Targeting the Translocator Protein (18 kDa) in Cardiac Diseases: State of the Art and Future Opportunities

Emma Baglini,[‡] Valeria Poggetti,[‡] Chiara Cavallini,[‡] Debora Petroni, Francesca Forini, Giuseppina Nicolini, Elisabetta Barresi, Silvia Salerno, Barbara Costa, Patricia Iozzo, Danilo Neglia, Luca Menichetti,* Sabrina Taliani,* and Federico Da Settimo

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ABSTRACT: Mitochondria dysfunctions are typical hallmarks of cardiac disorders (CDs). The multiple tasks of this energy-producing organelle are well documented, but its pathophysiologic involvement in several manifestations of heart diseases, such as altered electromechanical coupling, excitability, and arrhythmias, is still under investigation. The human 18 kDa translocator protein (TSPO) is a protein located on the outer mitochondrial membrane whose expression is altered in different pathological conditions, including CDs, making it an attractive therapeutic and diagnostic target. Currently, only a few TSPO ligands are employed in CDs and cardiac imaging. In this Perspective, we report an overview of the emerging role of TSPO at the heart level, focusing on the recent literature concerning the development of TSPO ligands used for fighting and imaging heart-related disease conditions. Accordingly, targeting TSPO might represent a successful strategy to achieve novel therapeutic and diagnostic strategies to unravel the fundamental mechanisms and to provide solutions to still unanswered questions in CDs.

TSPO: diagnostic and therapeutic target for CDs
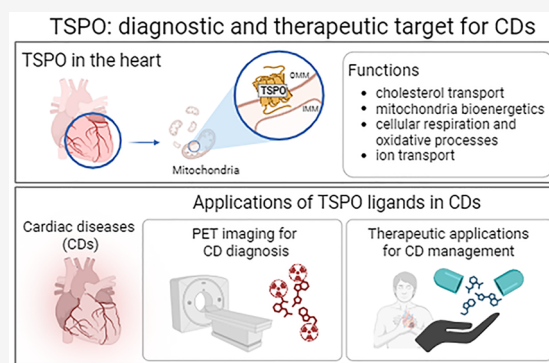
1. INTRODUCTION

Despite significant advances in the treatment of cardiovascular diseases (CVDs), the latter remain the leading causes of morbidity, disability, and death, with very high social and economic impact. CVDs are estimated to affect 471 million people worldwide with approximately 17.6 million deaths per year (32% of all global deaths), a trend that will increase to 24 million by 2030, which means 66 000 deaths per day.[1] In Europe, according to data from the fifth edition of the European Cardiovascular Disease Statistics, CVDs affect more than 80 million people (48% men and 52% women) and are responsible for 3.9 million deaths per year (45% of all causes of death). Among CVDs, heart failure (HF) is the leading cause of hospitalization in people over 65 and has very high mortality rates: 1 in 25 patients does not survive the first admission, 10% die within 30 days, and 30% die within a year of admission. Accordingly, it is always a challenge and a relevant priority to discover new diagnostic and therapeutic targets for cardiac disorders (CDs). Among them, the translocator protein (18 kDa), TSPO, is an attractive emerging candidate. TSPO is an integral membrane protein of 169 amino acids that is composed of five transmembrane α-helical domains. In most tissues, it is predominantly expressed in mitochondria, more specifically at the contact site between the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM).[2] The participation of mitochondrial TSPO in a multimeric complex known as mitochondrial permeability transition pore (mPTP) along with other proteins, including the 32 kDa voltage-dependent anion channel (VDAC) and the 30 kDa adenine nucleotide translocase (ANT), is supported by some,[3] but not all, studies.[4−7] A nonmitochondrial localization of TSPO has also been described, e.g., in the plasma membrane of erythrocytes, the nuclear and extranuclear fraction of breast cancer cells, the nuclei of human hepatocytes, and other organelle membranes of several cell types.[8]

TSPO is evolutionarily well-conserved, and it is present in almost all organisms, suggesting a critical role in biological processes.[9] Regarding tissue distribution, TSPO is ubiquitous, but it is mainly found in steroid-synthesizing tissues (including adrenal glands and gonads), kidneys, nasal epithelium, lungs, and the heart, while a lower expression is shown in the brain and liver.[8]
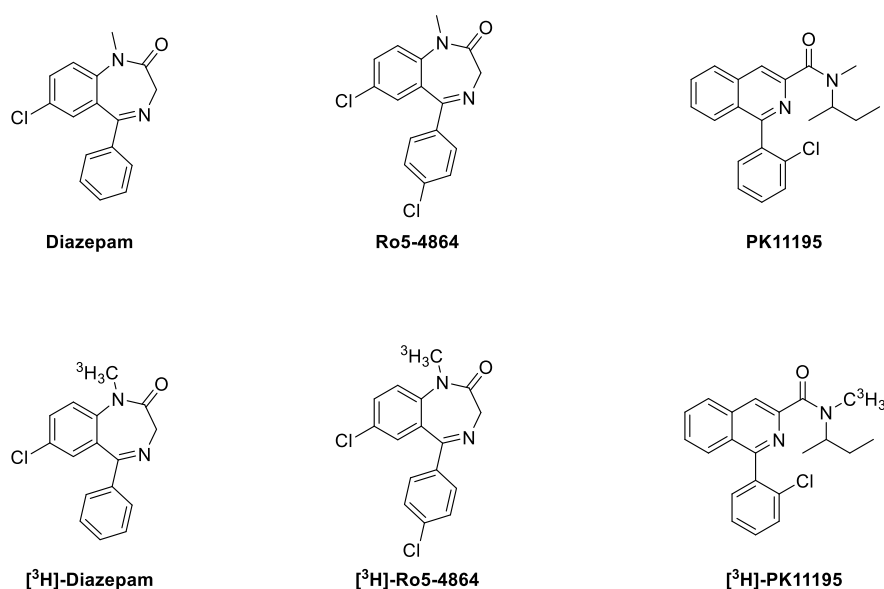
Figure 1. Chemical structures of diazepam, Ro5-4864, and PK11195 and their corresponding [³H]-radiolabels.

TSPO participates in several cellular functions, but one of the most frequently described is the regulation of the rate-limiting step of steroidogenesis, explaining its prominent localization at the level of steroid-synthesizing tissues. Specifically, TSPO is involved in the internalization of cholesterol into the mitochondrion. In the IMM, cholesterol is converted into pregnenolone by the enzyme CYP11A1 (cytochrome P-450 family 11 subfamily A member 1), then pregnenolone is transported to the sarcoplasmic reticulum where it is metabolized to steroid products.[8] It has been hypothesized that TSPO ligands stabilize the tertiary structure of the protein and consequently facilitate mitochondrial import of cholesterol, leading to increased steroidogenic efficiency in various steroidogenic *in vitro* models.[10] Interestingly, it has been demonstrated that the equilibrium thermodynamic parameter predicting the steroidogenic efficacy of a TSPO ligand is the residence time to the protein rather than the binding affinity.[11] TSPO is also involved in other processes related to mitochondrial bioenergetics, such as apoptosis,[12] cellular respiration and oxidative processes,[13] mitochondrial metabolism,[14] protein import,[15] ion transport,[16] immunomodulation,[17] porphyrin transport, and heme biosynthesis.[18]

TSPO is upregulated in several cancerous tissues, including lung, ovary, colon, prostate, and brain cancers,[19] as well as in activated microglial cells in neuropsychiatric pathologies such as neurodegenerative diseases (*i.e.*, Alzheimer's and Parkinson's diseases). Due to this feature, TSPO has been suggested as a biomarker of neuroinflammation[20] and the progression of these pathologies.[21] Conversely, TSPO is downregulated in the brains of patients with post-traumatic stress, anxiety, and obsessive-compulsive disorders[22] and upon repeated stress, including noise exposure.[23] Small synthetic TSPO ligands have been developed and biologically evaluated as potential tools for treating TSPO-related disorders, and TSPO has gained recognition as a marker and therapeutic target of neuro-psychiatric pathologies.

Biodistribution studies (*in vivo* and *in vitro*) using radiolabeled ligands, *e.g.*, [³H]-diazepam (rat and guinea-pig cardiac membranes),[24] [³H]-Ro5-4864 (7-chloro-5-(4-chlorophenyl)-1-methyl-1,3-dihydro-2*H*-benzo[*e*][1,4]diazepin-2-one), and

[³H]-PK11195 (*N*-methyl-*N*-(1-methylpropyl)-1-(2-chlorophenyl)isoquinoline-3-carboxamide) (Figure 1), demonstrated TSPO is also located in the heart, prevailing in the myocardial ventricles compared to atria.[25]

TSPO is up-regulated upon acute electroshock in cardiac ventricles, followed by an increase in TSPO density in the cerebral cortex.[26] Recently, TSPO has been found to be involved in CDs and, more specifically, in ischemia-reperfusion injury (IRI), but its exact role remains to be established.[27]

In the present report, an overview of the recent literature evaluating the effects of TSPO ligands on cardiac pathophysiology is presented, together with the most representative examples of the few TSPO radioligands studied so far for diagnosing CDs, particularly atherosclerosis, myocarditis, large vessel vasculitis (LVV), and myocardial infarction (MI). The discussion, moving from diagnostic to therapeutic applications, aims to shed light on the potential of TSPO as a target to be exploited by the scientific community to advance cutting-edge research in the field of CDs management.

2. THE ROLE OF TSPO IN CARDIAC PATHOPHYSIOLOGY

Several studies demonstrated that TSPO ligands may have different cardiac effects, indicating a possible involvement of TSPO in regulating heart physiology. Initially, TSPO has been proposed to influence cardiac contractility and heart rate by modulating calcium flux and ion transport, respectively.[27,28] TSPO ligands decrease the calcium current and thus lower calcium release from the sarcoplasmic reticulum, which might explain their observed negative inotropic effects.[29] The negative chronotropic effect of TSPO ligands has been ascribed to a decreased inward ion current during the fourth phase of the action potential in pacemaker cells, which leads to a hyper-polarization of the resting membrane potential.[30] Further studies have questioned the involvement of TSPO ligands in the regulation of cardiac electrophysiology because certain TSPO ligands are responsible for biological effects only at concentrations that exceed their affinity values for the target, and in these conditions they can also bind other molecular targets/receptors.[28] Thus, their resulting pharmacological effect might

hardly be attributable to their specific binding to TSPO. For example, the negative chronotropic effect of Ro5-4864 (Figure 1) might be a result of the direct interaction with calcium channels.[31] It is conceivable that inconsistencies may depend on different experimental models, species, types of TSPO ligands, and concentrations used.[27] It is important to remark that this complicated issue concerning synthetic TSPO ligands has been frequently ascribed to the complexity of this protein modulating numerous processes. It is not uncommon that a TSPO ligand can determine different effects with reference to a specific TSPO function depending on the experimental system employed. Thus, the classical concept of inhibitor (antagonist) and activator (agonist), commonly recognized for membrane ligand/receptor systems, cannot be applied to TSPO ligands and synthetic ligands, initially labeled as TSPO agonists or antagonists, often revealed to produce comparable effects if different experimental models are applied.

In addition, TSPO ligands have been proposed to exert a protective effect in CDs by reducing reactive oxygen species (ROS) production and preventing mitochondrial dysfunction and stress-dependent cardiomyocyte loss.[28] Together with these possible effects, the main physiologic mechanisms involving TSPO that have been investigated are related to the interference with cholesterol transport and the interaction with other mitochondrial components, as detailed below.

2.1. TSPO, Cholesterol Transport, And Oxidative Stress.

The first recognized and best-studied function of TSPO is to facilitate cholesterol transport from the cytosol to the IMM, a crucial step for steroidogenesis and other biological processes.[28] However, in a pro-oxidative condition, cholesterol within mitochondria is highly sensitive to ROS attack and autoxidation, resulting in the formation of oxysterols, which may promote mitochondria damage, such as membrane lipid peroxidation, increased membrane permeability and, ultimately, cell death.[32−36] The most abundant oxysterols in cardiac mitochondria are 7α- and 7β-hydroxycholesterol (OHC), 7-ketochol, and cholesterol epoxides, derived from 7-hydroperoxycholesterol. Among them, 7α-OHC, 7β-OHC, and 7-ketochol do not promote further lipid peroxidation, while hydroperoxycholesterol and cholesterol epoxides change the biophysical organization of lipids and proteins within membranes, thus affecting membrane fluidity.

During myocardial reperfusion following an ischemic event, cholesterol accumulates in the mitochondrion and generates self-oxidized oxysterols,[35,36] which may be involved in the myocardial IRI. Accordingly, Musman et al. demonstrated that in the diabetic hypercholesterolemic rat model (ZDF fa/fa), the increased accumulation of cholesterol, other sterols, and oxysterols inside cardiac mitochondria dramatically exacerbated mitochondrial impairment following ischemia reperfusion (IR).[36] A previous study showed hypercholesterolemia increases mitochondrial oxidative stress in the porcine myocardium.[37] Thus, the correlation between hypercholesterolemia and mitochondrial damage in these experimental models underlines the possible deleterious effects of cholesterol-derived oxysterols.[35,36] Besides propagating lipid peroxidation, oxysterols contribute to oxidative stress by depleting the antioxidant defense of cardiomyocytes, including glutathione. For example, 7-ketochol and 7β-OHC cause apoptosis via the mitochondrial pathway by reducing cell antioxidant activity. In addition, oxysterol can enhance the activity of superoxide-producing enzymes, such as NADPH oxidase and xanthine oxidase, further exacerbating oxidative stress.[34,38]

Interestingly, the inhibition of oxysterol accumulation as a consequence of cholesterol accumulation in the myocardial mitochondrial matrix through TSPO ligands was shown to exert cardioprotective effects and rescue oxidative phosphorylation in a lean and hypercholesterolemic murine model of IR, even if the underlying mechanism still needs to be established.[35,36] However, the available findings suggest that oxysterols, which are produced in pathological conditions, could represent a link between mitochondrial damage, cholesterol accumulation, and the potential therapeutic role of TSPO ligands in CDs.

2.2. TSPO-Interacting Mitochondrial Components.

TSPO has been shown to influence the activity of other mitochondrial components, such as the mPTP, VDAC, and inner membrane anion channel (IMAC), that play a crucial role in determining the fate of cardiomyocytes in acute and chronic CDs, such as acute MI and HF.[39,40]

2.2.1. mPTP. The mPTP supramolecular complex is a nonspecific channel with a cutoff of 1.5 kDa that is formed and opens under stress conditions, triggering the so-called mitochondrial permeability transition that is responsible for cardiomyocyte death.[41] The molecular identity of mPTP-forming units has long been debated, yet no definitive model has agreed upon.[4−7] Although the peptidyl-prolyl *cis−trans* isomerase (PPIase), or cyclophilin D (CypD), is the only component unambiguously involved in mPTP regulation, TSPO, among others, has been attributed at least an indirect facilitating role in channel opening. Cardiac IR is the main pathological condition able to trigger irreversible mPTP opening. Following ischemia, the lack of oxygen supply to the mitochondrial respiration chain blocks mitochondrial ATP synthesis. In the first few minutes of ischemia, anaerobic glycolysis copes with the lack of oxygen, but the ATP produced is insufficient to support cardiac activity. This process has several consequences, such as ionic imbalance and membrane depolarization. A cytosolic calcium overload occurs at the expense of a decrease in mitochondrial calcium concentration.[42] At first, the decreased mitochondrial Ca^{2+} amount is helpful as it causes acidosis, which protects the heart from ischemic damage[43] and prevents mPTP opening. However, conditions in favor of the latter are established, such as the decrease in mitochondrial membrane potential and the increase in ADP and Pi concentrations. If ischemia persists, the increased cytosolic Ca^{2+} concentration activates Ca^{2+}-dependent enzymes that cause membrane destruction and cell death. Rapid coronary reperfusion is the only therapeutic strategy used to date in hospitals to treat ischemic events. However, reperfusion paradoxically causes so-called reperfusion injury. Indeed, the sudden supply of oxygen following a period of hypoxia promotes an excessive production of ROS, which is also increased by the calcium accumulated in the cytosol during ischemia. ROS are then transported into the mitochondrion once the membrane potential is restored. The increase in ROS and augmented calcium concentrations lead to an increase in mitochondrial membrane permeability, resulting in mPTP opening. As a consequence, the proton motive force is dissipated because of the inability of the IMM to act as a barrier toward protons; this causes the uncoupling of oxidative phosphorylation, which in turn results in ATP depletion and, therefore, in the exhaustion of cellular energy.[44] The increased permeabilization of the IMM because of mPTP opening causes mitochondrial swelling,[45] which is in turn augmented by several processes accompanying postischemic heart reperfusion, such as adenine nucleotide depletion, high phosphate concentration, and oxidative stress.[44]

The main driving force of mitochondrial swelling is the equilibration of all low-molecular-weight osmolytes between the cytosol and mitochondria, while proteins are retained within their respective compartments.[45] Thus, the highest protein concentration in the matrix exerts an osmotic pressure resulting in swelling of the matrix compartment.[45] Mitochondrial swelling, followed by OMM breaking, in turn leads to the release of pro-apoptotic signaling molecules and irreversible mitochondrial damage.[41] However, if insufficient ATP levels are maintained, apoptotic death predominates over necrotic cell death.[45]

The possible role of mPTP in CDs was first studied by Crompton et al.,[46] who showed that preventing the opening of the pore could represent a potential target for cardioprotection against myocardial IRI.[47] Accordingly, many drugs used in cardioprotection trials, such as sangliferin A (SfA),[48] cyclosporin A (CsA),[49,50] 6-MeAla-CsA, 4-methyl-val-CsA, *N*-methyl-4-isoleucine-CsA (NIM811), and D-3-MeAla-4-EtVal-CsA (Debio-025), prevent mPTP opening during reperfusion.[51,52] Further works showed that the beneficial effect induced by CsA is mediated by the inhibition of CypD,[53] the 18 kDa matrix protein encoded by the nuclear PPIF gene.[49] In detail, CypD has been proposed to sensitize mPTP by interfering with the function of ATP synthase, a putative component of the mPTP.[54]

Several studies, which will be discussed in more detail below, have reported that certain TSPO ligands act as cardioprotective agents by preserving the physiological function of mitochondria and preventing cell death.[55−57] Other TSPO ligands have been shown to promote mPTP opening and apoptosis regardless of TSPO,[58−60] thus suggesting that this protein does not directly intervene in pore regulation.[7] Accordingly, it has emerged that TSPO ligands play an indirect role in mPTP opening by acting on oxidative stress and the production of ROS as pivotal triggers of pore opening.

2.2.2. VDAC. The VDAC, also known as a mitochondrial porin, is found in the OMM, and one of its primary roles is the regulation of both the input and output of mitochondrial metabolites, ions, and nucleotides controlling the exchanges between mitochondria and the rest of the cell. The anion channel is closely associated with TSPO at the contact sites between the IMM and OMM.[61] VDAC isoforms 1 and 3 actively participate in intrinsic cell death by forming a large flexible pore that allows the release of pro-apoptotic proteins such as cytochrome c (Cyt-c), apoptosis-inducing factor (AIF), Smac/DIABLO, and endonuclease G.[62] Furthermore, VDAC is considered a target for proteins of the Bcl-2 family[62] and promotes apoptosis by favoring ROS overproduction in conjunction with TSPO activity.[63,64] VDAC is also involved in mitochondrial damage and rupture since its closure causes a defect in ATP/ADP exchange, leading to mitochondrial swelling and breakage followed by the release of several proapoptotic factors such as Cyt-c into the cytosol, as observed during mPTP opening.[62]

TSPO ligands may exert their cardioprotective action by increasing the stabilization of the antiapoptotic Bcl-2 in the mitochondrial membrane at the expense of the proapoptotic Bax, thus restraining cell death.[56] In particular, TSPO ligands hinder the interaction of pro-apoptotic proteins with VDAC at contact sites between IMM and OMM, where TSPO, VDAC, ANT and other proteins are located,[65] thus preventing Cyt-c release, but also limiting the production of ROS and therefore the permeabilization of the OMM.

2.2.3. IMAC. The IMAC is a partially anion-selective channel of the IMM found in both the heart and liver. Although its molecular identity is still unknown, its presence was first characterized in 1986 by Garlid and Beavis, who demonstrated that anions like Cl^-, Br^-, SO_4^{2-}, PO_4^{3-}, *etc.*, could cross the mitochondrial membrane.[66] The IMAC is mainly involved in mitochondrial volume homeostasis and also plays a role in the contractile and electrical functions of the heart. It has been implicated in postischemic damage by promoting the generation of arrhythmias during the reperfusion period.[67,68] This occurs due to ROS production, particularly due to a mechanism known as "ROS-induced ROS release" (RIRR). Briefly, RIRR is a process in which an initial release of ROS by a cellular compartment due to oxidative stress triggers the production and the release of ROS in other compartments.[69] Regarding cardiac damage, IMAC is thought to be activated by the production of ROS at the mitochondrial level, which in turn causes the efflux of superoxide anions.[70,71] In a study conducted in 2003,[72] it was shown that RIRR can trigger oscillations of mitochondrial NADH and oscillatory depolarization of the IMM in cardiomyocytes, which in turn may cause arrhythmias in the reperfusion phase following ischemia by altering myocyte excitability.[67] In addition, it has been shown that IMAC opening due to RIRR also influences mPTP opening.[73,74] In particular, the pore opening is triggered by the oxidation of thiols in cardiomyocytes.[74] IMAC does not participate in forming complexes with TSPO. However, TSPO ligands limit mitochondrial ROS production avoiding the threshold for IMAC opening to be reached, which represents an additional mechanism for indirect modulation of mPTP and limitation of arrhythmias.[67,68,72,75,76] TSPO ligands have also been shown to inhibit IMAC activation in swelling assays on isolated mitochondria,[77] strengthening the assumption that they are functionally related to each other, even though through a still incompletely understood mechanism. However, some controversy emerged, especially considering the concentrations of ligands necessary to inhibit IMAC and perform cardioprotective action (>30 μM), which far exceed far the affinity range in the nanomolar order.[66,67]

3. CARDIAC IMAGING VIA TSPO RADIOLIGANDS

In clinical practice, molecular cardiac imaging serves a significant role in clinical cardiology.[78,79] It enables a detailed evaluation of the pathophysiology of heart injury and the subsequent remodeling, which is essential in developing new and effective therapies.[80] One of the well-established clinical applications of positron emission tomography (PET) in cardiac imaging is the assessment of myocardial perfusion with high accuracy in the absolute measurement of myocardial blood flow and coronary flow reserve. This application promises to be expanded thanks to the availability of novel compounds like [^{18}F]-flurpiridaz.[81] Another established clinical application of cardiac PET is the assessment of myocardial and vascular glucose metabolism with the use of the radiopharmaceutical leader in PET imaging, *i.e.*, [^{18}F]-FDG (2-[^{18}F]-fluoro-2-deoxy-D-glucose), even if the quality of the PET signal, in particular in the wall of vessels, is still limited. Nevertheless, PET has multiple potentials in cardiac and vascular molecular imaging.

TSPO is one of the most interesting inflammatory biomarkers exploited for PET imaging.[82] Nonetheless, thus far there are just a few TSPO radioligands employed for cardiac imaging purposes, representing a new and valuable opportunity to

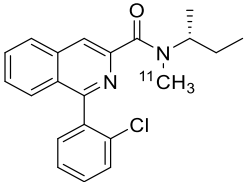**Chart 1. TSPO Radiotracers Tested in PET Cardiology Imaging**
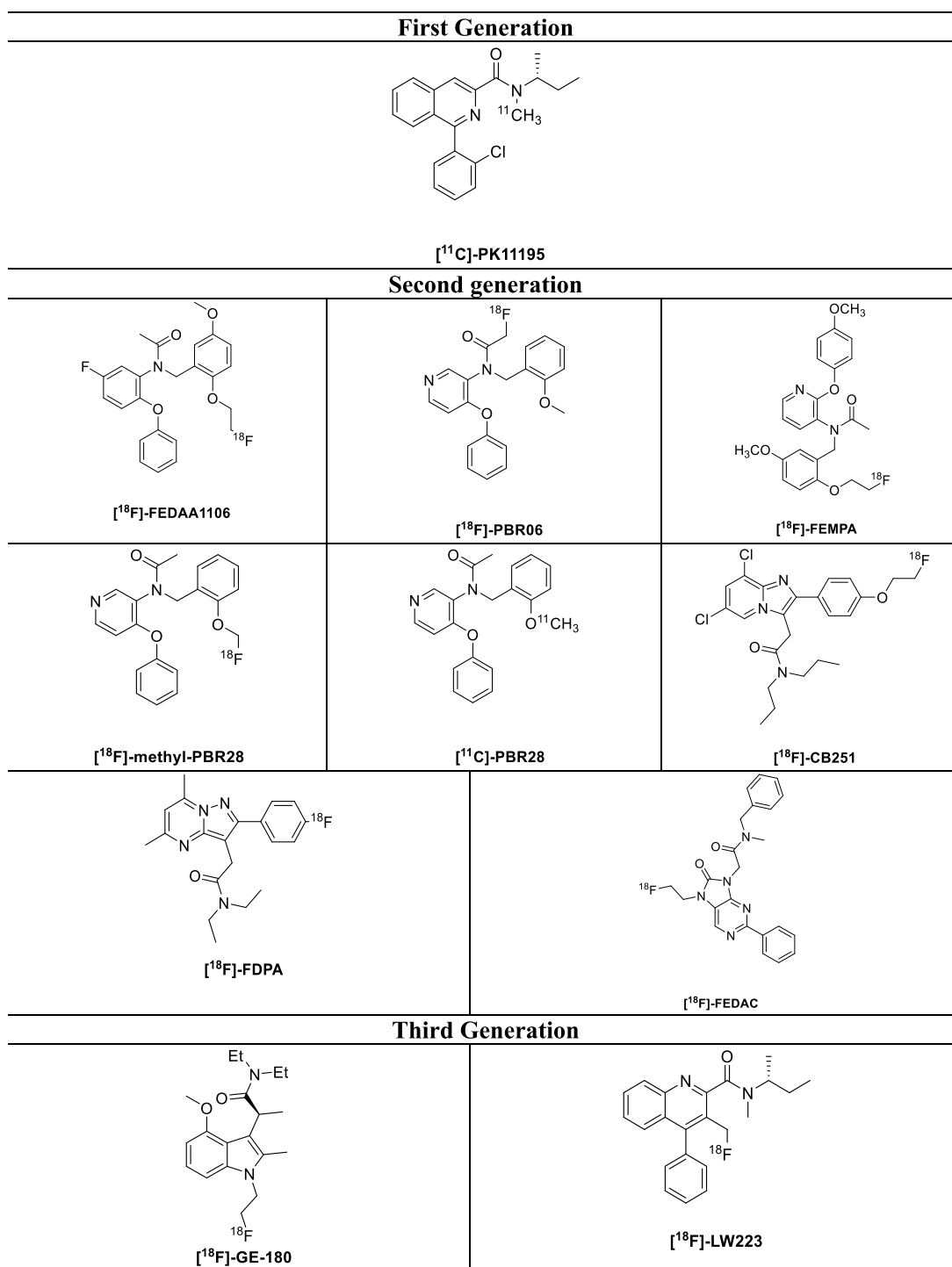
exploit the potential of PET imaging, particularly thanks to the quantification capabilities.

A list of TSPO PET radiotracers tested in cardiology imaging is reported in Chart 1. Most of these were initially developed for neuroimaging applications due to TSPO's high expression in activated microglial cells characterizing neuroinflammatory diseases.[82,83]

3.1. First-Generation PET Tracers for TSPO. PK11195 and Ro5-4864 (Figure 1) belong to the so-called first-generation TSPO ligands.[84] In 1984, PK11195 was radiolabeled with carbon-11[85] to give [^{11}C]-PK11195 (Chart 1, Table 1), the first

radiotracer for TSPO used in PET imaging. This radiotracer was used for imaging atherosclerosis,[86] a chronic disease of large and medium-sized arteries involving inflammatory processes that leads to major CVDs, such as ischemic heart disease, stroke, and peripheral vascular disease.[87] Macrophages play a crucial role in atherosclerosis onset, since they are recruited in the vessel wall from the beginning of the inflammatory process and participate in plaque progression and/or rupture;[88] in a nutshell, they are the primary inflammatory cell types in atherosclerotic plaques. Because activated macrophages express high TSPO levels,[89] TSPO radioligands could represent valuable tools for detecting

Table 1. First-Generation PET Tracer for TSPO

Radioligand	Chemical Class	Chemical structure	Properties	Pathologies	Applications	Ref.
[^{11}C]-PK11195	Isoquinoline carboxamides		Ki= 9.3 nM (rat) Ki = 2.1-28.5 nM (human) LogD = 3.97	Atherosclerosis/vascular injury Large vessel vasculitis	Mice Human	94–96

inflammation associated with atherosclerosis by PET. Experiments performed in an atherosclerotic mouse model showed [^{11}C]-PK11195 uptake in inflamed plaques but similar accumulation in unaffected arterial walls, discouraging a potential application in the clinical field.[90]

In addition, a clinical study performed in patients with abdominal aortic aneurysms showed that chronic inflammation of the vessel wall was not detectable with [^{11}C]-PK11195.[91]

More promising results were obtained in a proof-of-concept clinical study regarding the imaging of intraplaque inflammation with the dual-modal PET/[^{11}C]-PK11195/contrast-enhanced computed tomography (CT) angiography, where the possibility to distinguish recently active and symptomatic plaques from asymptomatic plaques was highlighted.[86]

The same dual modality was used for imaging vascular inflammation in patients with LVV (e.g., giant cell arteritis or Takayasu's arteritis),[92] a chronic granulomatous inflammatory condition occurring in the aorta vessel wall or its main branches.[93]

The radiotracer allowed the assessment of arterial inflammatory activity in LVV patients, distinguishing patients with active and nonactive or more quiescent disease.[92] PET/CT angiography could detect [^{11}C]-PK11195 uptake in the vascular wall, providing anatomical details on vessel wall thickening and excluding atherosclerotic disease.[92]

Nonetheless, the promising results of these experiments were accompanied by some drawbacks. The high level of nonspecific binding,[94] due to the high lipophilicity of the compound (Table 1), involves a weak signal-to-noise ratio that hampers its quantification, and the short half-life of carbon-11 (20 min) limits the clinical application of [^{11}C]-PK11195. To overcome these obstacles, alternative TSPO probes were developed.

3.2. Second-Generation PET Tracers for TSPO.
These new tracers were conceived to obtain compounds with lower lipophilicity than the previous generation, maintaining a high affinity for TSPO (Chart 1, Table 2). The labeling was performed mainly using fluorine-18, having a longer half-life (109.7 min) than carbon-11 and therefore being more easily manageable.

One of these new compounds is [^{18}F]-FEDAA1106 [N-(5-fluoro-2-phenoxyphenyl)-N-(2-^{18}F-fluoroethyl-5-methoxybenzyl) acetamide] (Chart 1, Table 2), which was used to image vascular inflammation in vivo in a murine model.[97] Furthermore, the same authors compared this TSPO radioligand with [^{18}F]-FDG for PET imaging of vascular inflammation, showing that the [^{18}F]-FEDAA1106 signal was significantly higher at the inflamed, disturbed flow region than the noninflamed, uniform flow regions. In contrast, differences in [^{18}F]-FDG uptake were less distinct.

Significant tracer uptake in lesion areas was observed; however, the murine model has an induced and intense local inflammation in the vessel wall, which may not appear equally in a clinical situation of atherosclerotic patients. Thus, subsequent studies used alternative approaches to better capture variability in inflammatory activity in plaques, as seen in clinical atherosclerosis.

When one benzene ring of DAA1106 was replaced with a pyridine ring, a series of phenoxyarylacetamide derivatives with a lower lipophilicity was produced (Chart 1, Table 2).

In 2016, Hellberg et al. investigated [^{18}F]-FEMPA (N-{2-[2-^{18}F-fluoroethoxy]-5-methoxybenzyl}-N-[2-(4-methoxyphenoxy)pyridin-3-yl]acetamide) (Chart 1, Table 2) as a radiotracer for the detection of atherosclerotic plaque inflammation[98] in the mouse aorta. Similar to [^{11}C]-PK11195, the [^{18}F]-FEMPA uptake ratio between the atherosclerotic plaque and the nonatherosclerotic vessel wall was not favorable; therefore, the authors proposed that this radiotracer may be more suitable for imaging intense areas of inflammation, such as LVV.

[^{18}F]-Fluoromethyl-PBR28 (N-(2-((fluoro-^{18}F)methoxy)-benzyl)-N-(4-phenoxypyridin-3-yl)acetamide) and [^{18}F]-CB251 (2-(2-(4-(2-[^{18}F]fluoroethoxy)phenyl)-6,8-dichloroimidazo[1,2-a]pyridin-3-yl)-N,N-dipropylacetamide) (Chart 1, Table 2) were used in a comparative study to evaluate their suitability for myocarditis diagnosis.[99,100] Myocarditis is an inflammatory myocardial condition that, in the acute and/or chronic form, involves variations in the number and function of lymphocytes, macrophages, and antibodies. This disease features cardiomyocyte abnormalities, which determine regional or global contractile impairment, chamber stiffening, or conduction system disease.[101] It is a relatively low-incidence pathology for which early diagnosis is hampered by the lack of specific and differential symptoms compared to other heart diseases, being easily confused with different cardiovascular pathologies, i.e., MI.[102,103] The comparative study was performed in a rat experimental autoimmune myocarditis (EAM) model. Regarding specificity, [^{18}F]-CB251 showed superior TSPO uptake in the EAM rat heart compared to [^{18}F]-fluoromethyl-PBR28, which did not significantly differ from healthy controls. Results support [^{18}F]-CB251 imaging for the noninvasive detection of myocarditis.

The potentiality of [^{18}F]-PBR06 (^{18}F-N-fluoroacetyl-N-(2,5-dimethoxybenzyl)-2-phenoxyaniline) as TSPO radiotracer to image atherosclerotic plaques in ApoE-knockout mice provided promising results. A few years later, this ligand and [^{11}C]-PBR28 (N-acetyl-N-(2-^{11}C-methoxybenzyl)-2-phenoxy-5-pyridin-amine) (Chart 1, Table 2) were used in an in vitro and in vivo pilot study in humans to evaluate the potential of these compound for the imaging of inflammatory vascular disease.
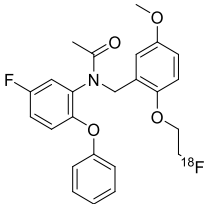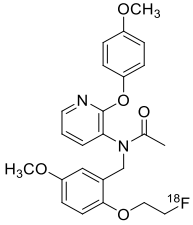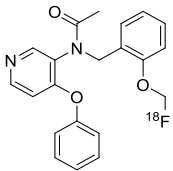
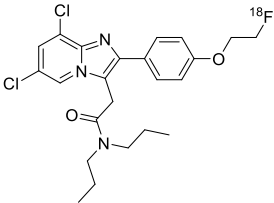**Table 2. Second-Generation PET Tracers for TSPO**
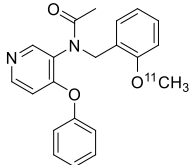
Radioligand	Class	Chemical structure	Properties	Pathologies	Applications	Ref.
[^{18}F]-FEDAA1106	Phenoxyarylacetamides		Ki= 0.078 nM (rat) logD = 3.81	Atherosclerosis/vascular inflammation	Murine	96,110
[^{18}F]-FEMPA	Phenoxyarylacetamides		PSL/mm^2= 760 ± 520 (CD68-positive areas) PSL/mm^2= 230 ± 220 P = .091 (α-SMA-positive areas) In the presence of 100 x molar excess of PK11195 PSL/mm^2= 160 ± 65; P = .15 (CD68 positive areas) PSL/mm^2= 38 ± 21, P = .14 (α-SMA-positive areas)	Atherosclerosis	Rat Human	98
[^{18}F]-FMPBR28	Phenoxyarylacetamides		Ki= 8.27 nM* (rat) *The half maximal inhibitory concentration (IC$_{50}$)	Myocarditis	Rat Human	111
[^{18}F]-CB251	Phenoxyarylacetamides		Ki = 0.27 ± 0.09 nM Log D = 3.00 ± 0.03	Myocarditis	Rat	99
[^{11}C]-PBR28	Phenoxyarylacetamides		Ki= 0.68 nM (rat) Ki= 0.94 nM (monkey) Ki= 2.2 − 52 nM (human) Log D = 3.01 ± 0.11	Inflammatory vascular disease	Human	96,112,113

Table 2. continued

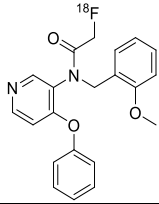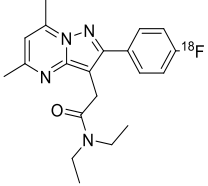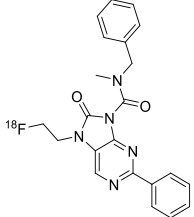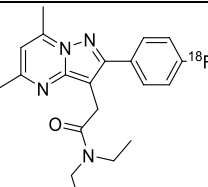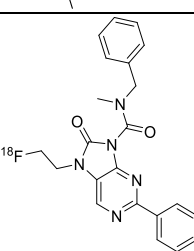| Radioligand	Class	Chemical structure	Properties	Pathologies	Applications	Ref.
[18F]-PBR06	Phenoxyarylacetamides		Ki = 0.30 nM (monkey) Ki = 0.180 ± 0.007 (rat) Ki = 0.997 ± 0.070 nM (Human) logD = 4.01	Inflammatory vascular disease	Rat Human	84,96,114
[18F]-FDPA	Pyrazolopyrimidines		Ki = 2.0 ± 0.8 nM (rat) LogD = 2.34 ± 0.05	Myocarditis	Rat	115
[18F]-FEDAC	Aryloxodihydropurine		Ki = 1.34 ± 0.15 nM (rat) LogD = 3.1	Atherosclerosis	Rabbit Human (*in vitro*)	116

Table 3. Third-Generation PET Tracers for TSPO

Radioligand	Class	Chemical structure	Properties	Pathologies	Applications	Ref.
[18F]-FDPA	Pyrazolopyrimidines		Ki = 2.0 ± 0.8 nM (rat) LogD = 2.34 ± 0.0.05	Myocarditis	Rat	115
[18F]-FEDAC	Aryl-Oxodihydropurine		Ki = 1.34 ± 0.15 nM (rat) LogD = 3.1	Atherosclerosis	Rabbit Human (in vitro)	116

Despite good uptake on surgical samples *in vitro*, PET studies in patients showed no sign of inflammation *in vivo*, indicating that those two radiotracers failed to prove clinical relevance for imaging inflammatory vessel disease.

[18F]-FEDAC (*N*-benzyl-*N*-methyl-2-[7,8-dihydro-7-(2-18F-fluoroethyl)-8-oxo-2-phenyl-9*H*-purin-9-yl]acetamide) (Chart 1, Table 2) recently showed, in *in vivo* and *in vitro* studies, the potential to detect atherosclerotic plaques in rabbits and atherosclerotic lesions and high-risk coronary plaques in humans.[104] In another study, in a rat model of coronary occlusion, [18F]-FEDAC demonstrated TSPO is a promising biomarker for imaging mitochondrial dysfunction associated with myocardial ischemia by PET/CT, highlighting the potential of TSPO ligands as tracers for the imaging of ischemic injuries in the heart.[105] A preliminary evaluation of [18F]-FDPA

(*N,N*-diethyl-2-(2-(4-18F-fluorophenyl)-5,7-dimethylpyrazolo-[1,5-*a*]pyrimidin-3-yl) acetamide) (Chart 1, Table 2) for cardiac inflammation imaging was investigated in rats after MI. The stability of uptake in the heart and the fast clearance from the other organs allowed a sufficiently large time window for cardiac imaging. Obtained data highlighted [18F]-FDPA as a potential radiotracer for cardiac inflammation.[106]

Second-generation TSPO radioligands possess higher TSPO-specific signals than first-generation radioligands. Conversely, unlike first-generation probes, they suffer from other drawbacks, such as sensitivity to TSPO single-nucleotide polymorphism (SNP) rs6971.[107] The rs6971 SNP influences the binding of TSPO radioligands in the TSPO gene that determines an amino acid substitution (Ala147Thr). Such polymorphism leads to modifications in the TSPO structure, such as the reduced

Table 4. TSPO Cardioprotective Agents
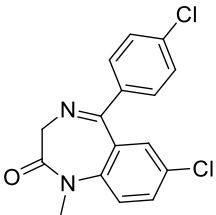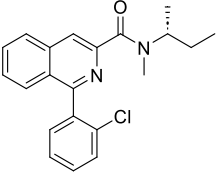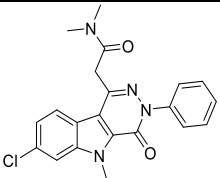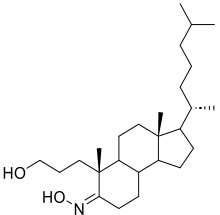
Ligand	Class	Chemical structure	Pathology	Properties	Animal	Ref
Ro5-4864	Benzodiazepines		IRI	Highly controversial:		
				Negative inotropic effect	Guinea pigs, rats, rabbits	76,119–121
				Decrease atrial rate Decrease contractile force	Dogs	122
				Modification of coronary flow rate	Rats	123
				Positive inotropic effect if induced by drugs	Rats, guinea pigs	124
				Positive inotropic effect Increase coronary blood flow	Rats	125
				Decrease atrial rate	Dogs	151
				Decrease calcium inward current	Rabbits, guinea pigs	119
				Limitation of mitochondrial membrane permeabilization Inhibition of mPTP opening	Rats	56
				Reduction ROS production Reduction xanthine and NADPH oxidase activity	Rats	126
				Decrease arrhythmias Reduction calcium overload Improve contractile performance	Rabbits	76,126
				Decrease lipid peroxidation Decrease oxysterols accumulation	Rats	35
				Counteracting cardiac ISO-inducted hypertrophy	Rats	129
				Reduction SOD and GPx activity	Rats	131
				Inhibition doxorubicin-induced contractile dysfunction	Human	132
				Reduction "no-reflow" area Reduction infarct size Improvement global systolic function	Rats, pigs	128
PK11195	Isoquinoline carboxamides		IRI	Modulation effects induced by other compounds:		
				Inhibition negative inotropism	Guinea pigs	125
				Inhibition positive inotropism Increase coronary blood flow	Rats	125
				Decrease aortic flow Decrease dP/dtmax (baroinometry) Decrease end-systolic pressure Decrease stroke	Rats	120
				Inhibition BayK8644-induced myocardial ischemia	Rats, guinea pigs	29
				Counteracting bad effects caused by verapamil	Dogs	134

Table 4. continued

Ligand	Class	Chemical structure	Pathology	Properties	Animal	Ref
				Dose-dependent (50, 100, 200 μM) mPTP opening	Rats	135
				Inhibition dose-dependently RIRR response Stabilization mitochondrial membrane potential Protection against post-ischemic injury	Dogs	138
				Limitation ROS release Limitation ROS-induced cell death	Mice	139
SSR180575	Indole-acetamides		IRI, possibly HIV (study conducted on leukocytes)	Inhibition of toxic effects produced by ROS	Rats	55
				Reduction contractile dysfunction Reduction infarction size	Rats, rabbits	55
				Reduction peroxidative damage Reduction expression pro-apoptotic proteins	Rats	57
				Dose-dependent protection leukocytes from TNFa-induced apoptosis	Human blood	141
				Dose-dependent restoration left ventricular function Recovery 50% cardiac function Prevention ischemia Prevention mitochondrial uncoupling Inhibition oxidative phosphorylation	Rabbits	57
TRO40303	Cholesterol-like TSPO ligand		IRI, selected for phase I and phase II studies	Reduction infarct size Reduction 38% pro-apoptotic proteins	Rats	143
				Reduction ROS release Inhibition 50% pore opening	Rats	143,145
				Reduction oxysterols production	Mice	147
				Prevention doxorubicin-induced changes in contractility Increase cardiomyocyte viability	Rats, mice	132

distance between the second and fifth transmembrane domains, resulting in a lower radioligand affinity. Notably, there are three different human subject categories, namely high-affinity binders (HABs), low-affinity binders (LABs), and mixed-affinity binders (MABs) that are, respectively, homozygous for wild-type TSPO, homozygous for the Ala147Thr TSPO, and heterozygous.[108,109] Thus, patients with the same TSPO density but different genotypes will provide different PET signals; therefore, TSPO genotyping is required to interpret imaging outcomes. For this reason, a third generation of ligands with low sensitivity toward rs6971 was established (Chart 1, Table 3).

3.3. Third-Generation PET Tracers for TSPO. [18F]-GE180 (S-*N*,*N*-diethyl-9−2-18F-fluoroethyl)-5-methoxy-2,3,4,9-tetrahydro-1*H*-carbazole-4-carboxamide (Chart 1, Table 3)[117] is a tricyclic-indole compound and has been used in the imaging of atherosclerotic plaque inflammation in a mouse model. [18F]-GE180 shares the same high level of TSPO specificity of second-generation ligands and the low sensitivity to SNP of the first-generation class. It showed similar binding characteristics compared to the previously described [18F]-FEMPA, displaying uptake in macrophage-rich areas in atherosclerotic lesions and lesion-free vessel walls in mice.[83]

Recently, [18F]-LW223 [(*R*)-*N*-(*sec*-butyl)-3-((fluoro-18F)-methyl)-*N*-methyl-4-phenylquinoline-2-carboxamide] (Chart 1, Table 3) was developed and tested in the detection of macrophage-driven inflammation in a rat MI model.[118] MI is a life-threatening condition caused by a lack of blood flow and oxygen supply to the heart muscle. It is due mainly to the development of plaques within arterial walls that may occlude the coronary vessels *in situ* or, quite commonly, by vulnerable plaque rupture and distal embolism. The binding of [18F]-LW223 to TSPO in the human brain and heart in *in vitro* assays highlighted that it is not susceptible to the rs6971 human genetic polymorphism. In addition, [18F]-LW223 detected and quantified the macrophage-driven inflammation in a rat MI model, holding promise in clinical translation for the prognosis of MI.[118]

4. TSPO LIGANDS AS CARDIOPROTECTIVE AGENTS

Several studies have been conducted on TSPO ligands, and their cardioprotective effects have been mainly associated with stabilizing mitochondrial function.

4.1. Ro5-4864 (4′-chlorodiazepam). Ro5-4864 (4′-chlorodiazepam, Figure 1, Table 4) is a TSPO ligand belonging

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to the class of benzodiazepines in which, basically, a chlorine atom was inserted at the *para* position of the 4-phenyl ring of diazepam, a clinically used benzodiazepine drug.

Regarding its role in the physiological activity of the heart (heart rate and contractility), the results reported in the literature have been highly controversial. In a first study, Ro5-4864 showed a negative inotropic effect in several models, for example, in the left and right ventricle papillary muscles of guinea pigs, rats, and rabbits.[76,119−121] In another study, Saegusa et al. demonstrated that injecting Ro5-4864 into the sinus node of an isolated canine right atrium decreased atrial rate and contractile force at concentrations between 100 and 1000 μg.[122] Grupp et al. questioned these findings, proving that Ro5-4864 did not show inotropic effects, either positive or negative, but only modified the coronary flow rate by producing a dose-dependent increase in isolated perfused rat hearts.[123] However, in a following study, Weissman et al.[124] reported that Ro5-4864 did not act as an inotropic agent by itself but incremented the pro-ischemic inotropic effect induced by a calcium channel activator (BayK8644). On the contrary, Leeuwin et al. demonstrated that Ro5-4864 has a dose-dependent positive inotropic effect and increases coronary blood flow in rat hearts when administered at concentrations greater than 24 μM.[125] These controversial findings may be due to different doses utilized for the tested ligand or the different animals/species. Leeuwin et al. also demonstrated that the effects mediated by Ro5-4864 were abolished by PK11195.[125] Also, PET studies highlighted a negative chronotropic action of Ro5-4864 when injected at 200 μg/kg or more,[27] while a further work demonstrated the role of the ligand in decreasing the calcium inward current during the second phase of the action potential in rabbit and isolated guinea pig cardiomyocytes at concentrations greater than 3 μM.[119]

With respect to pathological conditions, TSPO seems to be mainly involved in IRI. As recently demonstrated by Obame et al., Ro5-4864 was able to reduce cardiac injury and increase recovery during reperfusion following an ischemic event in rats.[56] This effect is probably associated with inhibition of both Cyt-c release and the activity of AIF. In particular, Ro5-4864 increases the resistance of mitochondria to calcium-induced pore opening by stabilizing the association of the antiapoptotic Bcl-2 with the mitochondrial membrane and hampering the association of the proapoptotic Bax.

To summarize, the cardioprotective effects of Ro5-4864 are related to its ability to limit mitochondrial membrane permeabilization and inhibit mPTP opening.[56] However, mPTP opening inhibition is not due to a direct action of the ligand on the pore, given that Ro5-4864 did not counteract mPTP opening in isolated mitochondria compared to cyclosporine A, an mPTP opening inhibitor used as reference. Paradoxically, Ro5-4864 induced pore opening at concentrations several orders of magnitude higher than those required to saturate the receptor. In the same study, it has been shown that Ro5-4864 administration resulted in the restoration of mitochondrial respiration and oxidative phosphorylation, which could be associated with the inhibition of mitochondrial Cyt-c release, making it more available for the electron transfer chain.[56]

In 2010, Xiao et al. confirmed these results, indicating that Ro5-4864 reduces ROS production caused by a sudden increase in oxygen during reperfusion and increases the activity of complexes I and III of the mitochondrial electron transport chain while blunting the activity of the ROS generating xanthine and NADPH oxidase.[126]

Ro5-4864 can also decrease the incidence of arrhythmias and reduce calcium overload. In addition, if administered at reperfusion, it protects against postischemic arrhythmias following reperfusion in rabbit hearts; if administered before ischemia, it improves the recovery of the post-IR contractile performance.[76,126] In a preclinical study conducted on pigs, Ro5-4864 intracoronary administration at the onset of reperfusion, after 60 min of coronary occlusion, improved faster ST-segment elevation resolution without hemodynamic complications and reduced microvascular damage, but it did not significantly reduce infarct size.[127]

As mitochondrial membrane fluidity appears to be impaired after cardiac IR, Paradis et al. investigated the causes of this membrane alteration on mitochondria isolated from rat hearts subjected to a half-hour ischemia followed by a quarter-hour reperfusion; the administration of Ro5-4864 appeared to ameliorate this condition.[35] Specifically, membrane fluidity was measured by evaluating the change in steady-state fluorescence anisotropy of two fluorescent probes, namely 1,6-diphenyl-1,3,5-hexatriene (DPH) and hematoporphyrin IX (HP), that bound, respectively, to hydrophobic lipid regions and protein sites in mitochondrial membranes. The results indicated that cholesterol accumulation in the mitochondrial matrix in the post-IR setting indirectly affected membrane fluidity at lipid regions by promoting lipid peroxidation, which was prevented by the administration of Ro5-4864. Cholesterol accumulation, established during reperfusion, remains one of the major problems related to the cardiovascular system, as it leads to atherosclerosis, arterial stenosis, thrombosis, and myocardial ischemia. In this respect, Paradis et al. also showed that Ro5-4864 strongly inhibited the accumulation of cholesterol and cholesterol-derived oxidation compounds (oxysterols) during reperfusion, improved respiration parameters, and decreased the sensitivity of mPTP opening.[35] Collectively, these data demonstrate that one of the main cardioprotective effects exerted by Ro5-4864 is the limitation of dangerous effects exerted by cholesterol accumulation and lipid peroxidation to limit mitochondrial membrane derangements and functional impairments.

In a study conducted in 2016, the authors[128] compared the effects of intracoronary Ro5-4864 (2 μM) administered to pigs or rats just prior to or immediately after reperfusion, respectively. The treatment causes a reduction of the "no-reflow" area and guarantees long-term positive effects, such as a reduction in infarct size and improvement in global systolic function in rats. In large animals (pigs), closer to the human physiology, Ro5-4864 caused more or less the same effects with the addition of more rapid resolution of ST-segment elevation.

Another study conducted in 2010 concerning oxidative stress proved that Ro5-4864 could counteract cardiac hypertrophy in rats induced by isoproterenol (ISO),[129] a nonselective β-adrenergic agonist associated with an increase in oxidative stress, fibrosis, and hypertrophy. ISO binding to β-adrenergic receptors causes positive inotropic and chronotropic effects; specifically, it causes both peripheral vasodilatation and cardiac hypoxia, resulting in relative ischemia and calcium overload leading to excessive ROS production, oxidative stress, infarct-like cardiomyocyte necrosis, and myocardial fibrosis. The results showed that Ro5-4864 decreased myocyte hypertrophy, fibrosis, and necrosis, which were previously increased by the high levels of ROS. Concerning myosin heavy chain (MHC) isoform

expression in murine models, in physiological conditions the β-isoform is less expressed than the α-isoform, but in the case of cardiovascular problems the former is upregulated; in fact, β-MHC is considered a marker of hypertrophy. Administration of Ro5-4864 resulted in the downregulation of β-MHC expression and the reduction of left ventricular size. Important data emerged from this study regarding Ro5-4864 doses. Indeed, while the decrease in isoprenaline-induced production of thiobarbituric acid reactive substances (TBARs) did not change with increasing doses, different data were found on reducing two endogenous antioxidants, glutathione peroxidase (GPx) and superoxide dismutase (SOD). In fact, Ro5-4864 at 0.1 mg/kg inhibited the decrease in both antioxidants, but when the doses were increased to 0.5 mg/kg the effect was maintained only toward the SOD.[129] The explanation for this trend is not clear from this study, but Ro5-4864 has already demonstrated to have such paradoxical effects in terms of concentrations. Indeed, from a previous *in vitro* study conducted by Kenyon et al., it emerged that nanomolar concentrations of Ro5-4864 caused aldosterone release, but this effect is not retained by increasing concentrations to a micromolar range. This could be due to inhibitory effects involving enzyme competition.[130] Clearly the cardioprotective effects of the TSPO ligand are complex and need more focused studies.

In a more recent study, the possible cardioprotective effects of Ro5-4864, alone or in the presence of the NO synthase (NOS) inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME), were evaluated in a model of ISO-induced rat MI.[131] Lower levels of circulating and myocardial markers of ischemic injuries were measured in the Ro5-4864-treated group, which was partially prevented by L-NAME, thus suggesting that NO should be an important mediator of the TSPO ligand action.[131]

In another work, the role of Ro5-4864 against doxorubicin-induced cardiac damage has been evaluated. Doxorubicin is an antineoplastic antibiotic of the anthracycline family with a broad antitumor spectrum. However, it causes contractile dysfunction and the development of cardiomyopathies related to mitochondrial dysfunction, proved by the fact that the mitochondrial respiratory chain is the primary source of ROS during the treatment. In this context, the effects of Ro5-4864 have been studied on adult isolated-paced cardiomyocytes. The results evidenced that Ro5-4864 inhibits the doxorubicin-induced contractile dysfunction, which initially manifests as acute myocardial injury and then advances to a chronic congestive HF by augmenting ROS production and mPTP opening.[132]

4.2. PK11195. PK11195 (Figure 1, Table 4) is a TSPO ligand belonging to the class of isoquinoline carboxamides.[133] It should be remembered that PK11195 influences neither heart contractility nor coronary flow rate by itself. Still, it modulated Ro5-4864 effects, even if the two ligands display a similar affinity for TSPO. In fact, PK11195 was shown to inhibit the effects produced by Ro5-4864 on the heart, including the negative inotropism on papillary muscle from the right and left ventricles of guinea pigs and the dose-dependent positive inotropism together with the increase of coronary blood flow in rat hearts.[125] Nevertheless, PK11195 resulted in diminishing aortic flow, dP/dt_{max} (baroinometry), end-systolic pressure, and stroke work more than Ro5-4864;[120] this may be due to the interactions with the target that are influenced by the nature of the ligand in terms of enthalpy and entropy, which are the basis of the binding equilibrium.[133]

Cytosolic calcium concentrations play a crucial role in ischemic processes. Therefore, it is thought that the interaction

of TSPO with L-type voltage-dependent calcium channels located on the plasma membrane may contribute to the development of this pathological condition.

As previously explained in section 4.1, Ro5-4864 can increase BayK8644-induced myocardial ischemia; in contrast, PK11195 has been shown to inhibit it.[29] Both Ro5-4864 and PK11195 could also have a direct action on calcium channels, in addition to that via TSPO. In fact, in a study conducted in dogs treated with verapamil, an antihypertensive calcium antagonist that induced heart toxicity, PK11195 proved to counteract some effects caused by the drug. For example, PK11195 could restore sinus activity lost during verapamil treatment but could not prevent or cure hemodynamic changes.[134]

In a study conducted in 2006,[135] the ability of PK11195 to promote mPTP opening, leading consequently to Cyt-c release and mitochondrial uncoupling, was investigated. The results showed that PK11195 causes dose-dependent (50, 100, and 200 μM) mPTP opening. In general, a calcium concentration above 100 μM is required to cause pore opening,[136] but the results of this study showed that PK11195-induced opening does not require calcium, consistent with the fact that it can interact directly with voltage-dependent calcium channels located on the plasma membrane.[137] This study also revealed that CsA prevented PK11195-induced opening, inhibiting pro-apoptotic protein release.

Regarding oxidative stress, PK11195, at concentrations from 5 to 25 mg/kg, resulted in the dose-dependent inhibition of the RIRR response, stabilizing mitochondrial membrane potential and protecting against postischemic injury and early and delayed ventricular arrhythmias induced in dogs by 20 min of ischemia.[138]

In a more recent study conducted in 2020, PK11195 was shown to have a unique mechanism to limit the RIRR phenomenon in rabbits. Indeed, when administered at the moment of reperfusion, PK11195 limited ROS release and ROS-induced cell death.[139] In more detail, rabbit ventricular myocytes were subjected to 20 min of ischemia and then to 3 h of reperfusions in both the absence and the presence of 50 μM PK11195 treatments, the first after 15 min, the second after 1 h, and the third after 3 h from reperfusion. Myocyte death was assessed by lactate dehydrogenase (LDH) assay, while other effects, such as changes in calcium concentration, membrane potential, and ROS release, were examined by confocal microscopy combined with fluorescent indicators. An important finding emerged from the results; it was shown that PK11195, administered at the onset of reperfusion, normalized succinate oxidation and glutamate utilization. However, when administered earlier during ischemia, it did not exert cardioprotective effects. These findings highlighted the importance of the timing of PK11195 administration during myocardial ischemia.[139]

4.3. SSR180575. SSR180575 (7-chloro-N,N,5-trimethyl-4-oxo-3-phenyl-3,5-dihydro-4H-pyridazino[4,5-b]indole-1-acet-amide, Table 4) is a TSPO ligand that features both neuroprotective and cardioprotective effects.[57] As mitochondria not only represent an energy source but also regulate the cell life—death cycle in case of pathological conditions, ROS production represents one of the major problems in IRI; in particular, H_2O_2 causes a massive reduction of mitochondrial membrane potential, inhibition of oxidative phosphorylation, and release of pro-apoptotic proteins such as caspase 3. In several *in vitro* and *in vivo* models of cardiac IRI, SSR180575 prevented the ROS-dependent decrease of mitochondrial membrane potential, reduced oxidative phosphorylation

capacities, Cyt-c release, caspase 3 activation, and DNA fragmentation.[55] In more detail, administering SSR180575 in perfused rat hearts (100 nM to 1 μM) or by oral pretreatment (3−30 mg/kg), reduced contractile dysfunction caused by reperfusion. Furthermore, a marked reduction in infarction size was noted in both isolated rabbit hearts and anaesthetized rats undergoing left coronary artery occlusion followed by reperfusion; this is probably related to the coexistence of necrosis and apoptosis during ischemia, which diminishes in case of treatment with SSR180575.[140] Still, administration of SSR180575 both preventively and therapeutically restored left ventricular function in a dose-dependent manner following an ischemic event in a model of rabbit hearts. SSR180575 also improved recovery of cardiac function by 50% and prevented ischemia-induced reperfusion, the primary cause of cardiac damage.[55] Regarding oxidative stress, it was noted that SSR180575 prevented mitochondrial uncoupling and inhibition of oxidative phosphorylation in treated mitochondria subjected to H_2O_2. Therefore, it is thought that it may act directly on mitochondria involved in calcium homeostasis, one of the critical points of ischemic damage.[57]

Another study has determined TSPO's role in signaling pathways that lead to cell death. In particular, the study focused on renal dysfunction in a rat IR model. Similar to what was observed for the heart, ischemia followed by reperfusion caused both apoptosis and tubular necrosis in kidneys due to increased production of ROS, thus determining peroxidative damage, high expression of the proapoptotic protein Bax, low expression of the antiapoptotic protein Bcl-2, and caspase 3 release. Treatment with SSR180575 significantly reduced these damaging effects.[57] Another study on apoptosis was conducted in 2010 on polymorphonuclear leukocytes (PMNs), as TSPO is highly expressed in blood cells. Specifically, administration of SSR180575 protects leukocytes from TNFα-induced apoptosis in a dose-dependent manner, thus leading to the conviction that targeting TSPO could represent a potential strategy for the treatment of pathologies characterized by an increased blood cell apoptosis, such as HIV (human immunodeficiency virus).[141]

4.4. TRO40303. TRO40303, namely 3,5-seco-4-nor-cholestan-5-one oxime-3-ol (Table 4), is a cholesterol-like TSPO ligand that binds specifically to the cholesterol site of TSPO; it was initially identified as a neuroprotective agent[142] but was later investigated in more detail for its roles in cardioprotection, as [^{14}C]TRO40303 accumulates rapidly in rats' hearts after a single intravenous administration (2 mg/kg).[143] In the same study, rats were subjected to 35 min of ischemia and then reperfused for 24 h. Administration of TRO40303 (2.5 mg/kg) immediately before reperfusion reduced infarct size by 38%, and this was associated with a reduction in pro-apoptotic proteins, especially AIF, but not Cyt-c release, which is a major cause of cell death.[144] TRO40303 showed no effect on calcium retention but still caused a delay in mPTP opening in rats subjected to 2 h of ischemia followed by 2 h of reperfusion and inhibited pore opening in cardiomyocytes of neonatal rats treated with H_2O_2. It is therefore thought that the delayed mPTP opening is not due to an action on calcium retention but to a reduction in ROS production. Comparing the effects of TRO40303 with those of CsA on pore opening and oxidative stress, it was noted that TRO-dependent effects occur earlier than those caused by CsA and, in particular, mPTP opening inhibition promoted by CsA coincides with its effects on ROS production and calcium overload. In addition, it was found that effects mediated by both CsA and TRO40303 on mitochondrial and cytosolic calcium

concentration increases are similar, demonstrating that Ca^{2+} increase is a secondary and a pore-opening-dependent effect. By subjecting cardiomyocytes to H_2O_2-induced oxidative stress with subsequent ROS production, different effects were observed for TRO40303 and CsA. Specifically, TRO40303 drastically reduced ROS release, while CsA caused a much smaller ROS reduction; however, both TRO40303 and CsA inhibited pore opening by 50%. This effect could be explained by the fact that while TRO40303 inhibits mPTP opening by reducing the production of ROS, CsA acts by binding and inhibiting CypD, a protein that acts as a gate within the pore, decreasing the release of Cyt-c that causes programmed cell death.[143,145] It is worth mentioning that TRO40303 is a TSPO ligand that binds to the cholesterol site, unlike the other ligands discussed above. Therefore, it could also exert its action by binding directly to specific components related to TSPO, such as VDAC, promoting its interaction with a hexokinase that leads to the maintenance of the required ATP concentrations and augmenting glycolysis.[146] Furthermore, TRO40303 also reduced the oxysterol production leading to a minor cholesterol accumulation into the mitochondrial membrane during reperfusion, diminishing the related injury and the complications due to the presence of comorbidities such as dyslipidemia and hypercholesterolemia.[147] Still related to ROS production, TRO40303 prevented the doxorubicin-induced changes in contractility and augmented cardiomyocyte viability, as previously reported for Ro5-4864.[132] It is of great importance that TRO40303 was selected as a cardioprotective agent for a randomized phase I study carried out double-blind. Specifically, the study was based on the treatment of healthy male subjects, together with postmenopausal and hysterectomized women, with TRO40303 administered intravenously from 0.5−13 mg/kg with a flow rate of 0.04−35 mL/min. The results showed that the doses were tolerated without causing any major adverse effects, clarifying that the active dose is 6 mg/kg.[148] As previously mentioned, timely revascularization following an ischemic event is currently the only accepted therapy in cases of MI, despite causing the so-called reperfusion injury. For this reason, TRO40303 was selected as a cardioprotective agent for a phase II study (MITOCARE), a double-blind trial in which its safety and efficacy in limiting reperfusion injury were evaluated. In particular, reperfused patients treated for acute ST-elevation myocardial infarction (STEMI) with percutaneous coronary intervention (PCI) or thrombolysis were treated with TRO40303 or a placebo in addition to their current standard cardiac drugs. Efficacy was assessed primarily by measuring infarct size expressed as area under the curve (AUC) for plasma creatine kinase (CK) and troponin I over 3 days and secondarily by measuring infarct size normalized to the myocardium at risk using cardiac magnetic resonance (CMR) together with the evaluation of left ventricular function, echocardiography, ST-segment decrease, microvascular obstruction, and extension of the infarct after PCI.[149] Unfortunately, data showed no major differences in the reduction of reperfusion damage and infarction between TRO40303-treated and placebo-treated subjects. These results raised a more provocative question of whether reperfusion injury occurs in humans, which calls for further and more in-depth studies.[150]

5. FUTURE PERSPECTIVES AND CONCLUSIONS

Cardiovascular diseases are the leading causes of morbidity, disability, and death in Europe and worldwide. In the last two years, SARS-CoV2 (severe acute respiratory syndrome

coronavirus 2) infection has been an aggravating factor, enhancing cardiovascular frailty due to inflammation and stress. The current evidence suggests that TSPO ligands are promising cardioprotective candidates, primarily acting at the level of mitochondria to contrast oxidative stress and optimize the flux and fate of cholesterol away from the accumulation of toxic cholesterol catabolites in mitochondria. In this way, TSPO may contribute to the modulation of inflammation. However, the exact molecular mechanisms of TSPO action in the heart remain largely debated and unclarified, together with the clinical outcomes of TSPO-based drug agents. We reviewed the characteristics of several pharmacological agents in detail. So far, studies have been controversial on the outcomes of TSPO-based drugs on cardiac contractility and perfusion under physiological conditions, which may be partly attributed to the variable models or drug dosage adopted in different studies. More consistent findings support a beneficial role in alleviating IRI, in which a variety of targets have been proposed, including apoptosis-inducing factor (AIF), mitochondrial respiration and reactive oxygen species (ROS) production, calcium load (affecting cardiac rhythm), and lipid peroxidation (affecting membrane stability). However, the reduction of infarct size was only observed sporadically in specific models and selected circumstances.

In principle, molecular imaging by PET represents an ideal tool to characterize the *in vivo* sites of action of TSPO. PET imaging of radiolabeled TSPO ligands has been more traditionally investigated in the brain, where an elevation in TSPO binding reflects the activation of microglial cells and is considered a biomarker of neuroinflammation. Building on this inheritance, the imaging paradigm has been translated into the cardiovascular area with an initial focus on the inflammatory states accompanying CVDs, namely atherosclerotic plaque vulnerability, vasculitis, and myocarditis. Three generations of TSPO-targeted PET tracers have been synthesized and tested. First-generation tracers proved to be insufficiently sensitive to stratify plaque vulnerability but may still prove useful in diagnosing more severe inflammation in vasculitis. Second-generation probes were able to capture myocarditis or cardiac inflammation following MI; some of them hold promise in the diagnosis of inflamed plaques in animals, and one ($[^{18}F]$-FEDAC) also holds promise in humans. However, their binding and imaging signal is influenced by gene polymorphisms, hampering the interpretation of images unless genotyping is contextually performed. Third-generation probes appear to be less sensitive to genetic variability, showing a similar capacity to detect atherosclerotic plaque inflammation compared to second-generation radioligands as well as promising sensitivity to detect macrophage-driven inflammation in MI.

In conclusion, the study of TSPO in the heart has opened new diagnostic and therapeutic leads to address CDs. A better mechanistic understanding of such a multisite-acting protein requires systematic studies comparing disease models, doses, responses, and mechanisms in a consistent manner. TSPO-PET imaging has been so far used to seek inflammation; however, in light of the above-summarized mechanisms and their known involvement in different phases of CD damage, repair, and remodeling, it would seem appropriate to image the heart and vessels along disease phases, from insult to dysfunction to symptomatic disease. In fact, it is tempting to speculate that TSPO binding may signal different mechanisms in different phases. The development of organelle-selective PET tracers would greatly enhance the potential of molecular imaging to

contribute to understanding the described complexity and contradictions.

In this respect, the authors believe that the present overview on the advantages and limitations of TSPO ligands will help to design and develop new therapeutic and/or diagnostic tools with better efficacy, which will lead to unraveling the fundamental mechanisms and providing solutions to still unanswered questions in CDs.

■ AUTHOR INFORMATION

Corresponding Authors

Sabrina Taliani — *Department of Pharmacy, University of Pisa, Pisa 56126, Italy;* ⓘ orcid.org/0000-0001-8675-939X; Email: sabrina.taliani@unipi.it

Luca Menichetti — *Institute of Clinical Physiology, National Research Council of Italy, CNR Research Area, Pisa 56124, Italy;* ⓘ orcid.org/0000-0002-3293-9616; Email: luca.menichetti@cnr.it

Authors

Emma Baglini — *Institute of Clinical Physiology, National Research Council of Italy, CNR Research Area, Pisa 56124, Italy;* ⓘ orcid.org/0000-0002-8378-4052

Valeria Poggetti — *Department of Pharmacy, University of Pisa, Pisa 56126, Italy*

Chiara Cavallini — *Institute of Clinical Physiology, National Research Council of Italy, CNR Research Area, Pisa 56124, Italy*

Debora Petroni — *Institute of Clinical Physiology, National Research Council of Italy, CNR Research Area, Pisa 56124, Italy*

Francesca Forini — *Institute of Clinical Physiology, National Research Council of Italy, CNR Research Area, Pisa 56124, Italy*

Giuseppina Nicolini — *Institute of Clinical Physiology, National Research Council of Italy, CNR Research Area, Pisa 56124, Italy*

Elisabetta Barresi — *Department of Pharmacy, University of Pisa, Pisa 56126, Italy;* ⓘ orcid.org/0000-0002-9814-7195

Silvia Salerno — *Department of Pharmacy, University of Pisa, Pisa 56126, Italy*

Barbara Costa — *Department of Pharmacy, University of Pisa, Pisa 56126, Italy;* ⓘ orcid.org/0000-0002-7598-1275

Patricia Iozzo — *Institute of Clinical Physiology, National Research Council of Italy, CNR Research Area, Pisa 56124, Italy*

Danilo Neglia — *Fondazione CNR/Regione Toscana Gabriele Monasterio, Cardiovascular and Imaging Departments, CNR Research Area, Pisa 56124, Italy*

Federico Da Settimo — *Department of Pharmacy, University of Pisa, Pisa 56126, Italy;* ⓘ orcid.org/0000-0002-7897-7917

Complete contact information is available at:
https://pubs.acs.org/10.1021/acs.jmedchem.3c01716

Author Contributions

Notes

The authors declare no competing financial interest.

Biographies

Emma Baglini gained her M.Sc. in Pharmacy (2018) *summa cum laude* and Ph.D. degree in Science of Drug and Bioactive Substances (2022) from the University of Pisa. She has been a postdoctoral researcher in the group of Professor Da Settimo at the University of Pisa, working on the design, synthesis, and characterization of small molecules as anticancer agents. In 2023, she was awarded for the best doctoral dissertation by the University of Pisa. Currently, she is a researcher, III° level, at the CNR Institute of Clinical Physiology in the context of the project "SEE LIFE - StrEngthEning the ItaLIan InFrastructure of Euro-bioimaging". She is the author of 20 papers in high-impact international journals in the field of medicinal chemistry and communications to congresses.

Valeria Poggetti graduated with a degree in Chemistry and Pharmaceutical Technology in April 2021 at the University of Pisa. After a three-month fellowship, in November 2021 she got a position in the frame of the Doctoral School Science of Drug and Bioactive Substances at the Department of Pharmacy, University of Pisa, with a project entitled "Development of small molecules as modulators of cell life death pathways". She is currently at the second year of her Ph.D. program. She is the author of five papers in high-impact international journals in the field of medicinal chemistry and communications to national congresses.

Chiara Cavallini holds a Ph.D. in Science of Drug and Bioactive Substances from the University of Pisa, where she also obtained her master's degree in Chemistry and Pharmaceutical Technology. She has been a postdoctoral researcher at the Institute of Clinical Physiology of the National Research Council in Pisa, where she contributes to several research projects that focus on molecular imaging. Her research was geared towards testing innovative chemical entities and nanosystems for multimodal imaging and therapy in cancer and regenerative medicine. Currently, she is a Researcher at CNR Nanoscience Institute in Pisa, where she works in the frame of the "THE − Tuscany Health Ecosystem" Project. She has authored 17 scientific articles published in high-impact international journals and communicated her findings at several national and international congresses.

Debora Petroni graduated with a degree in Chemistry at the University of Pisa in 1996. In the following years, she worked as a radiochemist at the Institute of Clinical Physiology of the National Research Council in Pisa, where currently is a researcher. She was always involved in the radiopharmaceutical field, both in preclinical research and the production of radiopharmaceuticals for clinical, carrying out her activity in a "Good Manufacturing Practice"-compliant facility. Her research interests concern the development of new radiopharmaceuticals for nuclear imaging, mainly for positron emission tomography.

Francesca Forini graduated in 1996 with a degree in Biological Science at UNIPI and in 2002 gained a Ph.D. degree in Pathophysiology of Ischemic Heart Disease at Scuola Superiore S. Anna of Pisa. Currently she is a senior researcher at the CNR Institute of Clinical Physiology where she directs the Translational Cardiovascular Endocrinology group. The research activities include: (i) identification of thyroid hormone-dependent cardioprotective mechanisms in *in vitro* and *in vivo* models of post ischemic cardiac injuries, (ii) identification of molecular regulatory networks underlying the pathogenesis of idiopathic pulmonary artery hypertension, and (iii) characterization of the role of thyroid hormone dyshomeostasis in the development of diabetic retinopathy. The main pathophysiological processes investigated are mitochondrial function and quality control, cell death, adverse tissue remodelling, and gene regulation by noncoding RNA.

Giuseppina Nicolini graduated with a degree in Biological Sciences at the University of Pisa (1996). Currently she is a Senior Researcher at the Translational Cardiovascular Endocrinology group at the Institute of Clinical Physiology at CNR Pisa. Her research interests concern myocardial ischemia and reperfusion injury, cardiac remodeling, cellular and molecular biology, mitochondrial pathophysiology, thyroid hormone signaling in cardiac disease, and miRNA/gene regulatory networks. The main focus of her research is on the identification of T3-dependent cardioprotective molecular mechanisms *in vitro* and the *in vivo* model involved in cardiac remodeling processes, which could be the target of T3 replacement treatment in patients with heart failure and "Low-T3 Syndrome".

Elisabetta Barresi gained her Ph.D. degree in Medicinal Chemistry at the University of Pisa in 2014. Since 2023 she has been an Associate Professor in Medicinal Chemistry at the Department of Pharmacy, University of Pisa. Her scientific and research activity has focused on the study of molecules properly functionalized to bind several targets, including adenosine receptors, translocator protein (TSPO), and topoisomerases. She is also involved in the synthesis of probes for imaging A_2B receptor and TSPO, H_2S-releasing agents and molecules capable of modulating p53 pathways. In 2018, she was awarded for her relevant research in medicinal chemistry with the "Premio della Divisione Famaceutica". She is the author of more than 50 papers published in international journals and communications to congresses.

Silvia Salerno graduated with a degree in Chemistry and Pharmaceutical Technology in 1996 and gained a Ph.D. degree in Medicinal Chemistry in 2000 at the University of Pisa. Since 2006, she has been University Researcher in Medicinal Chemistry at the Department of Pharmacy of the University of Pisa. She has published about 60 scientific papers on international journals, in collaboration with Italian and international teams. Her research interests involve the areas of medicinal chemistry and, in particular, the development of suitably decorated heteropolicyclic compounds to interact with several targets mainly involved in cancer and neurodegenerative diseases. These targets include not only enzymes such as topoisomerases, tyrosine kinases, and carbonic anhydrases but also receptors such as TSPO, and adenosine receptor and macromolecules as DNA. She is a member of the Italian Chemical Society.

Barbara Costa gained her PhD (2000) in Biotechnologies Applied to Pharmacology and Cellular/Molecular Biotechnologies Applied to Biomedical Sector at the University of Milan. Currently, she is Full Professor at the University of Pisa. The main research fields include: (i) the development of novel synthetic ligands with therapeutic/diagnostic potential through the study of their thermodynamic parameters at equilibrium and investigation of their biological activities; (ii) mechanisms underlying cell functionality, life/death balance, differentiation, and communication; and (iii) the role of single nucleotide polymorphisms in psychiatric disorders. In the context of these issues, she focuses on the 18 kDa translocator protein with the goal of elucidating its role in behavioral alterations and fundamental cellular processes in both physiological and pathological conditions. She is the author of 105 scientific publications and one patent.

Patricia Iozzo (Ph.D., Specialist in Endocrinology-Metabolism and in Nuclear Medicine) is the Research Director at the CNR Institute of Clinical Physiology and Docent at the University of Turku. She has 30 years of experience in the study of obesity, type 2 diabetes, and related cardiovascular, neurodegenerative, and hepatic diseases using multimodal imaging in preclinical and clinical research, more recently in combination with omics technologies addressing gut-organ axes. She has conducted research at foreign centres in USA, UK, FI, building long-lasting collaborations, and has pioneered novel PET-CT- and

MRS-based approaches, translating them into human research use. She is the recipient of multiple project grants, a member of many national and international expert panels and scientific committees, and active in the supervision of Master and Ph.D. students in Italy and Finland.

Danilo Neglia has been a senior cardiologist and nuclear medicine specialist at Fondazione CNR/Regione Toscana in Pisa since 1997. He is an Affiliate Researcher at the Institute of Life Sciences and a Faculty Member of the Ph.D. Program in Translational Medicine of Scuola Superiore Sant'Anna in Pisa. He has coordinated national and international collaborative research projects in multimodal cardiac imaging and is currently the coordinator of the ESC-EORP EURECA Registry. He is Vice-President of the European Society of Cardiology Working Group on Nuclear Cardiology and Cardiac CT. He is an Italian Association of Cardiologists' Imaging Committee member. He has authored over 160 peer-reviewed publications and several book chapters, and he is Associate Editor of the *European Heart Journal CVI* and the *Journal of Nuclear Cardiology*.

Luca Menichetti received his Ph.D. in Design, Drug Development, and Bio-Pharmaceutical Testing and a specialization in Clinical Biochemistry from the Faculty of Medicine at the University of Pisa. Starting from a background in the radiochemistry of Transition Metals at the JRC-Institute for Transuranium Elements (Karlsruhe), he moved his interests to tracer chemistry for medical applications for the development of radiopharmaceuticals. He is a Senior Researcher at CNR–IFC in Pisa and a Contract Professor at the Universities of Pisa and Siena. He has developed novel tracers for imaging modalities such as magnetic resonance spectroscopy and nanostructured multifunctional materials. He is the scientific coordinator of national and European projects in the field of theranostics and has authored over 110 ISI publications.

Sabrina Taliani graduated with a degree in Chemistry and Pharmaceutical Technology (1994) and gained a Ph.D. degree in Medicinal Chemistry (1998) at the University of Pisa. Since 2015 she has been Associate Professor in Medicinal Chemistry at the Department of Pharmacy, University of Pisa. Her research interests, carried out in collaboration with Italian and international teams, focus on the development of heteropolycyclic aromatic derivatives appropriately decorated to interact with several targets, including the central benzodiazepine receptor, adenosine receptors, and translocator protein, as well as DNA intercalators, topoisomerase, and enzyme inhibitors. She also develops new molecular probes for imaging TSPO, H_2S-releasing agents, and small-molecules able to modulate p53 activity. She is the author of more than 135 papers in high-impact international journals and one patent.

Federico Da Settimo graduated *summa cum laude* in Chemistry in February 1985 at the University of Pisa. Since 2001 he has been a Full Professor in Medicinal Chemistry and in since 2016 has been the Director of the Pharmacy Department of the University of Pisa. President of the Medicinal Chemistry Division of the Italian Chemical Society, in 2019 he was the recipient of the Luigi Musajo Medal for his commitment in the Division and for his research and didactic activity. He contributed to the definition of a novel class of BzR ligands, the indolylglyoxylamides (IGAs), and a new class of high-affinity and high-selectivity TSPO ligands, the 2-phenylindolglyoxylamides (PIGAs). He is the author of 200 publications in international journals with high impact factors and several patents.

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ABBREVIATIONS USED

AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocase; AUC, area under the curve; CDs, cardiac disorders; OHC, hydroxycholesterol; CK, creatine kinase; CMR, cardiac magnetic resonance; CsA, cyclosporin A; CT, computed tomography; CTR, cervico-thoracic ratio; CVDs, cardiovascular diseases; CypD, cyclophilin D; CYP11A1, cytochrome P-450 family 11 subfamily A member 1; Cyt-c, cytochrome-c; DPH, 1,6-diphenyl-1,3,5- hexatriene; EAM, experimental autoimmune myocarditis; GPx, glutathione peroxidase; HABs, high-affinity binders; HF, heart failure; HIV, human immunodeficiency virus; HP, hematoporphyrin IX; IMAC, inner membrane anion channel; IMM, inner mitochondrial membrane; IR, ischemia reperfusion; IRI, ischemia-reperfusion injury; ISO, isoproterenol; Laboratories, low-affinity binders; LDH, lactate dehydrogenase; L-NAME, N(ω)-nitro-L-arginine methyl ester; LVV, large vessel vasculitis; MABs, mixed affinity binders; MHC, myosin heavy chain; MI, myocardial infarction; mPTP, mitochondrial permeability transition pore; NIM811, *N*-methyl-4-isoleucine-CsA; Debio-025, D-3- MeAla-4-EtVal-CsA; NO, nitric oxide; NOS, NO synthase; OMM, outer mitochondrial membrane; PBR, peripheral benzodiazepine receptor; PCI, percutaneous coronary intervention; PET, Positron Emission Tomography; PMNs, polymorphonuclear leukocytes; PPIase, peptidyl-prolyl *cis*−*trans* isomerase; RIRR, ROS-induced ROS release; ROS, reactive oxygen species; SARS-CoV2, severe acute respiratory syndrome coronavirus 2; SfA, sangliferin A; SNP, single nucleotide polymorphism; SOD, superoxide dismutase; STEMI, ST-elevation myocardial infarction; TBARs, thiobarbituric acid reactive substances; TSPO, translocator protein (18 kDa); VDAC, voltage-dependent anion channel; β-MHC, β-myosin heavy chain

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This paper was originally published ASAP on December 19, 2023. Due to a production error, the Greek letters in Table 2 were not displayed correctly. The updated version was reposted on December 20, 2023.