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## **Overview of the Components of Cardiac Metabolism**

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## Acronyms

adherens junction (AJ)

carnitine palmitoyltransferase (Cpt)

connexin (Cx)

cytochrome P450 (CYP)

delayed rectifier K<sup>+</sup> channel receptor (IKr)

dihydroxyeicosatrienoic acid (DHET)

epoxyeicosatrienoic acid (EET)soluble epoxide hydrolase (EH)

facilitative glucose transporter (GLUT)

human ether-a-go-go-related gene(hERG)

hydroxyeicosatrienoic acid (HETE)

Madin Darby canine kidney(MDCK)

organic anion transporter (OAT)

organic anion transporting polypeptide (OATP)

organic cation transporter (OCT)

organic carnitine transporter (OCTN)

pannexin (Panx)

sodium-glucose cotransporter (SGLT)

tight junction (TJ)

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## Abstract

Metabolism in organs other than liver and kidneys may play a significant role in how a specific organ responds to chemicals. The heart has metabolic capability for energy production and homeostasis. This homeostatic machinery can also process xenobiotics. Cardiac metabolism includes expression of numerous organic anion transporters, organic cation transporters, organic carnitine (zwitterion) transporters, and ATP-binding cassette transporters. Expression and distribution of the transporters within the heart may vary depending upon age, disease, endocrine status, and various other factors. Several cytochrome P450 (CYP) enzyme classes have been identified within the heart. The CYP hydroxylases and epoxygenases within the heart produce hydroxyeicoatetraneic acids (HETES) and epoxyeicosatrienoic acids (EETS), metabolites of arachidonic acid, which are critical in regulating homeostatic processes of the heart. The susceptibility of the cardiac CYP system to induction and inhibition from exogenous materials is an area of expanding knowledge as are the metabolic processes of glucuronidation and sulfation in the heart. The susceptibility of various transcription factors and signaling pathways of the heart to disruption by xenobiotics is not fully characterized, but is an area with implications for disruption of normal postnatal development as well as modulation of adult cardiac health. There are knowledge gaps in the timelines of physiologic maturation and deterioration of cardiac metabolism. Cross-species characterization of cardiac-specific metabolism is needed for nonclinical work of optimum translational value to predict possible adverse effects, to identify sensitive developmental windows, for the design and conduct of informative nonclinical and clinical studies and to explore the possibilities of organ-specific therapeutics.

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## Introduction

The extensive and expanding volume of information on the components of hepatic metabolism is now accompanied by a new perspective on extra-hepatic drug metabolism. In addition to drug metabolism in the kidneys, lungs, and gastrointestinal tract, it is recognized that other organs, including the heart, have the capacity for xenobiotic metabolism as well as the extensive processes involved in the endobiotic transformations necessary for homeostasis (Gervasini, 2004; Pavek, 2008; Stegman, 1982; Wu, 1997). While this is a minor portion of overall systemic metabolism, there is potential for local drug metabolism to be an important factor in both desired pharmacology and unwanted cardiotoxicity. This is especially important when considering populations that may be more vulnerable to adverse drug effects, such as children or the elderly.

Tremendous efforts have been devoted to characterizing the physiologic differences between infants, children and adults, but much remains to be investigated. Incomplete understanding of pediatric development may lead to significant difficulties in providing safe and effective medications for children. Simply extrapolating doses or systemic drug exposures for children based upon the adult clinical experience may lead to either lack of efficacy or increased risk from dosing that is inappropriate for the pediatric physiology. While dose extrapolation may include consideration of differences in maturation and metabolism between the two populations, it may not be possible to estimate effects upon developing body systems, especially when some effects may not become apparent for years or even decades after drug exposure.

Postnatal development of the heart encompasses changes in structure, proportions of the components, mechanisms of growth, metabolism for energy generation, local innervation, central nervous system regulatory pathways, local receptor density and distribution, local transmitter synthesis and storage, ion channel development, electrical conduction development, and the interface with renal (maturation of renin-angiotensin-aldosterone axis) and endocrine development (involvement of the thyroid axis and insulin in cardiac maturation). The same nexus of development that

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integrates signaling pathways from spatially separated organs primarily processes endogenous substances such as hormones, fatty acids, glucose, prostaglandins, and other substances for homeostasis. The receptors, transporters, sensors and enzymes of metabolism may be developmentally regulated, and they may in turn help to coordinate postnatal development by their roles in communication between organ systems.

At any age, variability of expression and/or function of transporters and metabolic enzyme systems may contribute to observed variability of response to a therapeutic agent. Another important factor is the role of the heart in several endocrine axes. The heart demonstrates endocrine function in the regulation of blood pressure and volume involving distant communication with kidneys, adrenal glands, and vascular smooth muscle cells through the renin-angiotensin-aldosterone system as well as cardiac-initiated signals from the natriuretic peptides and endogenous neural signals (Brownsey, 1997; McGrath et. al., 2005). Insulin and thyroid hormones have been indicated in the postnatal development of the heart as well as having effects on enzymes, transporters, and protein expression throughout life (Barreto-Chaves, 2009; Ock, 2016; Krüger, 2008; Jonker and Louet, 2016). Many of the molecules involved in metabolism within the heart appear to work in concert with protein partners, a phenomenon that appears to create and maintain coordination within and between cells (Meens, 2015; Brownsey, 1997). The insulin receptor is one example of a coordination of signaling proteins with the receptor producing a localized cardiac effect (Brownsey, 1997). Another example is the role of the thyroid in most aspects of cardiovascular function. Thyroid hormone effects are mediated directly by thyroid hormone receptors in the heart and indirectly via factors including the autonomic nervous system, the renin-angiotensin-aldosterone system, and renal function. The phosphorylation/activation of phosphoinositol 3-kinase, protein kinase B (akt) and mammalian target of rapamycin (mTOR) are mediators of thyroid hormone involvement in cardiomyocyte protection, growth and maturation, including developmental protein isoform shifts (reviewed in Grais and Sowers, 2014; Kuzman et. al. 2005; Krüger et. al. 2008; Kenessey and Ojamaa, 2006; Dillman, 2010; Chattergoon et. al. 2012). The multitude of proteins within the heart and the developmentally regulated shifts that occur throughout life, make the morphologic status of the heart a component of the metabolic milieu.

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In drug development, the biological fate of a xenobiotic is determined by absorption, distribution, metabolism, and excretions, topics that we address in this paper based upon the current literature findings. In addition to this paradigm, there are many factors to be considered in choosing an animal species for nonclinical studies in cardiac physiology and pharmacology. The relevance of those results in guiding clinical studies or drug development depends on knowledge of the similarities and differences across species. Detailed understanding of cardiac metabolism offers the possibility of targeted, organ specific therapy, or conversely, avoiding cardiac-specific toxicology. This includes both direct adverse effects of a therapeutic as well as indirect effects caused by disruption of metabolism needed for homeostasis or normal development. Thus, knowledge of cardiac metabolism has the potential to improve therapeutic safety for both children and adults. Finally, nonclinical studies conducted with a clear understanding of cardiac metabolism in different species will enhance both the translational value of such work supporting the standards of the three Rs: reduce, refine, and replace. The focus of this manuscript is compilation of the current understanding of metabolism endogenous to the heart. Part of this endeavor includes identification of data gaps.

## **Absorption**

### *Endothelium*

Any systemically available xenobiotic and associated metabolites will circulate in the blood to some extent and pass through at least the right atrium and right ventricle. The drugs not absorbed in the lungs, as well as the metabolites generated there and released into circulation, will pass through the left atrium and left ventricle. Absorption of xenobiotics into the heart will be regulated by the same principles that apply elsewhere, such as the cellular structure of the heart, presence of transporters, receptors and enzymes, the functional or maturational state of the heart, and the

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physicochemical properties of the drug. Two important considerations for cardiac metabolism are the role of the endothelial junctions and tight junctions in intracellular signaling pathways and the role of developmentally regulated protein isoforms in endothelial regulation of permeability.

The endothelium is one of the controlling factors for the movement of plasma components to the interstitium, with a number of factors contributing to the efficacy as a selective barrier. That is, while there will be molecular and time-frame variations across species, the shared, non-species-specific features of the absorption barrier include characteristics of the endothelial cells themselves, the interendothelial junctions, transport pathways, and their role in myocardial metabolism (Bazzoni and Dejana, 2004). Endothelial cells in general, across species, are heterogenous populations, varying with location. Within the heart, the different groups of endothelial cells include those of the endocardium, coronary arteries and veins (not discussed here as this is beyond the scope of this manuscript), and myocardial capillaries.

Endothelial cells of the endocardium are larger than endothelial cells of other cardiac locations, with increased surface area comprised of microvilli. The intercellular clefts are deeper, more tortuous, have more gap junctions and fewer vesicles compared to the endothelial cells of the myocardial capillaries. As described by Katz (2013), Poiseuille's law regulates molecular movement through the endothelium. Hydraulic conductivity is dependent upon fluid viscosity and pore radius of the permeability pathways. The permeability pathways include the small pores (4 nm) of capillary endothelium, large pores (25-80 nm) occurring between endothelial cells, endothelial vesicles, and transendothelial pores (Katz, 2013).

The capillaries of the myocardium have continuous endothelium with tight junctions between the endothelial cells and close contact with cardiomyocytes. There is a ratio of 3 myocardial endothelial cells to 1 cardiomyocyte, separated by a distance of 1 $\mu$ m. The high proportion of endothelial cells to cardiomyocytes and close apposition facilitates transfer of blood-borne substances (Hsieh, 2006).

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In addition to the endothelial cells there are other components contributing to selective passage of molecules to and through the heart. These components include the tight junctions(TJ), adherens junctions(AJ), connexins, and pannexins. There is ongoing characterization of the roles of each of these in cardiac metabolism. The totality of understanding is therefore still evolving.

Across mammalian species, the primary components of interendothelial junctions are tight junctions and adherens junctions, promoting adhesion and restricting passage of substances circulating in the blood. These differ from the cardiac gap junctions that facilitate passage of solutes and small molecules between cells, discussed below in

**Distribution.** The endothelial connections of AJ and TJ require interaction with transmembrane proteins. These proteins bind to cytoskeletal and signaling proteins, ultimately connecting to actin filaments, linking junctional porosity to some degree with contractility (Dejana, 2004; Aird, 2007; Hsieh, 2006). Developmentally regulated protein isoforms and the maturational state of gap junction complexes determine myocardial contractility.

Various signaling pathways, described in detail elsewhere and beyond the scope of this paper, also contribute to regulation of endothelial junction permeability. Modulators to permeability include lipopolysaccharide, thrombin, and vascular endothelial growth factor (VEGF). The endothelial junctions, AJ and TJ, have been shown also to be involved in cell growth, apoptosis, and gene expression (Yoon, 2014; Vandenbroucke, 2008).

Passage across or into an endothelial cell includes a transcellular pathway (caveolae-mediated transcytosis or active transport) or the paracellular pathway of passive movement through the intercellular space between adjacent endothelial cells, e.g., lipophilic molecules traverse primarily by passive diffusion. The transcellular/caveolae pathway may require the involvement of receptors, pumps, or transport proteins, described below.

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*Connexins*

In addition to endothelial cells, connexins (Cx) are also components of the blood vessel wall. Cxs, found in both gap junctions and the blood vessel wall, are membrane proteins found throughout the body. Six Cx subunits can form a connexon or hemi-channel in the plasma membrane of the cell. This connexon can then appose to the connexon of an adjacent cell to form a gap junction channel. In blood vessels, the connexons allow for intercellular communication between endothelial cells, smooth muscle cells and myoendothelial coupling. Within the heart overall, connexons are primarily recognized for permitting cell to cell, nonspecific passage of ions (Makowski, 1984; de Wit and Griffith, 2010). Transgenic mice have been used for elucidation of the role of connexins in arrhythmias and conduction disorders (Verheule and Kaese, 2013). Recent work examines the role of connexins in disturbances of the cardiac electrical conduction system (Santa Cruz et. al., 2015).

Several different Cxs have been identified to date in human, mouse, rat, dog, and rabbit hearts (Coppin, 1999; Coppin, 2001). A recent addition to this list is Cx26 (Moscato et.al., 2018). Cx40, Cx43, Cx44, and Cx45 are the primary signature proteins of gap junctions, endocardium, coronary vessels and aorta, but have not been identified in myocardial capillaries. Cx37 has been detected in endothelial cells (Gros and Jongma, 1996). Various studies have shown that the temporal and spatial expression of each protein as well as abundance of each protein within the cardiac tissue may vary among species. Coppin and colleagues (1999) investigated the spatial expression pattern of Cx40, Cx43, and Cx45 in rabbits using dual-channel scanning confocal microscopy and determined that Cx40 and Cx45, but not Cx43 were expressed in the central SA node. Another investigation of immunolabeled Cx40, Cx43, and Cx45 in BALB/c mice, from embryonic day 12.5 to adult, determined that Cx45 is the earliest detectable connexin in the central conduction system and, in addition, is the only connexin expressed throughout the entire conduction system (Coppin, 2001). Another study found Cx43 to be the predominant Cx throughout the rat heart based on quantity, found on myocytes of both atria and ventricles (Van Kempen, 1996). This study also found that expression of Cx43 in the rat is uniform at birth and changes to punctate during the postnatal period. There is also some expression in fibroblasts and the electrical conduction system of humans (Purkinje fibers and intercalated discs), mice and rats (Chen, 1994;

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Gourdie, 1993). Cx43 is also found in cardiomyocyte mitochondria of mouse, rat, pig, and human origin, possibly associated with cell death and survival (Boengler, 2005; Miro-Casas, 2009; Jovic, 2012; reviewed in Rodríguez-Sinovas, 2012). An association between connexin 43 and myosinVI, one of the sarcomeric motor proteins, has been demonstrated in mice to be necessary for gap junction maintenance of morphology and intercellular communication (Waxse, 2017).

In the rat, Cx40 has been reported in the atria prior to birth and is highly expressed in the ventricle early in development, declining to minimal or no ventricular expression in adulthood (van Kempen, 1996). This is similar to humans where early studies showed Cx40 to be several-fold more abundant in fetal hearts compared to pediatric or adult hearts (Chen, 1994). Recent work using samples from humans has indicated that normal atrial conductance requires similar levels of expression of Cx40 and Cx43 (Gemel et. al., 2016). Studies in patients with atrial fibrillation indicate decreased expression of Cx40 and the ion channel KCNA5 in atrial myocytes. This possible connection between structure and electrophysiology of atrial fibrillation has been extended into examination of genetic variants of Cx40 and also Cx37 (Carballo et. al., 2018; Zhang et. al., 2017). Connexin 40 is also expressed preferentially in the vascular endothelium of human coronary arteries and rat ventricular vascular endothelium (Chen., 1994; Bastide, 1993). Connexin 45 is reported to be widely but not highly expressed in the human, dog, rabbit, mouse and rat heart, possibly helping to define the developing conduction system (Kanter, 1993; Coppen, 1999; Coppen, 1998; Kim et. al., 2016). In vitro work in a rat liver epithelial cell line suggests that expression of Cx43 with coexpression of inducible levels of Cx45 may modulate the size of the gap junction, a feature of importance across age groups (Grikscheit et. al., 2008).

Recently, Cx26 mRNA and protein was found to be expressed in rat, pig and human cardiomyocytes. The protein was described in the cardiomyocytes of human atrium, rat atrium and ventricle and the left ventricle of the pig. Unlike other cardiac connexins, the authors described the protein distribution as not at the intercalated discs but distributed in the cytoplasm at the level of the mitochondria, myofibrils and cytoplasmic vesicles (Moscato et. al., 2018).

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Various studies have shown that remodeling of connexin expression and gap junction organization occur in adult heart conditions such as arrhythmia, ischemic heart disease and sudden cardiac death (Severs, 2008; Molina et. al., 2018; Visoná et. al., 2018). Alterations can occur in the distribution, amount and type of connexin expression during different types of heart disease in humans. Pharmacological modulation of connexins has been proposed as a possible therapy for arrhythmias in patients with ischemic heart disease (deVuyst et. al., 2011). The therapeutic possibilities of cardiac connexins have been considered in a proposal for a modernization of the Vaughan Williams classification of antiarrhythmic drugs. A new category of drugs acting on connexin-associated channels was suggested for several reasons, supported by the investigations with agents that block or open the Cx channels, carbenoxalone and the peptide analog rotigaptide (ZP-123) respectively. The authors note also the changes in gap junction that can accompany alterations in other action potential modifiers such as remodeling, exemplified by fibrotic change. Changes in Cx43 expression were noted to occur in both dilated and hypertrophic cardiomyopathy (Lei et. al., 2018).

### *Pannexins*

Pannexins (Panx) are a protein family with three members identified to date (Panx1, Panx2, Panx3) that have a different functional role from connexins. Based on their similar sequence homology with the invertebrate gap junctions and predicted similar topology to the gap junction proteins, it was originally suggested that they may form gap junction-like structures. To date it has been shown that, unlike the connexin gap junction intercellular channels, pannexin oligomers form large pore channels that are functional in single plasma membranes but not as intercellular channels in appositional membranes (Sosinsky, 2011). When open, they provide a conduction pathway between the cytosol and extracellular space. Panx1-triggered ATP release has been associated with pathologic fibrosis in canine cardiomyocytes, sympathetic activation in murine cardiac slices, atrial fibrillation in murine tissue, and possibly other processes (Sridharan, 2010; Dolmatova, 2012; Johansen, 2011; Petric, 2016; Dong, 2016). The Panx1 channel has been shown to be inhibited by a peptide mimicking a sequence of trovafloxacin, potentially explaining the side effects of this antibiotic (Poon, 2014). The role of pannexins in physiologic and pathologic processes is not completely understood.

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## Transporters

Transporters are given a separate section due to their function in both absorption and excretion of drugs, xenobiotics, and multiple endogenous compounds. The transporters are also involved in homeostatic functions and postnatal development of several organs including the heart (Koepsell, 2013). Transporters are susceptible to many additional chemicals that can act as low or high affinity inhibitors (Koepsell, 2007). We briefly list the solute carrier transporters that have been identified in the heart.

The solute carrier family (SLC22A) includes organic anion transporter (OAT) transmembrane proteins, organic cation transporters (OCT), and organic carnitine (zwitterion) transporters (OCTN), all of which classes have been identified to varying extents in the heart.

### *Organic Anion Transporters (OAT) and Organic Anion Transporting Polypeptides (OATP)*

The organic anion transporting polypeptides (OATP) and organic anion transporters (OAT) are widely distributed in tissues throughout the body, including the heart. The OATs and OATPs are capable of transporting a range of structurally unrelated compounds, including endogenous substances such as thyroid hormone, steroid conjugates and xenobiotics. OATPs transport mainly large, hydrophobic organic anions, while OATs transport smaller and more hydrophilic anions. Despite the name, OATPs can transport both cationic and neutral compounds (Hagenbuch, 2003; Roth, 2012).

Expression of OATP4A1 mRNA is found throughout the human body, including in the adult human heart (Fujiwara, 2001). Compared to other OATPs, OATP4A1 has narrower substrate specificity, with estrone 3-sulfate, benzylpenicillin, and thyroid hormone reported as substrates (Tamai, 2000).

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Messenger RNA for OATP2A1, originally identified as the prostaglandin transporter, is expressed in the adult human heart as well as numerous other tissues (Lu et al., 1996). The activity of this transporter is needed for terminating prostaglandin signaling via prostanoid receptors. OATP2A1 can also function as an organic anion exchanger (Obaidat, 2012). SLCO2A1 has been identified in pig heart (Van Poucke, 2009). Kamo and colleagues (2017) examined the effects of 636 FDA-approved drugs on OATP2A1 transport activity using HEK293 cells expressing OATP2A1. The activity of OATP2A1 was strongly inhibited by 51 approved drugs and stimulated by 10. Inhibitors included suramin, pranlukast, olmesartan, zafirlukast and losartan, all with IC<sub>50</sub> (inhibitor concentration where the response or binding is reduced by half) values less than 2 μM. While it is difficult to speculate the specific clinical consequences of inhibiting or stimulating the activity any of these receptors, it is not unreasonable to assume an effect of organ-specific impact.

OATP2B1 protein expression has been described in healthy human atria and ventricles, and hearts with dilated and ischemic cardiomyopathy (Grube, 2006). Messenger RNA expression was found in all samples, localized to the vascular endothelium. Samples from those treated with atorvastatin exhibited decreased OATP2B1 compared to those who had not taken the medication. Other xenobiotics, including aliskiren, bosentan, and pravastatin, have been demonstrated as substrates for OATP2B (Roth, 2012).

OATP3A1 is expressed in the human heart as well as other organs, including testes, brain, lung, and spleen.

Transported substrates include prostaglandin E1, thyroxine, and a non-specified cyclic oligopeptide endothelin receptor antagonist (Huber, 2007). Adult human ventricular cardiomyocytes maintained in culture demonstrated that simvastatin modulated OATP3A1 expression in the cardiomyocytes and HEK293 cells transfected with the OATP3A1 gene.

Simvastatin uptake was modulated by the pH of the cultures and the presence of other substrates of the OATP3A1 transporter suggesting the potential for drug-drug interactions at the level of the heart (Atilano-Roque, 2017).

#### *Organic Cation Transporters (OCT) and Organic Carnitine Transporters (OCTN)*

Organic cation transporters (OCT) are involved in the cellular uptake of endogenous monoamines (e.g., epinephrine), neurotransmitters (e.g., acetylcholine, serotonin), metabolites (e.g., choline, creatinine) and a variety of drugs (e.g.

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metformin, cimetidine, famotidine, prazosin, verapamil). This SLC22 family includes organic carnitine transporters (OCTN) (reviewed in Koepsell, 2007).

OCTN1 (SLC22A4) expression at the DNA and protein levels has been identified in adult human atria and ventricular surgical samples, and is localized to cardiomyocytes. A similar distribution is reported for mouse, rat, and rabbit (McBride, 2009; Iwata, 2008; Wu, 2000; Lamhonwah and Tein, 2006). Co-expression of this transporter in Chinese hamster ovary cells with the human ether-a-go-go-related gene (hERG) channel potentiated a delayed rectifier K<sup>+</sup> channel receptor (I<sub>Kr</sub>) drug block (McBride, 2009). Other investigators have provided data supporting mitochondrial localization of OCTN1 using a variety of transfected human cell lines as well as mouse hearts (Lamhonwah and Tein, 2006). Drugs that have been demonstrated to be transported by OCTN1 include quinidine, pyrilamine, verapamil, ipratropium, and the chemotherapeutic drugs mitoxantrone and doxorubicin (Yabuuchi, 1999; Nakamura, 2010; Okabe, 2008).

The OCTN2 (SLC22A5) has been identified in mouse (expressed on the plasma membrane), rat, pig, and human (Sekine, 1998; Iwata, 2008; Luo, 2014; Tamai, 1998). OCTN2 is expressed in several tissues including the myocardium (human atria and ventricles), skeletal muscle, renal tubules, and intestine (Grube 2006). The expression of this transporter has been demonstrated to be selectively decreased in human cardiomyopathy (Grube, 2011). OCTN2 is the primary high affinity sodium-dependent L-carnitine transporter in humans (Srinivas, 2007). L-Carnitine is critically involved in the movement of long chain fatty acids into the mitochondria for purposes of  $\beta$ -oxidation and removal of fatty acyl-CoA metabolites out of the mitochondria. The role of carnitine in postnatal development, and by implication the role of OCTN2, was examined in rat pups from postnatal day 4 to 20. The concentration of L-carnitine in both serum and cardiac tissue increased from postnatal day 4 to postnatal day 20, with increases of 20% and 50% respectively. The expression of cardiac OCTN1 mRNA remained constant except for an increase at postnatal day 8 that was greater than all other time points. The expression of cardiac OCTN2 mRNA increased postnatally by 100% while OCTN3 mRNA was approximately 200% higher at postnatal day 8 than postnatal day 4 and remained constant thereafter. These increases are also paralleled by increases in L-carnitine acyl transferases, carnitine

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palmitoyltransferase (Cpt) 1b and Cpt2 mRNA expression (Ling, 2012). These results are consistent with the postnatal increase in fatty acid oxidation rates reported for the heart, attributed in part to maturation of mitochondrial systems and increased L-carnitine. Cell culture evaluation of human and rat OCTN2 transporters indicated that the antibiotics cephaloridine and cefepime produced 40-90% competitive inhibition of carnitine transport, with rat OCTN2 showing somewhat less affinity for the antibiotics than the human transporter (Ganapathy 2000).

Recent genetic analysis has linked a human biallelic variant in the SLC22A5 gene encoding the OCT2 with pediatric cardiomyopathy that is responsive to exogenously administered carnitine (Larouchi, 2017). Others have found variants of SLC22A17 and SLC22A7 as predictive markers of anthracycline cardiotoxicity (Visscher, 2015).

OCT3 (SLC22A3) cDNA and/or protein has been identified in mouse, rat, and human tissues (Verhaagh, 1999; Iversen, 1965; Gründemann, 2002). This transporter has been demonstrated to be expressed in the heart as well as skeletal muscle, aorta, and human liver. This transporter is involved in monoamine uptake in the heart, demonstrated in OCT3-null mice (Zwart, 2001). When examined in adult human failing and non-failing hearts, immunostaining showed that OCT3 expression in the left ventricles was localized with endothelial cells and partially colocalized with gap junction protein connexin-43. There was no difference in OCT3 expression between the failing and non-failing hearts (Solbach, 2011).

Madin Darby canine kidney (MDCK) II cells overexpressing the cardiac transporters OCT1, OCT3, OCTN1 and OCTN2 were used to examine the interaction of these transporters with cardiovascular drugs. OCT1 and OCTN1 were each inhibited to less than or equal to 50% residual transport activity by 11 of the 21 tested drugs. OCT3 residual transport activity was reduced to 30% or less by nifedipine, propranolol, and verapamil. OCTN2 was essentially unaffected, with transport activity remaining at equal to or greater than 70%. Drugs tested were amiodarone, atenolol, atorvastatin, atropine, bisoprolol, carvedilol, digoxin, diltiazem, flecainide, ipratropium bromide, lidocaine, metoprolol, molsidomine, nadolol, nifedipine, propafenone, propranolol, sotalol, spironolactone, talinolol, and verapamil (Grube, 2011).

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## **Distribution**

Distribution may be seen from several different perspectives, such as distribution of a chemical to the heart from the general circulation (distribution to the heart) as well as movement throughout the heart once the endothelial barrier has been crossed (distribution through the heart). Cardiomyocytes communicate and are physically linked by the intercalated disc (ID), a structure unique to the heart. The gap junction component of the ID is involved in the rapid electrical transmission between cells as well as passage of small molecules. Relatively recently, the interactions of components of the ID have been re-described as transitional junctions, perinexus, and area compositae (also called composite junctions or connexomes). Myriad affiliated proteins have also been identified. (Forbes and Sperelakis, 1985; Vermij, 2017). The overall function of the ID is that of communication, providing passage of ions as well as signaling molecules. There is little easily accessible information as to potential involvement in xenobiotic distribution throughout the heart.

The gap junction provides direct communication between cardiac cells primarily for electrical communication by allowing ions to pass between cells, and thus helps coordinate depolarization. The passage of ions occurs within the pore of a gap junction that is formed by an assembly of connexin molecules.

The transitional junction is a recently recognized subcellular functional domain of the ID (Bennett, 2006). Its location in the ID is where the myofibrils lead into the adherens junction. It is hypothesized that this domain may allow for direct communication between the ID and contractile apparatus by acting as an anchor point for titin, the largest known protein, described as the third filament system for the sarcomere and important in the process of myofibrillogenesis (Bennett, 2006; Myhre and Pilgrim, 2014). It has also been proposed that the transitional junction is the site where new sarcomeres are added to the myofibril, providing elongation or growth of the cardiomyocyte during increased cardiac load (Vermij, 2017).

The perinexus, another functional region of the intercalated disc, is the area around the plaque of functional gap junctions in which free connexons (connexin hemichannel) interact with zonula occludens-1 (ZO-1). The ZO-1 regulates size, number and localization of the gap junctions, thus suggesting a potential regulatory role in their

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distribution. Named for its proximity to bordering gap junctions, the perinexus has been described as a microdomain, enriched in connexons, and containing sodium channels  $Na_v1.5$ ,  $Na_v1.3$ , several potassium channels including  $K_v$  and  $K_{ir}$ , connexin 43 and other protein-protein interactions, suggesting facilitation of distribution to several ion channels. Another role may be in the contribution to electrical coupling between cardiomyocytes and, thus, cardiac conduction (Rhett, 2011; Rhett, 2012, Rhett, 2013).

The adherens junctions (AJ, sometimes called fascia adherens) occur at cell-cell junctions and help to transduce and transmit contractile forces by anchoring myofibrils and connecting actin filaments from adjacent cells (Vermij, 2017). As reviewed by Henderson et al. (2017), AJs have a transmembrane component composed of cadherin proteins and a cytoplasmic plaque component comprised of catenin proteins. The N-cadherin protein homodimerizes with N-cadherins from adjacent cells, creating points of calcium-dependent intercellular connection.

During development, gap junctions appear at the ID after formation of the adherens junctions. Vreeker (2014) examined the time frame of human postnatal assembly of the adherens junction proteins N-cadherin and ZO-1 and the desmosomal proteins plakoglobin, desmoplakin and plakophilin-2. They found that these proteins were initially diffuse and located laterally, then were localized to the ID at approximately one year after birth. They also reported the detection of the  $Na_v1.5$  channel in the ID at 2 years after birth and the connexin 43 protein at 7 years after birth in humans, consistent with the findings of other investigators.

The area composita or adhesion junction is a term proposed by Borrmann (1999) and Franke (2006). An intriguing feature of the area composita and the ID region overall is the relatively prolonged period of postnatal maturation consistently found in rats, dogs, and humans (Pieperhoff and Franke, 2007). In rats, gap junctions, as determined by the location of connexin 43, and adherens junctions, as determined by the locations of desmoplakin and N-cadherin, went from a dispersed distribution across myocyte cell membranes at birth to a polarized distribution at the cell termini, that is, the developing intercalated discs, by postnatal day 20. From postnatal day 20 to postnatal day 90, the gap junctions

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became progressively concentrated in these zones. Similar findings have been reported for 1- and 3-month old dogs.

The progression of the process in 3-month old dogs was similar to that in a 20-day old rat (Angst, 1997).

A study of material obtained from pediatric surgical patients from 4 weeks to 15 years of age showed the neonatal human to have a multifocal distribution of connexin 43 gap junctions over the entire surface of the ventricular myocytes. With increasing age, the distribution of the gap junctions became localized to the IDs, reaching an adult pattern by approximately 6 years of age (Peters, 1994). The similarity of results in humans, rats, and dogs suggests that this change in the distribution and organization of gap junctions and cellular adhesion is an important process in cardiac maturation (Angst, 1997). Within the muscle fibers of the heart, the majority of desmosomes and adherens junctions integrate into composite junctions. However, in the conduction fibers, there is less integration of these two components. A study of Purkinje fibers indicated the presence of discrete desmosomes, discrete adherens junctions and composite junctions similar to those of the ID (Pieperhoff, 2010).

Once a drug moves through the blood vessel wall, it may traverse the cardiomyocytes and the extracellular matrix depending upon properties of the xenobiotic and the intrinsic properties of the developing animal. This may include the proportion of extracellular water to body fat and the maturity of transport mechanisms. In general, a newborn infant has a higher proportion of water as body weight (70-75%) compared to an adult (50-55%), approximately 5% less fat tissue, and 25% less muscle tissue. These relative differences in body composition will affect the volume of distribution of drugs that are distributed within these tissues (Koren, 1997).

Distribution of drugs to the heart will be, in part, due to the movement of the blood, contact with the endothelium, residence time (or plasma half-life) and the chemical properties of the material. Distribution of a drug is characterized by the ratio of total concentration of compound in the tissue to total concentration of compound in the plasma at steady state ( $K_p$ ). In a recent review article of the drug distribution based upon human samples from cardiac surgeries and forensic medical studies,  $K_p$  values for different types of drug were summarized (Tylutki, 2015). In general, the ratios

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of drug concentration in pericardial fluid and cardiac tissue to the plasma concentration for antibiotics were below 0.5.

For amiodarone, an antiarrhythmic compound, the ratio had a mean value of 23, with the highest concentration demonstrated in cardiac fat, possibly creating a slow release depot. On average, the myocardial concentration was 10 times higher than that in the plasma. Similarly, concentrations of digoxin were higher in the myocardium than in the plasma.

Physico-chemical attributes of the drug itself must be considered for the distribution phase. Lipophilic drugs and organic bases (e.g., tricyclic antidepressants, digoxin, cocaine) tend to accumulate in the myocardium with high affinity. Intra-individual variation in tissue concentrations from one region of the heart to another has been reported. As this is a developing area, there is reasonable speculation that multiple mechanisms are involved in distribution of drugs throughout the heart (Tylutki, 2015).

## **Metabolism**

### *Phase I*

Phase I metabolism is generally viewed as adding or unmasking a functional group, by oxidation, reduction, or hydrolysis. The main actors in these transformations are the cytochrome P450 families and the cytosolic aldehyde oxidases.

### *Cytochrome P450*

Cytochrome P450 (CYP) enzymes are recognized as critical agents of xenobiotic metabolism, important for the metabolism of foreign and endogenous compounds. Several CYP isozymes have been identified in the human heart (Table 3), To date, the majority of cardiac CYP exploration has been in adult hearts. Very little information was found in the literature for a developmental time-line, or ontogeny, for the cardiac CYPs.

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CYP2J2 is a predominant heart isoform and has been determined to be constitutively expressed in the adult human heart, with marked interindividual variation (Wu, 1996). Its primary function in the heart is drug metabolism, biosynthesis of epoxyeicosatrienoic acid (EET), and nitrous oxide production by metabolism of NCX-4016, a nitric oxide-releasing aspirin. EETs are endogenous molecules from metabolism of arachidonic acid (AA), important in the regulation of cardiovascular homeostasis. An examination of the mRNA and protein levels in the adult human heart, aorta and coronary arteries showed that CYP2J2 mRNA was highly variable but present in much greater abundance than either CYP2C8 or CYP2C9. Also, in non-diseased adult human hearts, CYP2J2 protein was identified in cardiomyocytes and endothelium of the cardiac vessels. A number of functional polymorphisms of CYP2J2 have been identified in humans, some of which may be associated with coronary artery disease, where overexpression may be one of several factors that contributes to protection (King et. al., 2002; Lee et. al., 2005; Spiecker et. al. 2004; Aliwarga, 2018). While cardiac CYP2J2 is relatively minor compared to hepatic metabolism, there are drugs where CYP2J2 has a significant role. One example is doxorubicin. Transgenic mice overexpressing cardiomyocyte-specific human CYP2J2 were administered doxorubicin either for 3 days or chronically for 5 weeks. Following the acute treatment, hearts of the transgenic overexpressers showed less doxorubicin-induced cardiomyocyte apoptosis compared with the wild-type hearts. After chronic treatment, cardiac function as assessed by echocardiography was significantly higher in the transgenic CYP2J2 overexpressers than in the wild type mice. Comparison of the microsomes from the WT and transgenic mice showed faster doxorubicin turnover in the CYP2J2 mice. A selective epoxygenase inhibitor, MS-PPOH, blocked the enhanced metabolism observed in the transgenic mice. (Zhang et. al.,2009). Very little is known about the developmental expression of CYP2J2. One study examined human fetal tissue for expression of CYP2J2 mRNA. This was found to be expressed ubiquitously in human fetal liver, heart (n= 5 samples), kidney, lung, intestine, and brain (Geadigk, 2006).

CYP2C9 cardiac isoform is inducible and involved with drug metabolism, biosynthesis of EET and reactive oxygen species production. CYP2C9 protein was identified in endothelial cells of aorta and coronary arteries of non-diseased

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human hearts (Askari, 2013; Delozier, 2007). In one ischemic heart, CYP2C9 mRNA was more abundant than either CYP2J2 or CYP2C8. Similar to many other studies of cardiac metabolism, this study had a relatively small number of samples (n= 8), all of which were from adult (>30 years) hearts (Delozer, 2007).

CYP11B2, aldosterone synthase, has been demonstrated to be constitutively expressed in the heart at low levels and is associated with local synthesis of aldosterone, corticosterone, and deoxycorticosterone. (Silvestre et. al, 1998). The mRNA for CYP11B2 has been identified in myocardium of stroke-prone spontaneously hypertensive at levels significantly greater than those of age-matched Wistar-Kyoto rats (Takeda et. al., 2000) Endomyocardial biopsy samples from patients with acute myocarditis and stable heart transplant recipients with no evidence of rejection (negative controls) were examined for evidence of CYP11B2 expression. Thirteen of 16 samples from myocarditis patients showed cytoplasmic staining of the cardiomyocytes compared to 2 out of 16 of the transplant patients, indicating local expression of aldosterone synthase protein (Cardona et. al., 2018).

The enzymes CYP2C8, CYP2C9, and CYP2J2 are the primary sources generating EETs, While the CYP2C and CYP2J enzymes are the major epoxygenases, CYP4A and CYP4F subfamilies function as hydroxylases, both of which subfamilies have been identified in human and canine hearts (Nithipatikom et. al., 2004; Zordoky and El-Kadi, 2003). As hydroxylases, the CYP 4A/4F isoforms are involved in 20-HETE synthesis by AA metabolism. Hydroxylases can convert AA to 16-,17-18-,19-,20-HETE, but CYP4a/F only converts AA to 20-HETE. As reviewed by Jamieson et. al. (2017), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may compete with arachidonic acid (AA) as substrates for these enzymes. Mono-oxygenation of EPA and DHA leads to the production of putative active metabolites known as resolvins and protectins, that are involved in the resolution of inflammation. The EETs and HETEs tend to have opposite biological effects. The EETs generally produce vasodilation and angiogenesis while the HETEs in general are associated with vasoconstriction and hypertension (Oni-Orisan et. al., 2016). Some in vitro work suggests that individual HETEs have unique properties. The effect of enantiomers of 19-HETE on the AA metabolites produced by cultured rat cardiomyoblast (H9c2) cells and human cardiomyocytes (RL-14) was compared. In rat and

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human cardiomyocytes, both the 19(R)-HETE and the 19(S)-HETE decreased the levels of several of the mid-chain HETEs. Neither enantiomer of 19-HETE produced significant effects on mRNA or protein for CYP2B6, CYP2C8, or CYP2J2. The S- enantiomer increased CYP4F2 and CYP4F11 mRNA and protein expression levels (Shoieb and El-Kadi, 2018). The opposite roles of the EETs and HETEs suggest that the ratios between the two classes are important. Further it appears that the proportions of EETs and HETEs within each class may also be important in cardiovascular health or development of pathologies.

Once produced by CYP2C or CYP2J, EETs are rapidly hydrolyzed to dihydroxyeicosatrienoic acids (DiHET) by soluble epoxide hydrolase (sEH) (Jamieson, 2017). A number of marketed therapeutic agents have been demonstrated to be CYP2J2 substrates, including amiodarone, tamoxifen, terfenadine, and thioridazine (Lee, 2010; Lafite, 2006). Telmisartan and metoprolol were both shown to be capable of selectively inhibiting CYP2J2 (Ren, 2013). Whether or not these xenobiotics affect EET production is not clear due to the complexity of overall regulation (Deng et. al., 2010).

Another class of substrates for CYP2J2 is  $\omega$ -3 (omega-3) and  $\omega$ -6 (omega-6) endocannabinoids, endogenously produced from polyunsaturated fatty acids. The metabolism of endocannabinoids produces endocannabinoid epoxides, reported to be vasodilatory and anti-inflammatory (McDougle et.al. 2017). Phytocannabinoids have recently also been shown to be substrates of CYP2J2. The different classes of phytocannabinoids have shown both competitive and noncompetitive inhibition of the endogenous molecule anandamide (Arnold et. al., 2018).

Rabbits, nonhuman primates and dogs are similar to humans in having CYP2J member identified within the heart. Polymorphisms of the CYP2J member(s) have been proposed to lead to altered organ function. In the case of polymorphisms associated with cardiovascular disease, the results are not consistent between studies (Aliwarga et. al., 2018). Rats and mice have multiple CYP2J enzymes and pseudogenes. Not all of the rat enzymes possess epoxygenase activity (Askari, 2013). Characterization of the postnatal functional activity of this group of metabolizing enzymes in the heart of any species was minimal to nonexistent. Recent publications by Solanki (2018) and Aliwarga et. al. (2018) provide detailed discussions of the role of CYP2J2 in cardiovascular biology.

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Dog hearts have been shown to express several CYP4A and CYP4F enzymes. CYP4A37, CYP4A38, and CYP4A39 have been shown to be present in Beagle hearts at relatively low expression levels. The amino acid sequences for these three enzymes share  $\geq 90\%$  identity to one another and are approximately 71 and 78% identical to rat CYP4A1 and human CYP4A11 respectively (Graham et. al., 2006). Studies by El-Sherbini (2013) indicate that CYP4A1 while expressed at a relatively low level, is the major  $\omega$ -hydroxylase in the rat heart. The HETEs have been demonstrated to have a number of actions in the heart including vasoconstriction and interaction with various cardiac ion channels (Aspromonte, 2014).

The identification of CYP enzymes within the heart suggests the possibility of tissue-specific metabolism, either beneficial or adverse. Modulating the activity of the cardiac CYPs, that is, induction or inhibition, is also possible. While the inhibition or induction of CYPs in liver or lungs has received extensive investigation, this area of work for the heart is developing. Mice overexpressing human cardiomyocyte CYP2J2 showed increased metabolism of doxorubicin in conjunction with decreased toxicity (Zhang et. al., 2009). Cocaine is another drug that has been demonstrated to induce CYP2J2 mRNA (Wang et. al., 2002). Studies by Zordoky et. al. (2008) demonstrated isoproterenol induction of CYP1A1, CYP1B1, CYP4A3 and inhibition of CYP2C11 and CYP2E1 in hypertrophied rat hearts.

### *Aldehyde Oxidase(AOX)*

Mammalian AOX is a group of soluble Phase I enzymes located in the cytosolic fraction of the cell. AOX has marked interspecies variability of expression that has been associated with profound differences in pharmacokinetics between nonclinical and clinical studies, leading to failure of drug development (Jensen et. al., 2016). The highest mRNA for AOX isoforms in human tissues has been located in the adrenal gland and liver. Essentially no AOX mRNA was identified in the heart of either healthy mice or humans (Terao et. al., 2016). Ghaffari et. al. (2012) demonstrated AOX

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activity in heart tissue from control and rats with diabetes mellitus. The enzymatic activity was localized to the organ but not to a cell type or structure. This is a rapidly expanding area of interest and further species-specific information is likely to be forthcoming.

### *Phase II*

There are several enzyme classes that constitute the predominant agents of what is referred to as Phase II metabolism. This has been historically viewed as a hydrophilic enhancement of a CYP-modified molecule. However, some of the “Phase II” enzyme classes may conjugate molecules that already have groups such as hydroxyls, carboxyls, or suitable amines. The major transforming enzymes are transferases: UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), N-acetyl transferases (NAT), and glutathione S-transferases (GSTs). Other transferases also exist, and cumulatively, comprise a fair proportion of metabolic events (Jancova et. al., 2010).

### *Uridine 5'-diphosphoglucuronosyltransferase (UDP-glucuronosyltransferases or UGT)*

These are the primary enzymes for glucuronidation. UGTs are membrane bound in the endoplasmic reticulum and similar to sulfotransferases, UGTs are important both in metabolism of xenobiotics and endogenous compounds such as bilirubin and steroid hormones. At this time, over 22 functional human UGT proteins have been identified, some with broad tissue distribution and others are tissue specific. In addition to the liver and gastrointestinal tract, kidney, brain, and nasal epithelium have been demonstrated to have UGT capacity, but there is little evidence of cardiac

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glucuronidation activity (Chen et. al., 2005; Sutherland et. al.,1993; King et. al.,1999; Lazard et. al. 1990). A study using adult (n=3) human tissues examined UGT mRNA in various tissues, including heart. Using RT-PCR, only trace amounts of mRNA for UGT1A1, UGT2A and 2B families were identified in the heart with no cellular type or location identified (Court et. al. 2012). An examination of five different strains of mice showed Ugt1a2 and Ugt2b5 protein to be specific to the hearts of male mice (Chen et. al., 2017).

The role of thyroid hormones in postnatal cardiac development has been demonstrated (reviewed in Li et. al. 2014). It has also been shown that UGTs glucuronidate both xenobiotics and endogenous substances, including the thyroid hormone thyroxine (Yamanaka et. al., 2007). It is speculative at this moment if there is some form of local, organ specific regulation of the thyroid involvement in postnatal cardiac development, an area that may be important in developmental toxicity or localized therapeutic effects.

### *Sulfotransferases(SULT)*

Cytosolic SULTs are involved in sulfonation of xenobiotics and endobiotics such as steroids, bile acids, and thyroid hormones, providing for an important role in endocrinology. The membrane bound SULTS are associated with the Golgi apparatus of the cell. Substrates for these isoforms include lipids, peptides, and proteins with sulfonation of the endogenous material causing structural and functional change (Li, 2014). The SULT enzymes are unique in significant expression early in life, in contrast to UDP-GST. Sulfotransferase activity has been demonstrated in fetal hepatic cytosol preparations, suggesting a role in early metabolism (Runge-Morris, 2013). Investigations by Alnouti and Klassen (2006) showed mRNA for Sult1a1, Sult1c1, and Sult1e1(trace amounts) in the hearts of 8-week old mice of both sexes. The mRNA for Sult3a1 and Sult5a1 were identified in male mouse hearts only, a phenomenon later characterized as androgen-dependent (Alnouti and Klassen, 2006 and 2011). RNA for SULT1C4 was found in fetal human heart (Sakakibara et. al. 1998) Sult1c1 mRNA expression after birth in humans has not been detected in cardiac tissues to any significant amount and only trace amounts of mRNA for Sult2b1. There appears to be somewhat greater

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expression of SULTs in hearts of male rats. Sex-related expression and distribution of SULT mRNA has been described for Sprague-Dawley rats. Messenger RNA for SULT1A1 was identified in the hearts of male rats as well as trace amounts of SULT1E2. No mRNA for SULTs was identified in the female rat hearts (Dunn and Klassen, 1998). Pigs have been demonstrated to be lacking or poor at sulfonation compared to other species. Minimal evaluation of porcine cardiac Phase II metabolism was found in the published literature (Helke and Swindle, 2013).

Mueller et. al. (2015) raise an interesting consideration in the active transmembrane transport needed for cellular influx of hydrophilic sulfated steroids. The SLCO and SLC22A superfamilies of SLC transporters have members associated with sulfated steroid transport. At this time, there is a paucity of information concerning the relationship or lack thereof of the SULTs and SLC22A in the heart. The multi-drug resistance protein (MRP) protein has been associated with cellular efflux of sulfated steroids, and has also been identified in the heart. As proposed by Mueller et. al. the relative tissue expression of OATP and MRP relates to total intracellular concentrations of steroids, suggesting a partnering effect of metabolic and transport systems. Overall regulation in general and coordinate regulation in particular is incompletely understood at this time.

### *N-acetyltransferases(NAT)*

NATs are cytosolic enzymes with at least two functional forms, NAT1 and NAT2, found in humans. A recent study examined the tissue expression patterns of NAT1 and NAT2 in Cynomolgus monkeys. Very low levels of mRNA for both were detected in the heart, with greatest expression located in the liver, followed by the kidney. The investigators point out that human variability of NAT activity, sometimes described in terms of fast or slow acetylators for drugs such as isoniazid or sulfamethazine, can be attributed in large part to genetic variants. The interanimal variability in NAT activity in nonhuman primates may similarly be due to genetic variants, information of potentially great importance, and not necessarily available (Uno et. al.,2018). Species variability is a critical consideration for this

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family of enzymes. For example, NATs are lacking in the dog but occurring at high levels in swine (described in the liver) and rodents (Trepanier et. al. 1997; Collins, 2001; Helke and Swindle, 2013). In addition to rat NAT1 and NAT2, a third rat enzyme, rNat3\*1 was identified. Evaluating tissue from F344 rats, cDNA for rNAT1 was found in the heart, followed by lower levels of expression of cDNA for rNAT2 and no detectable rNAT3 (Walraven et. al., 2008). Further work demonstrated mRNA concentration for all three rNATS in the heart of F344 rats. The levels of mRNA for rNAT3 were consistently lower than for the rNAT1 and rNAT2, suggesting lower constitutive expression but leaving open questions about tissue specific expression or linkage to a particular developmental stage or disease state (Barker et. al., 2008).

## Excretion

### *ATP binding cassette superfamily of transport proteins*

ATP-binding cassette (ABC) transporters, which use energy from ATP hydrolysis to move substances against chemical or electrical gradients, is one of the largest transporter superfamilies. Several ABC transporters, such as ABCB1 (P-glycoprotein), ABCC5 (MRP5), or ABCC9 (SUR2), are present in the human heart (Solbach, 2006). These transporters are involved in lipid metabolism and movement, as well as cardiomyocyte function via cyclic nucleotide efflux and metabolism, homeostasis, and potentially xenobiotic movement (Ichikawa et. al., 2012; Schumacher, 2017).

One of the best characterized ABC transporters is P-glycoprotein, also known as P-gp or multidrug resistance protein 1 (MDR1), the product of ABCB1 gene. P-gp is a drug efflux pump to protect the organism against toxic xenobiotic compounds. In the human heart, P-gp has been identified at both the mRNA and protein levels in endothelial cells of cardiac arterioles and capillaries. P-gp expression was greatly reduced in samples from dilated cardiomyopathy hearts (Meissner, 2002). Variable P-gp expressions were reported from human heart tissues (Meissner, 2004). Digoxin has been demonstrated to be a substrate for P-gp, as well as the beta-adrenergic antagonists talinolol and celiprolol (Karlsson, 1993; Westphal, 2000). Overexpression of P-gp has also been associated with multiple drug resistance, as

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well as decreased cytotoxicity of agents such as anthracycline chemotherapeutics (Krishna, 2000; Zhou, 2016).

Individual variability of expression of this transporter is a possible explanation for variable response to substrates.

The multidrug resistance protein 5 (MRP5/ABCC5), has been localized to the heart, in terms of both mRNA and protein expression. Using samples from adult humans presenting either for bypass surgery or transplantation, MRP5 was identified in three different cardiac cell types: vascular smooth muscle cells, cardiomyocytes, and vascular endothelial cells. MRP5 has been shown to mediate the cellular efflux of 3',5'-cyclic nucleotides, cAMP, and cGMP.

Therefore, it is suggested that MRP5 can affect NO/cGMP signaling by reducing its intracellular content in addition to its metabolic degradation by phosphodiesterases. A higher expression of MRP5 was observed in the ischemic cardiomyopathy tissues compared to the normal ventricular samples (Dazert, 2003).

In addition to chemical excretion, ABC transporters also play important roles in heart development, including the shift from fetal glycolytic to postnatal mitochondrial oxidative metabolism. For example, SUR2 (encoded by ABCC9) is a regulatory subunit of the major potassium-sensitive ATP ( $K_{ATP}$ ) channel in the heart. SUR2-containing  $K_{ATP}$  channels are enriched in the sarcolemma, where they control opening or closing of the potassium channel in response to the intracellular energy state. When *Abcc9*, the gene coding for ABCC9, is deleted from mice, the period from postnatal day 2 to postnatal day 8 is marked by a failure to develop adequate mitochondrial networks to support cardiac growth. These animals develop a fatal neonatal cardiomyopathy (Fahrenbach, 2014).

### **Energy Metabolism**

Continuous work means the heart requires a continuous energy supply. It is estimated that approximately 60-70% of the ATP generated in the heart is consumed in contraction and the remaining 30-40% is consumed by the sarcoendoplasmic reticulum calcium transport ATPase (SERCA) and other ion pumps (Stanley, 2005; Schramm, 1994). The profound energy demand makes this aspect of cardiac metabolism one where even small disruptions or alterations may have significant consequences. This is also an area of intense study for the connection to heart failure and cardiomyopathies.

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The mammalian fetal heart generates most of its ATP from glycolysis and lactate oxidation. In the newborn human, almost half of total ATP is produced from glycolysis. In the mature human heart, glucose generates approximately 25% to 30% of total energy. The postnatal energy transition is from glucose metabolism to fatty acids and mitochondrial oxidative metabolism (reviewed in Onay-Besicki, 2006). The time frame of this transition is described for several species. Early work by Breuer (1967, 1968) suggested that dogs from 7 to 12 days after birth and from 13 to 21 days after birth had different cardiac metabolic profiles from each other and from adult dogs, based upon the determination of glucose, lactate, and pyruvate from coronary arterial and venous blood flow (Breuer, 1967). Follow up studies on puppies from 7 to 13 days of age and a second group from 13 to 28 days of age suggested that the change from use of carbohydrates to use of fatty acids occurs at approximately the fourteenth day of life in dogs. Also in the third week of life, it was noted that the heart changed from lactate release to lactate uptake, coinciding with the beginning of free fatty acid uptake (Breuer, 1968). In rabbits, the contribution of glycolysis decreases from 44% on day 1 to approximately 10% of total cardiac ATP by day 7 after birth, which is similar to production in an adult human heart (Lopaschuk, 1991). Consistent with the decrease in glycolysis, isolated rabbit heart preparations demonstrated fatty acids as the main source of energy by two weeks of age (Itoi and Lopaschuk, 1993). Newborn rabbits with volume overload-induced hypertrophy had fatty acid oxidation rates 60% lower than control animals, and glycolysis rates increased by 246% ( $p < 0.05$ ). Overall, ATP production was significantly lower in the hypertrophied rabbit hearts (Okada et al., 2012).

It should be noted that growing/proliferating cells are usually characterized by immaturity of both the mitochondria and the mitochondrial networks (Lopaschuk, 2010; Tuomainen and Tavi, 2017). The endogenous metabolic development of the heart after birth includes a surge of mitochondrial biogenesis, followed by mitochondrial maturation, redistribution and packing of mature mitochondria along myofibrils. This stage of maturation includes increases in the processes of mitophagy (selective autophagous degradation of mitochondria), fusion and fission (Dorn, 2015). Ultimately, postnatal development of cardiac energy production depends upon mitochondrial maturation, with a complex interrelationship with the cardiac EETs (Singh et al., 2016).

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Another aspect of cardiac energy metabolism is the availability of substrate. Two classes of glucose transporters have been identified in the human heart: GLUTs (facilitative glucose transporters) and SGLTs (sodium-glucose cotransporters) (reviewed in Szablewski, 2017).

GLUT1 and GLUT4 have been identified in the rat heart. During fetal life of rats, GLUT1 (encoded by SLC2A1 and a member of the major facilitator superfamily) and hexokinase I are the predominant glucose transporters present in the heart of rats. Soon after birth in the rat, cardiac GLUT1/hexokinase I expression decreased while after postnatal day 10, GLUT4 and hexokinase II expression increased (Wang and Hu, 1991; Santalucia, 1992). The insulin-sensitive GLUT4 represents 70% of the glucose transporters in an adult human heart, with GLUT1 the next most prominent transporter. In resting (low insulin) conditions, GLUT4 is located primarily in intracellular membrane compartments. Stimuli such as ischemia, catecholamines, or insulin will cause acute translocation of GLUT4 to the cell surface, without transcription or translation, increasing glucose transport into cardiomyocytes by as much as 10- to 20-fold, as demonstrated in isolated rat cardiomyocytes, hearts of diabetic swine, and non-diabetic dogs (Fischer, 1996; Stanley 1994; Brosius, 1997a; Young, 1997; Mueckler, 2013). Expression of GLUT4 is also influenced by fatty acids and thyroid hormone (Finck, 2002; Gosteli-Peter, 1996). A study in adult dogs indicated a higher protein distribution of GLUT4 in atria compared to ventricles. During chronic heart failure in dogs induced by pacing, GLUT4 protein content was highest in the left ventricle while the amount in the right atrium was decreased (Ware, 2011).

GLUTs 1, 3, 8, 10, 11, and 12 have also been identified in the adult human heart. However, characterization of their physiological roles is incomplete. Transcriptional regulation has been determined to be the main mechanism for expression and activity of these molecules in the heart. GLUT1 in an adult may account for up to 40% of glucose transport, regulated by chronic hypoxia and long-term fasting (Brosius, 1997b; Kraegen, 1993). GLUT3 has been identified in both fetal and adult human heart (Grover-McKay, 1999; Kayano, 1988). The function of GLUT8 is unclear but it has been identified in the mouse heart along with lower levels of GLUT3, GLUT10, and GLUT12. Studies in paced mice given a long-term high fat diet suggest a role in regulating atrial activity (Aernie-Flessner, 2012; Maria, 2017). GLUT11 transports both glucose and fructose. There are three splice variants of this transporter,

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differentially expressed in heart, skeletal muscle, kidney, adipose tissue and pancreas (Doerge, 2001; Scheepers, 2005).

GLUT10 (SLC2A10) also has wide tissue distribution beyond the heart. Homozygous mutation of the SLC2A10 gene is linked to a disorder called arterial tortuosity syndrome in which morphological abnormalities of the arteries are present. Similar to the other GLUTs, GLUT12 is present in heart and skeletal muscle, as well as prostate and small intestine. In healthy and diabetic mouse heart, there was no difference in total GLUT12. Under the conditions of this particular study, GLUT12 translocation did not appear to be insulin-responsive, unlike GLUT4 (Waller, 2013).

The sodium/glucose transporter (SGLT) exists in several isoforms. The SGLT1 isoform is expressed in the small intestine, renal proximal tubule and heart (Turk, 1991; Zhou, 2003). The SGLT1 was expressed in healthy human and murine myocardial tissue and significantly upregulated in diabetes mellitus, ischemia and hypertrophy (Banerjee, 2009; Di Franco, 2017).

## Discussion and Conclusion

The heart contains metabolic machinery for processing endogenous material for energy production, physiologic development and homeostasis. The same machinery can also transport and process xenobiotics. The mitochondrial and nuclear regulation of these pathways is intricately connected and incompletely understood. The intricacy of metabolic machinery is made more complex when genetic variants of the components are considered. We found little information about the postnatal development of cardiac metabolism, with the exception of energy processes. Other than energy metabolism, there was little information as to ontogeny, time to full or mature function, and species differences. The last data gap is not surprising as much of the current information has been generated in human tissues. The limitation to this is that most of these human studies have had small sample sizes and tend to be mature or aged hearts. The data from non-human species included some information about development of metabolic machinery for energy production, but leaves gaps primarily for transporters, Phase I, and Phase II metabolism.

The relatively new concept of protein partners for coordination and communication of effects of receptors and transporters between distant sites is an intriguing one. Conceptually, this fits with an emerging systems biology view of receptors and transporters having roles in metabolite signaling pathways, inter-organ communication as well as

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neuroendocrine, growth factor and general homeostatic processes (Nigam, 2018). This also links development of local metabolic capability with the regulation of protein isoform shifts. The signaling pathways for both these phenomena, as well as the potential connections between the two, are incompletely described.

A detailed understanding of cross-species cardiac metabolism offers several possibilities. First, it may be possible to leverage local metabolic capabilities to create location specific conversion of prodrugs to active moieties. Second, an understanding of the natural progression of ontogeny followed by mature function and then physiologic deterioration may assist in understanding differences in drug effects between adults and children in general and special disease populations within age groups. With greater understanding of species similarities both in composition and ontogeny, it may be possible to design more informative nonclinical studies.

It seems intuitive that there may be developmental periods sensitive to xenobiotic perturbation or pathways susceptible at any age to chemical disruption. While there was some information about pharmaceuticals that were substrates or inhibitors for cardiac enzymes or transporters, there was little characterization of downstream effects or effects on signaling pathways. This has relevance for pediatric therapeutics: disruption of normal developmental pathways. Effects on cardiac function, reaching full adult cardiovascular capacity, or increased susceptibility to different forms of cardiovascular disease may not be perceptible for years to decades after exposure to a drug. By that time, many confounding factors have entered the picture. The same information about downstream effects on signaling pathways may also be informative for conditions such as heart failure. Events and signaling pathways unique to different ages, e.g. adult or geriatric, or physiologic stages may help to identify sensitive developmental windows and further to avoid unintended or adverse effects such as acceleration of naturally occurring cardiovascular disease.

Important areas for further exploration are cardiac metabolism related to drug toxicity, or targeted therapeutics, involvement in normal postnatal development, and modulation of adult homeostasis. There were some descriptions of cardiac-specific metabolism in the literature, but this is clearly an emerging area of research with regard to both

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therapeutics and toxicity. The possible association of xenobiotic modulation of postnatal development seems to be largely undescribed. This is an area where greater understanding of postnatal ontogeny becomes an issue of translational significance. Studies using human tissue have produced information for several disease states. However, the sample sizes tend to be small and it is unclear how much the results can be extrapolated to the population in general given the genetic diversity of the human race.

Comparisons of species effects, or translational value, were similarly limited with direct interspecies comparisons rare. Differences between both species, breeds within species and strains within breeds have been demonstrated. Studying postnatal development in different animal species is already complicated by the brevity of various physiologic windows and in some cases, the lack of similarity of the general developmental stage to the equivalent human developmental stage. Ideally hypothesis-driven studies could be designed with understanding of species differences, thus maximizing the translational value of the work. At this time, it is not possible or realistic to make generic recommendations of nonhuman species for investigative work or safety assessment based upon the available information. Clearly, understanding postnatal ontogeny of metabolism overall has the potential to significantly impact the development of pediatric therapeutics.

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This review is part of an effort of several working groups coordinated by the Health and Environmental Sciences Institute to try to identify, compile and integrate information concerning the postnatal development of various body systems, with emphasis on metabolism, the coordinated processes encompassing absorption, distribution, transformation (metabolism) and excretion (De Schaepdrijver et.al., 2018). This working group is composed of members from the federal government, industry, and academic communities.

### Authorship Contributions

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### Footnotes

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Table 1. Organic Anion and Cation Transporters Identified in the Heart

Class	Organic anion transporting polypeptides (OATP)				Organic cation transporters (OCT)		
Gene	<i>OATP4A1</i> ( <i>SLCO4A1</i> )	<i>OATP2A1</i> ( <i>SLCO2A1</i> )	<i>OATP2B1</i> ( <i>SLCO2B1</i> )	<i>OATP3A1</i> ( <i>SLCO3A1</i> )	<i>OCTN1</i> ( <i>SLC22A4</i> )	<i>OCTN2</i> ( <i>SLC22A5</i> )	<i>OCTN3</i> ( <i>SLC22A3</i> )
Protein	Solute carrier organic anion transporter family member 4A1	Solute carrier organic anion transporter family member 2A1	Solute carrier organic anion transporter family member 2B1	Solute carrier organic anion transporter family member 3A1	Solute carrier family 22 member4	Solute carrier family 22 member5	Solute carrier family 22 member3
Localization	N/A	N/A	mRNA in vascular endothelium, protein in human atria and ventricles	Expressed in ventricular cardiomyocytes	Human atrial and ventricular cardiomyocytes	Human atrial and ventricular cardiomyocytes	Localized in endothelial cells, partially co-localized with gap junction protein Cx43
Expression during postnatal development	N/A	N/A	N/A	N/A	N/a	Postnatal increase of OCTN2	N/A
Comments	Narrow substrate specificity	Mediates uptake and clearance of prostaglandins. Inhibited by multiple FDA-approved drugs.	Decreased expression after atorvastatin treatment	Increased uptake of simvastatin by OATP3A1 when combined with other OATP substrates	Transports drugs including quinidine, pyrilamine, verapamil, ipratropium, mitoxantrone, doxorubicin	L-carnitine transporter. Cephaloridine and cefepime produced competitive inhibition of L-carnitine transport.	Involved in cardiac monoamine uptake.
References	Fujiwara, 2001; Tamai, 2000	Lu et. al., 1996; Kamo, 2017	Grube, 2006; Roth, 2012	Huber, 2007 Atilano-Roque, 2017	Yabuuchi, 1999 Nakamura, 2010 Okabe, 2008	Srinivas, 2007 Ganapathy, 2000	Zwart, 2001
<b>N/A = not available</b>							

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Table 2. ATP-Binding Cassette and Facilitative Glucose Transporters Identified in the Heart

Class	ATP-binding cassette(ABC)		Facilitative glucose	
<b>Gene</b>	<i>ABCB1</i>	<i>ABCC5</i>	<i>SLC2A1</i>	<i>SLC2A4</i>
<b>Protein</b>	P-glycoprotein(P-gp) or multidrug resistance protein 1(MDR1)	Multidrug resistance protein 5 (MRP5)	GLUT1	GLUT4
<b>Localization</b>	N/A	N/A		
<b>Expression during postnatal development</b>	Endothelial cells of cardiac arterioles and capillaries.	Vascular smooth muscle cells, cardiomyocytes, vascular endothelial cells.	N/A	N/A
<b>Comments</b>	Drug efflux pump. Broad specificity includes digoxin, beta adrenergic agonists. Individual variability of expression contributes to variability of response.	Transports cyclic nucleotides and some nucleoside monophosphate analogues. Higher expression levels found in ischemic cardiomyopathy.		70% of the glucose transporters expressed in adult human heart. Insulin-regulated but also influenced by fatty acids and thyroid hormone.
<b>References</b>	Meissner, 2004; Karlsson, 1993; Westphal, 2000	Dazert, 2003	Wang and Hu, 1991; Santalucia, 1992	Wang and Hu, 1991; Santalucia, 1992
<b>N/A = not available</b>				

Table 3. Cytochrome P450 Identified in Cardiac Tissue

Family	Isozyme	Expression	Postnatal ontogeny	Functions	References
CYP1	CYP1A1	Moderate, Inducible	Expressed in mouse heart starting on E8.5	Drug metabolism; Metabolism of AA, CYP1A1 inducers increased heart size in chicken	Choudhary et al., 2003; Santes-Palacios et al., 2016
	CYP1A2	Inducible	N/A	AA metabolism; NO metabolism	Chaudhary et. al., 2009
	CYP1B1	Moderate	N/A	Biosynthesis of HETE and EET	Choudhary et al., 2003; Palenski et. al., 2014
CYP2	CYP2C8	Moderate	N/A	Biosynthesis of EETs, drug metabolism, e.g. verapamil	Delozier et.al. 2007
	CYP2C9	Low, Inducible	N/A	Metabolizes steroid hormones, fatty acids. Biosynthesis of EET; ROS production; drug metabolism	Aspromonte et. al., 2014; Delozier et.al. 2007
	CYP2D6	Low	N/A	Drug metabolism	Thum et al., 2000; Chaudhary et. al., 2009
	CYP2J2	High	Expressed in human fetal heart. Little information on developmental time line.	Predominant CYP in biosynthesis of EETs. Substrates include amiodarone, tamoxifen, terfenadine, and thioridazine; Inhibited by telmisartan and metoprolol. Mice overexpressing human CYP2J2 showed increased doxorubicin metabolism. mRNA in aorta and coronary arteries	Aspromonte et. al., 2014; Wu, 1996; Ren, 2013; Zhang et. al., 2009; Delozier et.al. 2007
CYP4	CYP4A1	Medium	N/A	Major hydroxylases for arachidonic acid; eicosapentaenoic acid(EPA) and docosahexaenoic acid(DHA) as substrates, compete with AA; major $\omega$ -hydroxylase in the rat heart	Thum and Borlak, 2000; El-Sherbini et al., 2013
	CYP4F12	Medium	N/A	Major hydroxylases for arachidonic acid; eicosapentaenoic acid(EPA) and docosahexaenoic acid(DHA) as substrates, compete with AA	Bylund et. al., 2001
	CYP4B1	Inducible	N/A	Drug metabolism e.g., verapamil	Chaudhary et. al., 2009
CYP11	CYP11B2	Low, Inducible	Not apparent in fetal tissues.	Low level constitutive expression. Associated with local synthesis of aldosterone, cortisone, deoxycorticosterone.	Silvestre et. al., 1998; Nebert et. al., 2013; Pezzi et. al., 2003

Note: AA = arachidonic acid; DHA = docosahexanoic acid; EET = epoxyeicosatrienoic acid; EPA = eicosapentaenoic acid; HETE = hydroxyeicosatetraenoic acid;

N/A = not available; NO = nitrous oxide; PFUA = polyunsaturated fatty acids; ROS = reactive oxygen spe

Table 4. Metabolic components identified in human heart

	component	Reference(s)
connexins	Cx26, Cx43, CX40, Cx45	Boengler, 2005; Miro-Casa, 2009 Chen, 1994;Moscato et. al.2018
OATP	mRNA for 4A1, 2A1 protein for 2B1, 3A1	Fujiwara, 2001;Lu et.al.,1996;Grube, 2005; Roth, 2012;Huber et. al., 2006
OCTN	Protein for OCTN1, OCTN2	McBride, 2009
OCT	Protein for OCT3	Verhaagh, 1999
Area composita and ID	Prolonged development after birth	Pieperhoff and Franke 2007
sulfotransferases	mRNA SULT1C4, SULT 2B1	Runge-Morris, 2013; Sakakibara et. al. 1998
CYP	mRNA and protein 2C8, 2C9, 2J2, 11B2, 4A, 4F	Aspromonte, 2014;King et. al., 2002;Lee et.al.,2005,S Piecker et. al., 2004;Aliwarga, 2018
ABC transporters	mRNA and protein P-glycoprotein, MRP5, ABCC9	Meissner, 2002;Meissner, 2004;Dazert, 2003
Glucose related	Protein GLUT1,3,4,8,10,11,12 and SGLT1	Brosius, 1997b;Kraegen, 1993;Grover-McKay, 1999;Kayano, 1988; Di Franco, 2017

Table 5. Metabolic components identified in rat heart

	component	Reference(s)
connexins	Cx26,Cx43, CX40, Cx45	Van Kempen, 1996; Moscato et. al.2018
OCTN	mRNA, Protein for OCTN1, OCTN2	Wu, 2000
OCT	Protein for OCT3	Verhaagh, 1999, Grundemann, 2002
Area composita and ID	Prolonged development after birth	Pieperhoff and Franke 2007
sulfotransferases	mRNA SULT1A1,SULT1E2, male rats only	Dunn and Klassen, 1988
CYP	mRNA 2J2, 2C11, 2E1, 4F, 11B2, 2B6,2C8,4A1	Takeda et. al., 2000;Shoieb and El-Kadi, 2018;Askari, 2013; El-Sherbini, 2013
Glucose related	Protein GLUT1,4	Wang and Hu, 1991;Santalucia, 1992
N-acetyltransferase	cDNA for NAT1 and NAT2 mRNA NAT1, NAT2, NAT3	Walraven et. al., 2009;Barker et. al., 2008

Table 6. Metabolic components identified in mouse heart

	component	Reference(s)
connexins	Cx43, CX40, Cx45	Coppen et.al., 2001;
OCTN	Protein for OCTN1, OCTN2	Iwata, 2008;
OCT	cDNA for OCT3	Verhaag, 1999
sulfotransferases	mRNA Sult 1a1, Sult1c1, Sult1e1 mRNA Sult3a1, Sult5a1 in males only	Alnouti and Klassen, 2006 Alnouti and Klassen, 2011
CYP	mRNA 2J2, 4A	
Glucose related	Protein GLUT1, SGLT1, GLUT3, GLUT8, GLUT10, GLUT12	Aernie-Flessner, 2012; Maria 2017; Banerjee, 2009;
UDP-glucuronosyl transferases	mRNA Ugt1a2, Ugt2b5- male mice only	Chen et. al., 2017
ABC transporters	ABCC9	Fahrenbach, 2014

Table 7. Metabolic components identified in canine heart

Identified Components in Dog Cardiac Tissue		
	component	Reference(s)
connexins	Cx45	Coppen,1999; Coppen,2001
Area composita and ID	Prolonged development after birth	Angst, 1997
CYP	Cyp4A, 4F, 4A37, 4A38,4A39	Aliwarga et. al. 2018; Graham et.al., 2006
Glucose related	GLUT4	Ware, 2011

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