down-regulated and cause reduced cardiac ATP levels (Hu et al., 2013). Similar results were obtained in rats following chronic ethanol intake that also exhibited decreased mitochondrial respiratory rates and phosphorylation efficiency, while the glucose metabolism, e. g. activities of glucose-6-phosphate dehydrogenase, aldolase, and glyceraldehyde phosphate dehydrogenase, were increased (Sardesai & Provido, 1978). However this finding is somewhat in contrast to a recent study that observed an impaired glucose metabolism, e. g. reduced activity of glyceraldehyde-3-phosphate dehydrogenase, following chronic ethanol exposure in mice (Yan et al., 2017). Others have found differential acute effect of ethanol on cultured neonatal rat cardiomyocytes, where levels of α and β subunits of ATP synthase are increased and ATP level remain unaltered (Mashimo, Arthur, & Ohno, 2015). Additionally, Li et al. showed altered cardiac glucose metabolism in chronic ethanol consumption, leading to dampened insulin signaling with glucose intolerance in general and reduced insulin-induced cardiac glucose uptake in mice (Li & Ren, 2008) and rats (Limin et al., 2009).

With respect to important recent findings on mitochondrial involvement in ethanol toxicity (especially ROS production), please refer to the section on oxidative stress. While changes in mitochondrial function have been reported in several studies, more recent studies did not replicate the mitochondrial injury upon chronic ethanol abuse that had previously been postulated (as nicely reviewed by Piano (2017)).

2.5. Ethanol induces autophagy and apoptosis

Previous investigations have found that also autophagy and apoptosis may be part of the cardiotoxicity of ethanol. AMP-activated protein kinase (AMPK) is known to be one of the central cellular energy sensors and regulators for glucose and lipid metabolism, but can also induce autophagy (Kim, Miller, & Young, 2009; Wang & Ren, 2018). Guo et al. observed increased AMP-to-ATP ratio after acute ethanol exposure and, consequently, AMPK was highly phosphorylated and activated in the murine myocardium (Guo et al., 2010). Interestingly, the impairment of isolated cardiomyocyte function induced by ethanol was strongly ameliorated in AMPK knock-out cells (Kandadi, Hu, & Ren, 2013) and absent upon AMPK-inhibition with Compound C (Guo et al., 2010).

In another follow-up study, Guo et al. could further delineate the cardioprotective effect of AMPK-inhibition after acute ethanol exposure (Guo & Ren, 2012). AMPK induced autophagy has been linked to inhibition of mammalian target of rapamycin complex 1 (mTORC1) by phosphorylation of the mTORC1-associated protein Raptor (Gwinn et al., 2008). Decreased activity of mTORC1 facilitates the activation of Unc51-like kinase (ULK1) and, as a consequence, autophagy induction (Kim, Kundu, Viollet, & Guan, 2011). Guo et al. found ethanol to stimulate Raptor and inhibit mTORC1, leading to an increased activity of ULK1 and thus autophagosome accumulation (Guo & Ren, 2012). These effects were attenuated by inhibition or knock-out of AMPK (Kandadi et al., 2013). Knock-down of ULK1 lead also to a reduced autophagosome formation and cell death (Kandadi et al., 2013).

Chronic exposure to ethanol leads to decreased phosphorylation of mTOR, Akt, AMPK, Notch1 and STAT3, further indicating an augmented autophagy formation (Ge & Ren, 2012). The precise cardioprotective effect of STAT3 is reviewed in (Leibing & Meyer, 2016). Interestingly, this signaling cascade was restored in ALDH2 overexpressing mice (see above) (Ge & Ren, 2012). In accordance, autophagy inhibition (chloroquine and 3-methyladenin) or ALDH2 agonism decreased ethanol-induced cardiac apoptosis and heart damage, offering therapeutic targets of ethanol-induced damage (Ge et al., 2011; Zhu et al., 2018).

Also, ROS elimination with *N*-acetylcysteine reduced ethanol-induced cardiac autophagy in vitro and in vivo (Zhu et al., 2018). Accordingly, ethanol-induced apoptosis could also be abrogated by NOX inhibition (apocynin) in H9c2 cells (Tan et al., 2012). Since metallothionein can scavenge both nitrative and oxidative stress, acute ethanol-induced autophagy and cell apoptosis is reduced in metallothionein overexpressing mice (Zhu et al., 2018). These mice were further protected against contractile dysfunction and Ca^{2+} mishandling following chronic ethanol consumption (Li & Ren, 2006).

Noteworthy, CaMKII, which appears to be intensely involved at least in acute ethanol effects as discussed above, is also involved in cardiac pro-apoptotic signaling (Feng & Anderson, 2017). Indeed, CaMKII was found to be a critical mediator of ethanol-induced cell death in neural progenitor cells in a chick embryonal model and cell death could be prevented by CaMKII-inhibition (Garic, Flentke, Amberger, Hernandez, & Smith, 2011). Yet, whether this also applies to adult cardiac cells remains to be shown and is currently under investigation by us.

3. Clinical outlook

Drinking cessation and prevention should obviously be the mainstay for the prevention and therapy of ethanol-associated diseases. Obviously, induction of *acute* arrhythmias and contractile dysfunction by ethanol would be prevented. There is however, also evidence that *chronic* pathologies involving ethanol strongly benefit if the patients then abstain. In alcoholic cardiomyopathy, left ventricular function was shown to recover following abstinence, but also when alcohol consumption was limited (20 to 60 g per day) (Masani et al., 1990; Guillo

et al., 1997; Nicolás et al., 2002). Moreover, patients with ACM had a reduced mortality after drinking cessation (Fauchier et al., 2000) and abstinence seems to be associated with a reduced frequency of arrhythmias (Fauchier, 2003).

However, drinking cessation is often times very difficult for patients and sometimes even more complicated due to genetic circumstances, like the variations and single nucleotide polymorphisms of the muscarinic acetylcholine receptor M2 gene that seem related to the severity of alcohol dependence (Jung et al., 2011; Luo et al., 2005). Also, while chronic ethanol abuse by patients with alcohol-dependence likely accounts for a large fraction of the patients with ethanol-induced cardiac disease, relatively moderate consumption and one-time only binge drinking can also cause arrhythmias and contractile dysfunction, as referenced earlier.

As of now, ethanol-induced cardiac pathologies are treated according to the general guideline standards for the phenotypic appearance of the condition (e.g. betablockers, ACE-inhibitors/AT2-antagonists, and mineralocorticoid receptor antagonists to treat heart failure), without addressing the underlying pathogenic mechanism. Thus therapies to prevent or specifically address cardiac pathologies induced by ethanol are needed.

While some experimental evidence suggest a therapeutic potential of AMPK inhibition (experimentally with Compound C (Guo et al., 2010)), such a strategy might be precarious in the face of the emerging role of AMPK in cancer progression (Chen et al., 2017). Also, the exact role of AMPK for cardiac pathologies is not well understood, as AMPK might in fact rather have a beneficial role in atrial fibrillation (Harada et al., 2015).

Promising strategies may include 1) reducing ACA levels (either by inhibiting its production or facillitating its metabolization), 2) the prevention of ethanol induced ROS-production, and 3) inhibition of cellular arrhythmias and contractile dysfunction by inhibition of CaMKII.

To achieve a reduction in ethanol toxicity through a reduction in ACA levels, inhibition of alcohol dehydrogenase (ADH), which results in a decreased production of ACA, may be a valuable therapeutic option (Leibing & Meyer, 2016; Liu et al., 2018; Ren et al., 1997; Zhang & Ren, 2011). However, this may prolong the toxicity of ethanol itself in the heart, but also in other organs. Presumably, this would also prolong the intoxicating effects of ethanol, with unknown consequences for ethanol addiction. Alternatively, activation of ALDH2 with Alda-1, leads to an accelerated ACA metabolism, ameliorating ethanol-induced cardiac damage (Münzel & Daiber, 2018). Additionally, there is also data demonstrating that ALDH3A1 activation with Aldo-89 may synergistically contribute to a decreased ethanol-induced damage (Chen, Cruz, & Mochly-Rosen, 2015). However, although there are multiple experimental studies showing beneficial effects of Alda-1 and cell-based therapy, there is still a lack of proof-of-concept in clinical studies for both (Münzel & Daiber, 2018). Therefore, more research is needed to find highly selective inhibitors with suitable bioavailability and to test them in vivo and in clinical trials.

As ethanol and ACA can induce ROS (Brandt et al., 2016; Mustroph, Wagemann, Lebek, et al., 2018), the specific mechanisms by which both induce ROS generation could be therapeutically targeted, e. g. by inhibiting NOX2. Novel small molecule inhibitors of NOX2 were recently described (Hirano et al., 2015), however, NOX2 inhibition, as of yet, remains a domain of basic research. Indeed, while in vitro studies have repeatedly suggested that ROS play criticial roles in cardiovascular pathologies, clinical trials employing antioxidative strategies (such as the HOPE trial (Lonn et al., 2002) or PHS II (Sesso et al., 2012)) have largely failed to show a clinical benefit (for an in-depth review of this discrepancy, refer to (Goszcz et al., 2015)). Yet, while, global systemic antioxidative therapeutic approaches with *N*-acetyl cysteine for pathologies involving ROS have often failed in the clinic (Null et al., 2015; Szakmany, Hauser, & Radermacher, 2012), *N*-acetyl cysteine in our opinion might still have some promise in ethanol induced heart disease. While acetylcysteine itself is only a mild antioxidant, its ability to enhance the generation of the endogenous antioxidant GSH, which is depleted in alcoholism (Hajnóczky, Buzas, Pacher, Hoek, & Rubin, 2005), might make it a promising candidate especially in ethanol abuse.

As data by Yan and our group show activation of CaMKII upon ethanol, leading to contractile dysfunction and arrhythmias (Mustroph, Wagemann, Lebek, et al., 2018; Yan, Thomson, et al., 2018), and data from neural progenitor cells suggests involvement of CaMKII in ethanol-induced cell death (Garic et al., 2011), pharmacological inhibition of CaMKII appears to be a promising therapeutic option. Of note, recent research suggests that CaMKII overactivity is unaffected by betablocker treatment and that directly inhibiting CaMKII could be an independent, additional therapeutic strategy (Dewenter et al., 2017). However, the established CaMKII-inhibitors AIP (tested by our group (Mustroph, Wagemann, Lebek, et al., 2018)), as well as KN-93 (tested by Yan et al. (Yan, Thomson, et al., 2018)) are not orally applicable (Mustroph et al., 2017; Pellicena & Schulman, 2014), have extensive side effects (Pellicena & Schulman, 2014), and/or low tissue permeability (Pellicena & Schulman, 2014), and thus serve solely as research tools. Novel CaMKII-Inhibitors, however, that are ATP-competitive and have sufficient oral bioavailability are approaching trials and may soon offer new therapeutic options in the treatment of ethanol-induced arrhythmias and contractile dysfunction (Lebek et al., 2018; Mustroph et al., 2017; Neef et al., 2017; Neef, Mann, et al., 2017). Still, while these direct CaMKII inhibitors will hopefully be a valuable therapeutic option, clinical development and trials will expectedly still take years. Moreover, as CaMKII emerges as an important physiological regulator in many tissues besides the heart (as reviewed in depth in e.g. (Beckendorf, van den Hoogenhof, & Backs, 2018; Dewenter et al., 2017)), regulating e.g. memory formation (Ataei, Sabzghabaee, & Movahedian, 2015; Lisman, Yasuda, & Raghavachari, 2012), the immune system (Colomer & Means, 2007), and fertility (Backs et al., 2010), potential adverse effects of chronic CaMKII inhibition will have to be very carefully considered (for an in-depth review on CaMKII physiological roles refer to e.g.. An intriguing alternative drug to modulate CaMKII activity (without directly inhibiting CaMKII) that is already clinically available could be empagliflozin. This compound is primarily an antidiabetic drug, but was moreover recently shown to consistently lower CaMKII activity also in isolated non-diabetic human and murine myocardium, leading to reduced SR Ca^{2+}-leak and improved contractility (Mustroph et al., 2018). Further basic research and clinical tests will, however, be necessary to evaluate empagliflozin in acute and chronic ethanol toxicity (while the use of empagliflozin in heart failure with reduced ejection fraction independent of the existence of diabetes is currently under investigation in the EMPEROR trial (NCT03057977).

Conflict of interest statement

The CaMKII inhibitor RA123456 is currently tested by the authors in cooperation with Sanofi R&D. Rimacalib (SMP-114) was provided to our group by Dainippon Pharma. AS105 (& follow-up compounds) was tested by the authors in collaboration with Prof. Howard Schulman. The CaMKII inhibitor GS-680 was tested by the authors in cooperation with Gilead Sciences.

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