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Mitochondrial Function in Aldosterone-Dependent Human Hypertension

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Abstract

Primary aldosteronism (PA), the most common form of secondary hypertension, is characterized by an increase in cytosolic intracellular Ca^{2+} concentrations $[\text{Ca}^{2+}]_i$ and excessive autonomous aldosterone productions.

Our aim is to investigate transients and oscillations of $[\text{Ca}^{2+}]_i$ and to assess mitochondrial function in a human model of CD56^+ cells isolated from aldosterone-producing adenoma (APA) and normal surrounding adrenocortical (AAC). Moreover, as PA is associated with abdominal aortic calcification, we will focus on measuring in vitro calcification propensity of PA and essential hypertensive (EH) plasma patients.

We assessed $[\text{Ca}^{2+}]_i$ in CD56^+ APA and AAC in basal conditions and after angiotensin II (Ang II) stimulation using Fura-2 dye, live confocal microscopy, and a developed custom-made pipeline to analyze $[\text{Ca}^{2+}]_i$ signals in terms of frequency and peak parameters as amplitude, full width at half maximum (FWHM) and area under the curve (AUC). As the two-pore domain K^+ TASK-2 channels are under expressed in APA, we used quinidine (20 μM), 20 seconds prior to $[\text{Ca}^{2+}]_i$ imaging acquisition to examine the effect of the pharmacological inhibition of this channel. In addition, we depleted extracellular Ca^{2+} to identify the source of Ca^{2+} transients.

In addition, we utilized the Biohybrid assay to assess PA and EH patient's plasma calcification propensity in vitro using 2 cell models-, namely induced vascular smooth muscle cells (iVSMCs) and human vascular smooth muscle cells (hVSMCs).

Resting $[Ca^{2+}]_i$ levels were higher in AAC (0.80 AU (25% percentile=0.65; 75% percentile=0.90)) compared to APA cells (0.53 AU (25% percentile=0.33; 75% percentile=0.79)) ($p=0.0212$). We detected spontaneous Ca^{2+} activity, during resting conditions, in both cell types without significant difference in peak parameters. Upon Ang II stimulation, AAC showed elevated FWHM, amplitude, and AUC peak parameters of first Ca^{2+} transients. Peak parameters of subsequent Ca^{2+} oscillations were variable between AAC and APA cells. Ca^{2+} oscillations frequency and degree of oscillating cells were significantly higher in APA cells than in AAC. In AAC, TASK-2 inhibition with quinidine tended to increase spontaneous activity and altered peak's parameters, but did not influence frequency of oscillation. Extracellular Ca^{2+} depletion did not diminish Ca^{2+} transients.

After 24 hours in culture, both AAC and APA cells developed rosette-like cell structure resembling the "glomeruli" of the intact zona glomerulosa clusters. Functional statistics and clustering analysis showed synchronous behavior of cells belonging to the same cluster without significant differences between AAC and APA.

Moreover, biohybrid assay results demonstrated elevated extracellular calcification deposition after the stimulation of both VSMC models with PA plasma compared to healthy volunteers. Further, using iVSMCs we observed a significant elevation in calcification deposition compared to EH plasma.

Quantitative assessment of $[Ca^{2+}]_i$ signals unveiled significant differences in $[Ca^{2+}]_i$ dynamics between human AAC and APA cells. These results suggest the concurrent modulation of frequency and amplitude of $[Ca^{2+}]_i$ subsequent oscillations as a potential driver of increased rate of aldosterone production. Moreover, we identified an in vitro propensity of plasma from PA

patients to drive mineralization deposition, providing a non-invasive diagnostic tool to be used for future personalized cardiovascular risk assessment medicine.

Introduction

1-Primary Aldosteronism

Arterial hypertension is a significant risk factor leading to global morbidity and mortality affecting more than 1 billion people [1][2]. Primary aldosteronism (PA) is the most common endocrine form of arterial hypertension comprising more than 11 % of the hypertensive patients referred for specialized centers for diagnosis and treatment[3]. It is characterized by an excess and autonomous production of aldosterone from the adrenal cortex that is renin independent [4]. The binding of secreted aldosterone to mineralocorticoid receptors results in sodium retention, potassium excretion, elevated blood pressure, and hypokalemia. However, findings from the PAPY study revealed that 50% of patients with aldosterone-producing adenomas (APAs) and 82% of those with bilateral primary aldosteronism remain normokalaemic [5]. Additionally, it is characterized by abnormally high plasma aldosterone levels, which is associated with low plasma renin levels and cannot be suppressed by sodium loading [4][6]. PA causes adverse and detrimental cardiovascular outcomes by increasing risk of cardiovascular diseases like stroke, arterial fibrillation, myocardial infarction, in addition to higher incidence of renal damage and metabolic diseases like diabetes[7][8]. The cardiovascular complications linked to PA syndrome are primarily driven by aldosterone. Beyond its harmful effects on serum potassium levels and its role in raising blood pressure, aldosterone contributes to a variety of adverse outcomes, including targeted organ damage. This damage manifests as fibrosis, inflammation, remodeling, hypertrophy, endothelial dysfunction, chronic fluid retention within blood vessels, and oxidative stress [8][9].

1.1-Forms of Primary Aldosteronism

The primary difference between the various classifications of PA lies in the recommended surgical or medical treatments required to address the syndrome [4]. The two major causes of PA are classified as either a bilateral form of aldosterone production due to mainly adrenal hyperplasia (idiopathic hyperaldosteronism) or a unilateral form of excess aldosterone predominantly due to APA (Figure 1) [10].

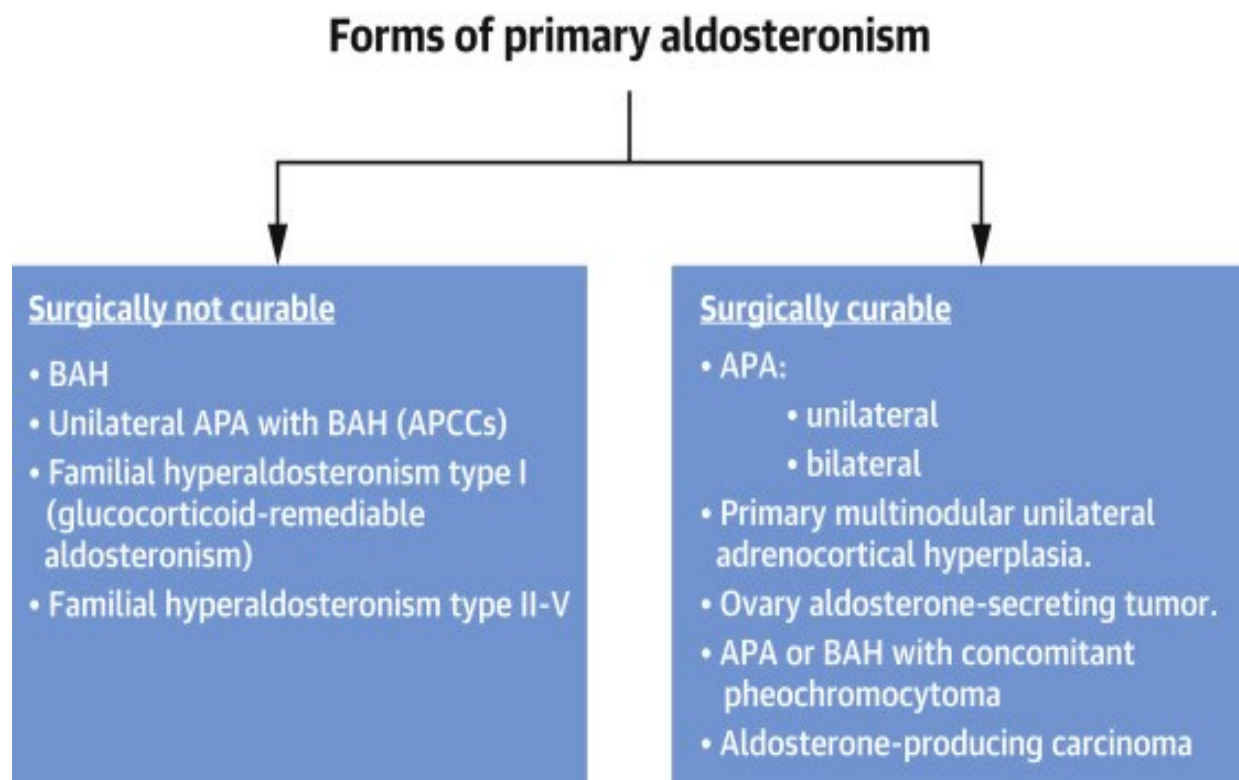


Figure 1: The Main Subtypes of Primary Aldosteronism Are Classified into Surgically Curable and non-surgically Curable[4].

Regardless of mode used to cure this pathology, most of total PA cases occur sporadically while only 6% of diagnosed PA patients carried familial origins of the disease [11]. By utilizing whole

exome sequencing and analyzing the underlying germline genetic abnormalities, four distinct variants of inherited forms of PA, known as Familial Hyperaldosteronism, were identified [12]. FH-I (glucocorticoid-remediable aldosteronism), the first detected form of FH, is characterized by an early onset of elevated blood pressure and is due to an unequal crossing-over of the regulatory regions of CYP11B1 encoding 11 β -hydroxylase to the coding sequence of CYP11B2 that encodes aldosterone synthase resulting in a chimeric gene. FH-II, the most common of FH forms with a prevalence of 1.2- 6% corresponding to the germline CLCN2 mutations [13]. FH-III, characterized by profound hypokalemia and severe hypertension, was found in patients showing germline mutations of KCNJ5 gene encoding the G protein-activated inward rectifier potassium channel GIRK4. FH-IV are characterized by germline mutations in gene in CACNA1H gene encoding the pore-forming α 1 subunit of the T-type voltage dependent calcium channel Cav3.2 [14].

1.2-Screening and Detection of PA

PA is hugely underdiagnosed even in high-risk hypertensive patients who show distinct symptoms meeting diagnostic tests indications [15] [16]. Due to detrimental complications in patients with PA, early diagnosis is recommended [17]. Current clinical guidelines for screening for PA recommend measuring the aldosterone–renin ratio (ARR) in those patients showing severe hypertension or an elevated blood pressure associated with hypokalemia, sleep apnea, or a detected adrenal mass [17]. Patients with elevated ARR levels proceed to the gold standard dynamic testing, such as intravenous saline loading, for a more definitive confirmation to identify and confirm the subtypes of PA [16][18][19][20]. AVIS-2, the largest international multi-center registry of PA patients pursuing surgical cure, highlighted the critical role of AVS in improving cure rates following adrenalectomy. [21].

It is essential to identify primary aldosteronism (PA) and determine its subtypes to achieve improved outcomes. This distinction is critical because unilateral forms of the condition can be treated surgically, whereas bilateral forms require treatment with mineralocorticoid receptor antagonists. Additionally, recognizing secondary hypertension is vital for implementing targeted management and addressing the underlying causes[22].

2- Steroid production in the human adrenal cortex

The human adrenal gland is a vital endocrine organ that regulates body fluids and volumes, maintains normal blood pressure, and manages metabolism. It consists of an outer cortex, responsible for steroid production, enclosed by a protective capsule, and an inner medulla, which produces catecholamines. The adrenal cortex is further divided into three functionally distinct zones: the zona glomerulosa (ZG), composed of tightly packed cells forming glomeruli-like structures; the zona fasciculata (ZF), characterized by cells rich in lipid droplets arranged in columns; and the zona reticularis (ZR), which contains smaller cells organized in a net-like pattern [23]. Aldosterone synthesis takes place in the adrenocortical ZG by aldosterone synthase enzyme, coded by the CYP11B2 gene. Of note, not all ZG adrenocortical cells express CYP11B2 enzyme, thus our team developed an approach based on immunomagnetic bead isolation of ZG cells expressing CD56 (neural cell adhesion molecule (NCAM)) to yield a pure population of ZG cells expressing CYP11B2[24]. The ZF is where glucocorticoid is produced, and the ZR where adrenal androgens are produced. Both the ZF and the ZR are regulated by Adrenocorticotrophic hormone (ACTH). The medulla cells secrete epinephrine and norepinephrine [25][26].

2.1-Aldosterone

Over 50 years ago the aldosterone hormone was first discovered and isolated [27]. Aldosterone is the main mineralocorticoid hormone responsible for the maintenance of fluid and electrolyte balance in mammals. It performs its function either through genomic effects mediated by mineralocorticoid receptors (MR), or via non-genomic effects that are mediated by membrane receptors and have a rapid onset of action [28].

Aldosterone regulates sodium excretion by binding to the inactive cytosolic mineralocorticoid receptor (MR) in the epithelial cells of the distal nephrons [29]. When aldosterone binds to its receptor (MR) in target cells, it activates the receptor. The ligand-bound MR then dissociates from a multiprotein complex and moves to the nucleus, where it targets specific gene promoters induced by aldosterone. In the kidneys, the MR promotes the expression of Serum and glucocorticoid-inducible kinase-1 (SGK1), which initiates a cascade that enhances sodium and water reabsorption via the epithelial sodium channel (ENaC) and facilitates potassium excretion. This process leads to an increase in blood volume and a rise in blood pressure (Figure 2) [30].

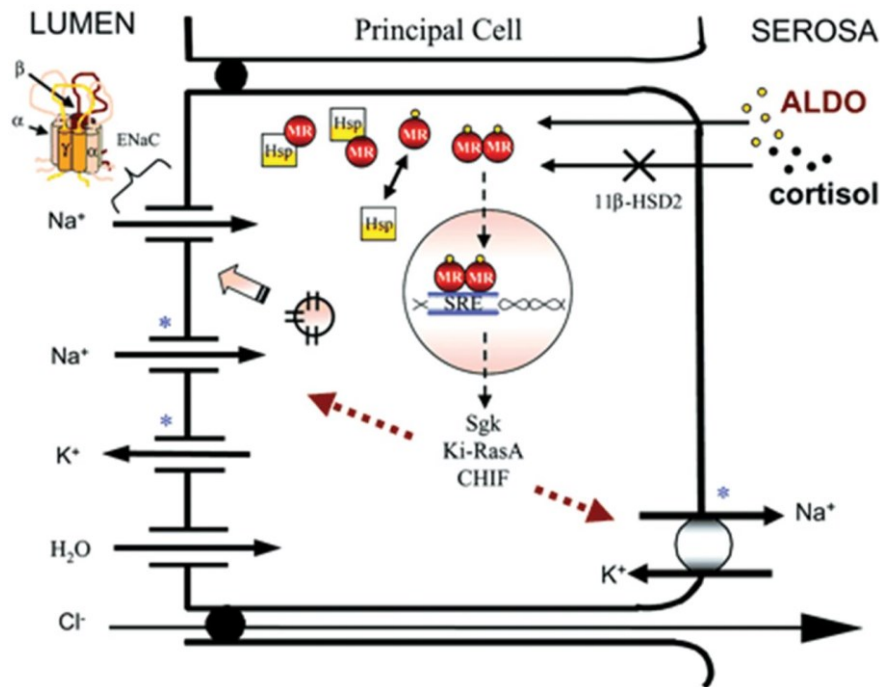


Figure 2: Classical MR-mediated effects of aldosterone in the principal cells of the kidney, resulting in increased potassium excretion and sodium reabsorption [30]

2.2-Ang II regulation of aldosterone synthesis

Various factors have been demonstrated to regulate aldosterone secretion, including adrenaline, vasoactive intestinal polypeptide, atrial natriuretic peptide, dopamine, endothelin 1 (ET-1), estrogens, adrenocorticotrophic hormone (ACTH), and urotensin II [31][32]. However, the major regulators of aldosterone production in the ZG are angiotensin II (Ang II), and the concentration of extracellular potassium [31][33].

Aldosterone biosynthesis is primarily regulated by the renin-angiotensin system (RAS). Renin is produced and released by the juxtaglomerular (JG) cells located in the afferent arterioles of the kidney [34]. Renin then catalyzes the hydrolysis of angiotensinogen, secreted by the liver, to

angiotensin I (Ang I). Ang I is then transformed to Ang II by the angiotensin converting enzyme (ACE) predominantly present in the lungs and vascular tissue[33].

Ang II stimulation can occur in both acute and chronic forms. The acute stimulation of aldosterone synthesis happens within minutes and involves rapid aldosterone production, primarily through the activation of the StAR protein. This process enhances the gene expression of aldosterone synthase (CYP11B2) and promotes ZG hypertrophy, along with an increase in cellular cholesterol uptake and StAR protein levels [35][36].

Angiotensin II (Ang II)-induced steroidogenesis is driven by the activation of the angiotensin type 1 receptor (AT1R), a G protein-coupled receptor (GPCR). This activation triggers various downstream signaling pathways involved in aldosterone synthesis. Ang II also regulates the increase in intracellular calcium (Ca^{2+}) from both extracellular and intracellular sources. In human adrenal glomerulosa cells, AT1R-mediated Ang II-stimulated aldosterone production controls calcium influx by inhibiting two key potassium channels: the G protein-coupled inwardly rectifying potassium (GIRK)4 channel and the Twik-related acid-sensitive K^+ (TASK) channels. This inhibition leads to membrane depolarization, which facilitates Ca^{2+} entry through the activation of both high-voltage (L-type, including $\text{CaV}1.1$ – 1.4 subtypes) and low-voltage (T-type, including $\text{CaV}3.1$ – 3.3 subtypes) calcium channels [31]. Additionally, Ang II raises cytosolic Ca^{2+} levels by triggering the activation of phosphoinositide-specific phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to produce the secondary messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Figure 3) [37].

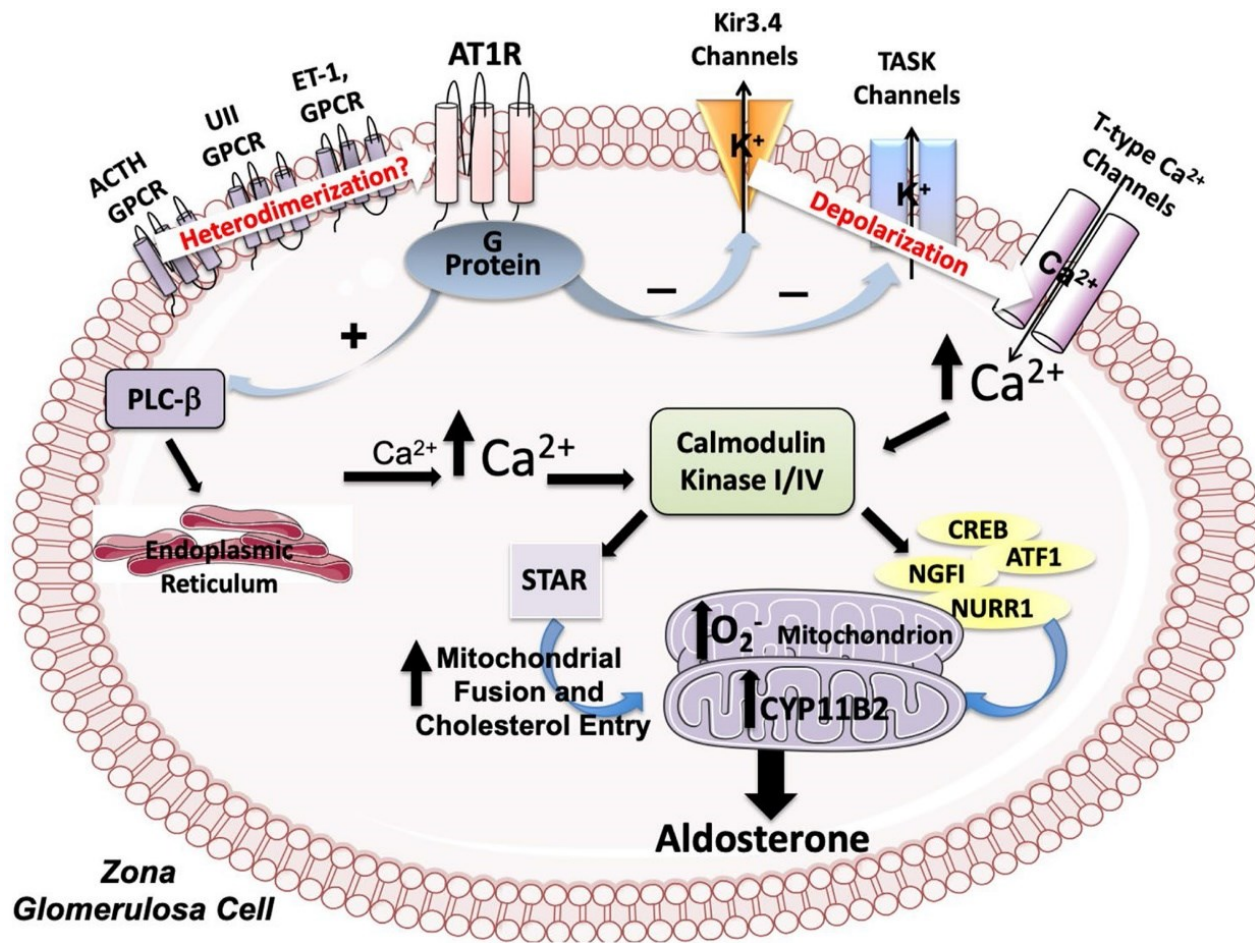


Figure 3: Molecular pathways modulated by Ang II in adrenal zona glomerulosa cells. Ang II, by acting through the AT1R stimulates the expression of the aldosterone synthase coding gene (*CYP11B2*) by increasing intracellular Ca^{2+} through the modulation of the activity of the PLC- β and of the GIRK4, and of the TASK channels. The increase of intracellular Ca^{2+} activates the calmodulin kinase (CaMK) cascade which acts on *CYP11B2* expression by phosphorylating the transcription factors Activating Transcription Factor 1 (ATF-1), cyclic adenosine monophosphate response element (CRE)-binding protein (CREB) and nuclear receptor related 1 (NURR1). Ang II also increases the aldosterone synthase activity by activating NADPH oxidase and, thereby, by elevating reactive oxygen species (O_2^-) in the mitochondria and by promoting fusion of mitochondria and translocation in these organelles of relevant steroidogenic acute regulatory. Finally, heterodimerization of AT1R with other G protein-coupled receptors (GPCRs) is supposed to be involved in aldosterone synthesis [37].

2.3- Role of Ca^{2+} signaling in adrenocortical cells

Ca^{2+} is a versatile and ubiquitous signaling molecule that plays a key role in regulating a wide range of cellular processes. Ca^{2+} behavior inside the cell could be arranged in spikes, bursts, rapid oscillations, or even adopt a chaotic undefined behavior[38][39]. The primary effect of the two aldosterone secretagogues, Ang II and K^+ , is the increase in cytoplasmic Ca^{2+} levels. Ang II raises cytoplasmic Ca^{2+} by releasing it from both intracellular and extracellular stores, as previously mentioned, while K^+ boosts cytoplasmic Ca^{2+} by stimulating the influx of Ca^{2+} from extracellular sources through the activation of L- and T-type calcium channels[40]. Moreover, it was reported that increased Ca^{2+} influx elevates aldosterone production and secretion in APA tumors [41].

The Ca^{2+} universal signal conveys information through its amplitude or frequency, which are interpreted by calcium-dependent proteins that control the subsequent signaling pathways [42]. However, despite the importance of this universal signal in the regulation of steroid biosynthesis [42], little is known about the dynamics of Ca^{2+} behavior in human adrenocortical aldosterone producing cells. What is well reported so far is the regulation of Ca^{2+} of the following processes for aldosterone production.

2.4- Aldosterone biosynthesis: Mitochondria and CYP11B2

The mitochondria are the central site for aldosterone synthesis as they house the steroidogenic enzymes needed for steroid biosynthesis [43]. The rate limiting step of steroidogenesis is the translocation of cholesterol, the metabolic precursor of adrenal steroids, through the outer mitochondrial membrane to the inner mitochondrial membrane where the first enzyme that converts the cholesterol to pregnenolone is located. This translocation is mediated by the key steroidogenic acute regulatory protein (StAR). Following that, aldosterone is converted through a

series of enzymatic steps involving two broad families of steroidogenic enzymes; three cytochrome P450 enzymes, involving the cholesterol side-chain cleavage (CYP11A1), 21-hydroxylase (CYP21) and CYP11B2, and one hydroxysteroid dehydrogenase, (Figure 4). The CYP11B2, which converts deoxycorticosterone (DOC) to aldosterone, is localized on the inner-mitochondrial membrane. Of note, the functional zonation of adrenal gland is due to specific expression of CYP11B2 in the ZG and of the CYP11B1 (11 β -hydroxylase) in the ZF [44][45][33]. Moreover, it was reported that Ang II increases the formation of mitochondrial matrix cyclic AMP (cAMP) through the activation of mitochondrial soluble adenylyl cyclase (sAC) upon stimulation of H295R cultured adrenocortical cells [46].

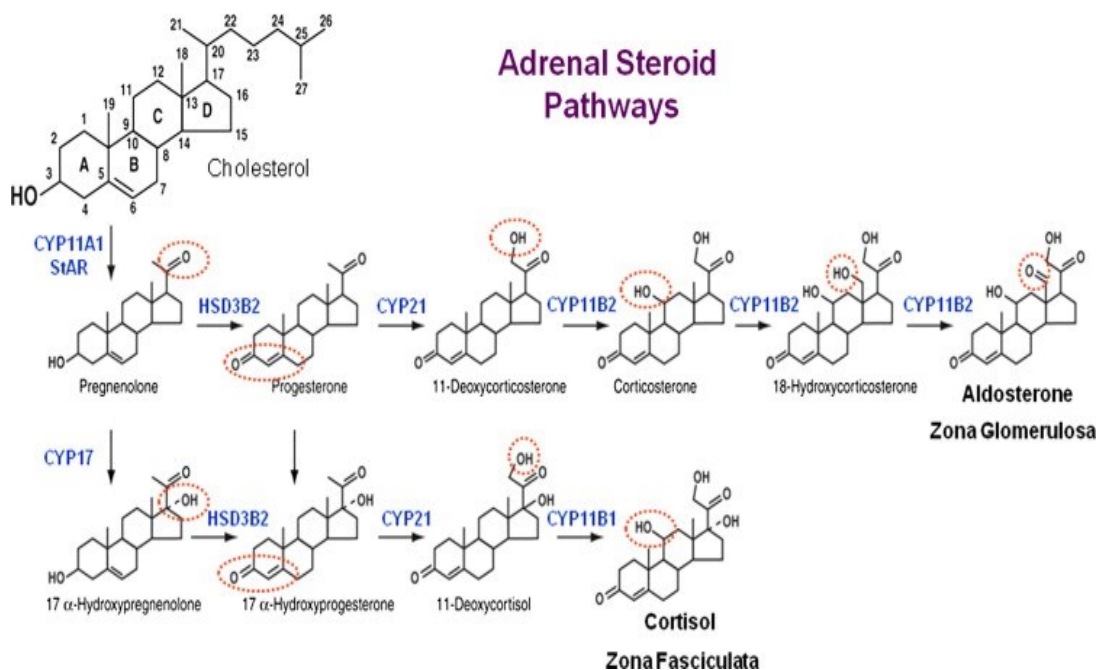


Figure 4: Adrenocortical steroidogenic pathways for the production of mineralocorticoids and glucocorticoids. The adrenal cortex produces zone-specific steroids as a result of differential expression of steroidogenic enzymes. In the initial step of steroidogenesis, steroidogenic acute regulatory (StAR) protein is needed for the rate-limiting step of movement of cholesterol to the inner mitochondrial membrane, where cholesterol is cleaved by cholesterol side-chain cleavage (CYP11A1) to pregnenolone. Further steps of the steroidogenic pathway include the enzymes 3 β -hydroxysteroid dehydrogenase type 2 (HSD3B2), 17 α -hydroxylase, 17,20-lyase (CYP17), 21-hydroxylase (CYP21), 11 β -hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2)[45].

2.5- Transport of cholesterol and increased Aldosterone Synthase expression

Adrenocortical cells synthesize steroids from the precursor cholesterol. It was reported that Ang II elevates the cholesterol content in the inner mitochondrial membrane (IMM) by activating its transport through the mitochondria[47]. Ang II evoked IP3 elicits cytoplasmic Ca^{2+} release from endoplasmic reticulum (ER) stores and activates Ca^{2+} /calmodulin dependent protein kinases (CaMKs). CaMK cascade phosphorylates STAR protein, thus increasing cholesterol transport into the mitochondria[47].

CaMKI and CaMKIV increase CYP11B2 expression by phosphorylating and activating different transcription factors, such as ATF-1, CRE-binding protein (CREB) that bind to CRE, a cAMP-response element in the promoter region of CYP11B2 gene, Nerve growth factor-induced clone B (NGFIB), and NURR1 that binds two additional *cis*-acting regulatory elements in the CYP11B2 promoter region, the Ad-5 and NBRE-1 (Figure 3). In addition, DAG molecules activate protein kinase c (PKC) that regulates StAR protein [37].

Of note, it was demonstrated that CaMKK2 knockdown in HAC15 cells leads to significant reduction in both CYP11B2 mRNA induction and aldosterone production after stimulation with Ang II [40][48].

The cytoplasmic Ca^{2+} signal described earlier is transferred to the mitochondria, where it functions independently of StAR translocation. It is known that Ca^{2+} plays a crucial role in the conversion of NADP^+ to NADPH [48], this process is crucial for all mitochondrial steroidogenic steps and occurs at the cost of NADH, which is required for ATP production. A substantial amount of evidence demonstrates the increased production of NADPH following Ang II stimulation[48]

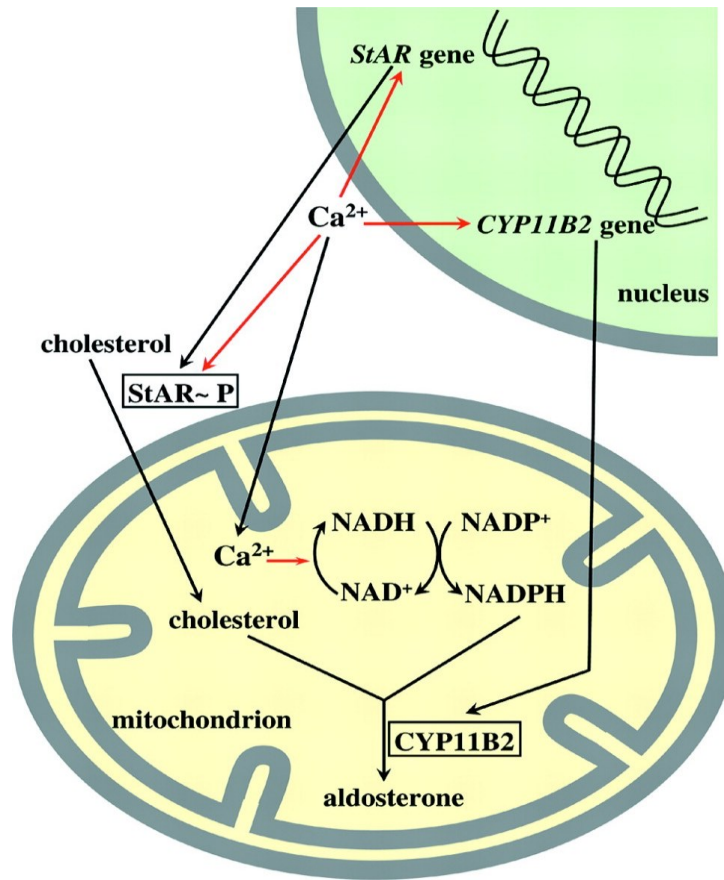


Figure 5: Steroidogenic actions of Ca^{2+} . The direct actions of Ca^{2+} are shown by red arrows, and the black arrows indicate metabolic transformations and transport processes. StAR, steroidogenic acute regulatory protein; CYP11B2, aldosterone synthase[48].

3-Genetic Alterations associated with PA

Mechanisms leading to aldosterone secretion are severely altered in PA. Most of the cases are sporadic cases due to recurrent somatic mutations identified in APA, as shown by whole exome sequencing [49]. The autonomous aldosterone production in APA is mainly due to hallmark somatic mutations in ion channels and pumps. Two pathogenic somatic mutations were detected in the K^+ channel *KCNJ5* (G151R and L168R) which alter the K^+ selectivity leading to Na^+ influx. Moreover, gain-of-function mutations in *CACNA1D* (voltage-gated Ca^{2+} channel CaV1.3) were

identified. These mutations shift the voltage dependence of activation to less depolarized potentials. Gain of function mutations were also detected in *CACNA1H*, encoding the T-type calcium channel $\text{CaV}3.2$ and in the gene *CLCN2* which encodes the ClC-2 chloride channel. Two mutations were detected in the P-type ATPase gene family (*ATP1A1* and *ATP2B3*). *ATP1A1* encodes the α -1 subunit of the Na^+/K^+ -ATPase, and mutations contribute to the permeability of the pump for Na^+ or H^+ ions rendering it functioning as a channel. Mutations in *ATP2B3*, the plasma membrane Ca^{2+} ATPase, lead to abnormal Na^+ and Ca^{2+} permeability, with a reduced Ca^{2+} transport capacity. *CTNNB1* (which encodes β -catenin) mutations were also detected in APA carrying other mutations on *CACNA1D* or on the *GNA11* and *GNAQ* genes, which encode G-protein α subunits G11 and Gq, respectively[22]. A recent discovery of a new mutation in APA was made in the neuronal cell adhesion gene *CADM1* which elucidated the importance of gap junction (GJ)-dependent regulation of aldosterone production[50].

A meta-analysis of 13 studies involving 1,636 patients found that *KCNJ5* mutations were present in 43% of APAs, with higher prevalence rates, up to 77%, observed in East Asian populations compared to European cohorts. Additionally, APAs with *KCNJ5* mutations were more common in women and were associated with higher plasma aldosterone levels and larger tumor volumes when compared to mutation-negative tumors. The *KCNJ5*-mutated APA tumors were characterized by ZF-like cells. Other mutations were more frequently observed in male patients and were linked to smaller tumors [51].

To detect genetic alterations in APA and aldosterone-producing regions, DNA was extracted directly from formalin-fixed paraffin-embedded (FFPE) tissues after identifying *CYP11B2*-positive areas via immunohistochemistry. Next-generation sequencing was then used to genotype the

targeted regions, revealing KCNJ5 mutations in 43% of samples, CACNA1D in 21%, ATP1A1 in 17%, ATP2B3 in 4%, and CTNNB1 in 3%[\[52\]](#). However, we recently demonstrated that IHC-guided sequencing protocols selecting CYP11B2-positive areas could furnish results that might not be representative of the entire mutational status of the adrenal tumor[\[53\]](#).

In APA, the genetic mutations mentioned above deregulate the mechanisms of aldosterone production via altering cellular Ca^{2+} homeostasis leading to pathogenic PA syndrome. In fact, ATP1A and KCNJ5 mutations alter the cell membrane potential which leads to the increase Ca^{2+} cytoplasmic whereas, ATP2B3 and CACNA1D mutations alter directly the intracellular Ca^{2+} signal [\[54\]](#)[\[55\]](#).

4-TASK-2 channels in APA

Adrenocortical cells selectively allow K^+ through TASK channels in their plasma membrane. These TASK channels are part of the two-pore-domain potassium (K2p) channel family, which includes six subfamilies (TWIK, THIK, TREK, TASK, TALK, TRESK) encoded by the KCNK genes. As leak channels, TASK channels are active across a wide range of membrane potentials, providing the necessary conductance to maintain the hyperpolarized resting potential of ZG cells. The KCNK5, KCNK3, and KCNK9 genes encode the TASK-2, TASK-1, and TASK-3 channels, respectively. In murine models, TASK-1 and TASK-3 are differentially expressed, while in normal human ZG cells, all three subtypes are expressed. Specifically, TASK-1 and TASK-2 are highly expressed in human ZG cells, with TASK-3 present at very low levels. It's also important to note that mutations in the KCNJ5 gene, found in about a third of APA cases, affect the inward-rectifying K^+ channel (GIRK4), which is structurally distinct from TASK channels[\[41\]](#)[\[56\]](#). The Kir3.4 K^+ channel allows potassium

outward current. However, mutations in or near the selectivity filter render the channel to preferentially carry depolarizing inward Na^+ currents[54][57]. Interestingly, our team demonstrated that APA exhibit a downregulated TASK-2 channels expression. Moreover, in vitro blunting of TASK-2 expression in adrenocortical cell lines (H295R and HAC15) demonstrated an increase of production of aldosterone and expression of CYP11B2[58].

5-Adrenal Rosettes

5.1-Structure and function

Rosettes are morphological multicellular structures that guide embryonic development, through epithelial remodeling, and organogenesis like branching of pancreatic duct [59]. These rosettes temporarily involve two processes: rosette formation, where at least five adjacent cells organize into a flower-like structure, and rosette resolution, where the structure disbands to allow for tissue growth extension [60]. However, rosettes postnatally persist in the mature mouse and human ZG [61].

The adrenal gland's various functional zones have unique patterns of development and function. The adrenal cortex's concentric zones continuously renew themselves, with cells from the ZG migrating inward to form the ZF cells. This process is referred to as zonal trans-differentiation (Figure 6) [62]. Extensive studies, done only on mature mice ZG cells, demonstrated that cells are arranged into multicellular rosettes made up of 10-15 cells. Cell-cell contact is formed by adherent junctions (AJ) proteins, mainly β -catenin and N-cadherin[61] and a basement membrane, composed by laminin subunit β 1 (Lamb1) and type IV collagen, enwraps each rosette unit [61][63].

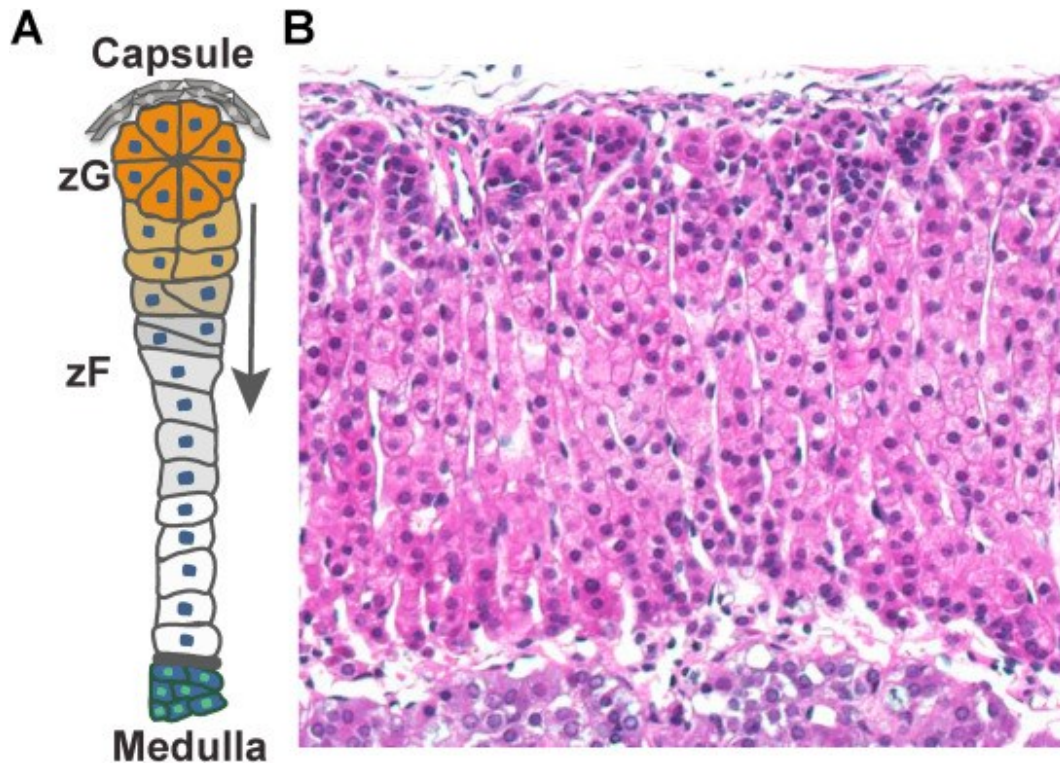


Figure 6: Adrenal cortex structure and histology. **A.** Schematic of a single adrenal cortex structural unit. Beneath the capsule, ZG cells are organized into rosettes and are in direct contact with ZF cells organized in cords. ZG cells migrate centripetally and transdifferentiate into ZF cells (red arrow) during homeostatic maintenance. **B.** H&E staining of an adult mouse adrenal cortex cross section. ZG cells are small and densely packed. ZF cells appear columnar and have a larger cytoplasmic volume[62].

5.2-Calcium Signaling in Rosettes

To investigate whether the structure of the multicellular rosettes influences the function of mature murine adrenocortical ZG cells upon Ang II stimulation, Ca^{2+} signals of ZG cells in the intact rosette's communities were recorded upon stimulation [64]. The authors demonstrated an increase in the oscillatory burst number of Ca^{2+} events that was proportional to the concentration of Ang II. Moreover, using two computational approaches, a synchronicity of Ca^{2+} oscillatory behavior in ZG cells localized in close proximity in the same rosette structure was shown [64]. Moreover, genetic deletion or acute pharmacological inhibition of TASK channels (TASK-1 and

TASK-3) resulted in robust spontaneous bursts of Ca^{2+} activity, that are Ang II independent, and in increased aldosterone production. Ca^{2+} imaging correlated among ZG cells within the same functional rosette upon analysis using computational approaches. This coordinated activity was AT1R independent[65]. Of note, rosette functional activity in human adrenocortical tissues was not investigated before.

6-Vascular Calcification

In the extracellular matrix of bones and teeth, calcium crystals are normally deposited as part of their formation and development. However, ectopic calcification (or ectopic mineralization) refers to the abnormal accumulation of calcium phosphate in soft tissues, which is considered a pathological condition[66]. Vascular calcification (VC) occurs when insoluble calcium phosphate crystals accumulate in the extracellular matrix (ECM) of blood vessel walls. It is a significant clinical feature of various conditions, such as atherosclerosis, diabetes mellitus, hypertension, aging, and chronic kidney disease (CKD), all of which contribute to the buildup of calcium phosphate in the vessel walls[67]. A meta-analysis illustrated the association of VC with a 3-4 fold higher risk of cardiovascular events and mortality[68].

6.1-Vascular Calcification Classifications

The localization of the ectopic calcific deposits determines the clinical presentation of the ectopic mineralization. VC is classified into 3 types: intimal calcification, media calcification, and cardiac valve calcification[67]. These types may overlap in terms of pathophysiology, but they have different clinical implications. Intimal calcification occurs in atherosclerotic diseases on the inner vessel walls and is associated with the stability of atherosclerotic plaques. It begins with early-stage microcalcification, which progresses into spotty calcification[69]. Inflammation is

considered a hallmark of intimal calcification in which there is a development of macrophage foam cells [70]. However, medial calcification is more frequently observed in individuals with diabetes and CKD, particularly affecting the internal elastic lamina of small and medium arteries. This condition is typically associated with reduced vessel elasticity and compliance, which in turn increases pulse wave velocity and contributes to systolic hypertension. This can lead to the development of arterial hypertension, ischemic heart disease, cardiac hypertrophy, or peripheral arterial diseases[71]. Cardiac valve calcification is seen in metabolic diseases like diabetes and is correlated with mechanical stress and inflammation[72][73].

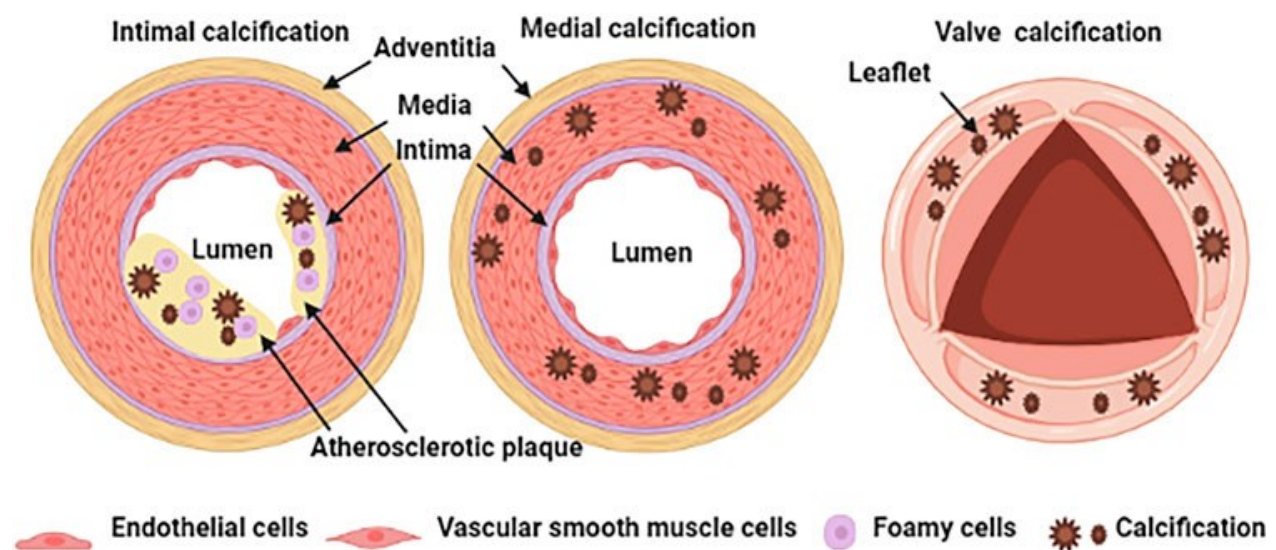


Figure 7: Schematic representation of vascular calcification classification[72]

6.2-Vascular Calcification initiation and progression

The process of mineral deposition in bones and mineralizing cartilage begins with the involvement of matrix vesicles (MVs), a type of extracellular vesicle (EV), which serve as nucleation sites for calcium deposition. EVs are classified into exosomes (50–150 nm), MVs (100-300 nm), microvesicles (100–500 nm),

and apoptotic bodies (1,000–5,000 nm) [72]. MVs are membrane bound vesicles formed by the budding process from chondrocytes, osteoblasts, and odontoblasts. These vesicles concentrate the key proteins for calcium-binding and phosphatases fundamental for the nucleation of hydroxyapatite[74]. Initiation and progression of VC is similar to the physiological process of bone formation described above[75]. Of note, key determinants of VC are the levels of extracellular calcium and inorganic phosphate (Pi), presence of collagen in ECM, and the quantity of calcification inhibitors in ECM. Both Ca^{2+} and Pi levels are regulated by phospho- and calcitropic hormones, parathyroid hormone (PTH), active vitamin D, and fibroblast growth factor 23 (FGF23) acting on the following organs, the kidneys, the intestine, and the bones [76]. Hormonal pathways that control calcium levels are more tightly regulated than those that govern Pi levels, with Pi being a key factor in VC. The concentration of Pi is primarily controlled by Pit1, a sodium-dependent phosphate transporter that mediates Pi uptake into cells, and by alkaline phosphatase (ALP), an important regulator of osteogenic calcification. ALP works by hydrolyzing pyrophosphate, thus influencing local Pi concentrations[77]. However, VC is a well-organized cell-active biological process mainly driven by vascular smooth muscle cells (VSMCs) and not a sole passive deposition of calcium phosphate crystals as it was originally thought to be[75].

VSMCs, the fundamental cells in VC, are non-voluntary contractile cells present in many tissue types including blood vessels. VSMCs roles include maintenance of blood pressure levels via the contractions and relaxations in opposition to the heart, and remodeling of blood vessel's ECM [75]. In adult tissues, VSMCs display a contractile phenotype characterized by slow proliferation, the ability to functionally contract, and the expression of contractile proteins like SM α -actin (SM α A), SM-22a, calponin, and smoothelin. However, VSMCs are not fully differentiated and

exhibit phenotypic plasticity[78]. Upon local stimulation, such as after an injury, VSMCs are capable of undergoing phenotypic transformation into synthetic state and act as hydroxyapatite nucleation sites[72]. This transformation encompasses downregulating contractile proteins, increasing the proliferation, facilitating migration by ECM remodeling[79]. It is important to note that VSMCs have the ability to differentiate into various other cell types, such as chondrocytes, osteoblasts, adipocytes, and macrophage foam cells (Figure 8) [70]. The osteo/chondrogenic phenotype involves the loss of contractile proteins in VSMCs (such as SM22a and SM α -actin) and the acquisition of osteochondrogenic markers, including Runx2, SP7, Osteopontin, osteocalcin, ALP, Sox9, and Type II and X collagen (Col II and Col X). This process is accompanied by the formation of calcifying vesicles, the downregulation of mineralization inhibitors, and the development of a matrix prone to calcification[70].

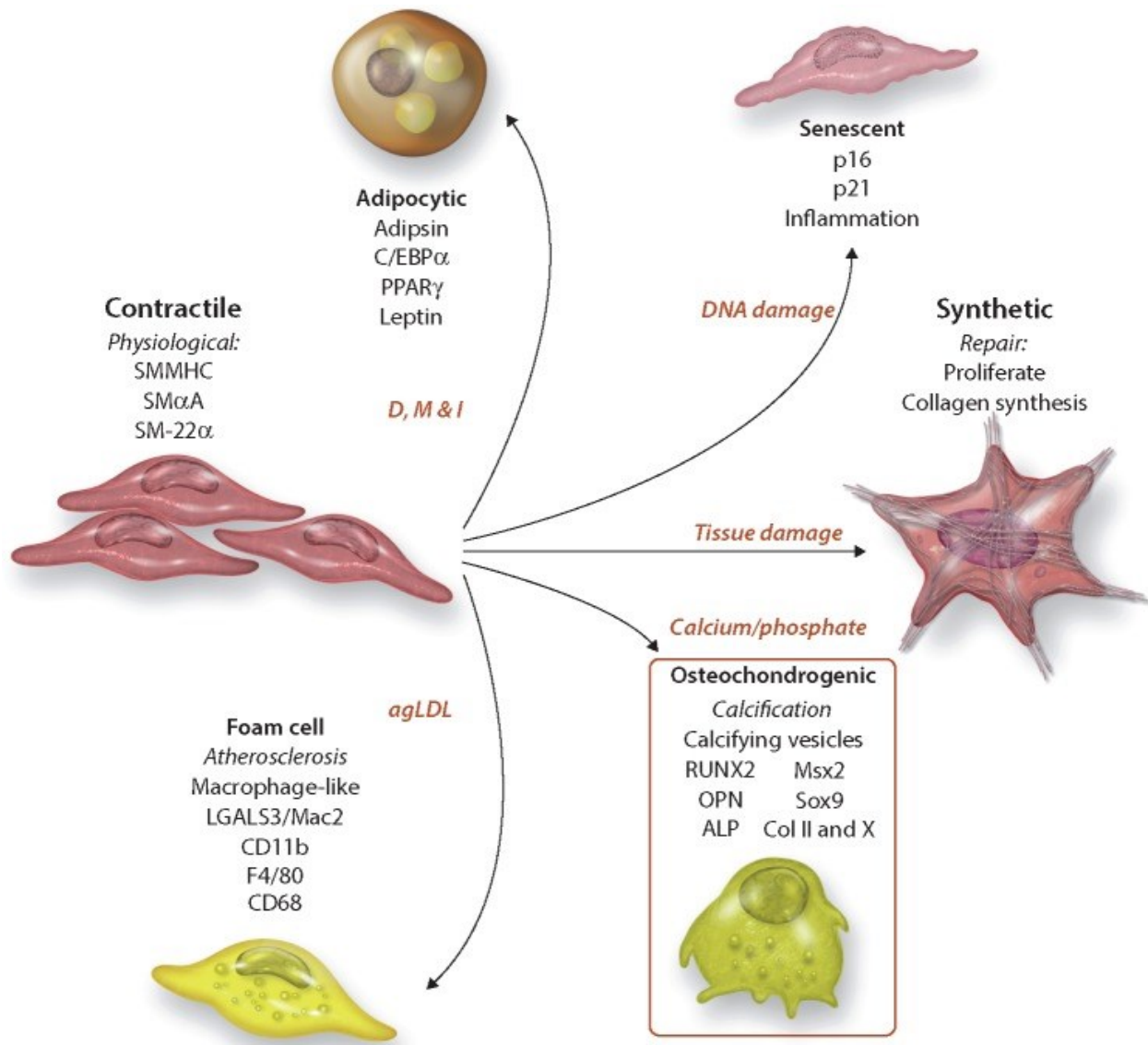


Figure 8: Diagram of possible VSMC phenotypes and their cellular/disease/organotypic origin. Diagrammatic representation of the spectrum of VSMC phenotypes identified during calcification (**bold**), their cellular markers and phenotypic drivers in red. Dexamethasone (D), Methylisobutylxanthine (M), and insulin (I)[70].

VC arises through critical mechanisms involving a disruption between osteochondrogenic signaling and anti-calcification processes (Figure 9) [80]. It is important to note that the phenotypic switch linked to VC is consistent in both medial and intimal calcification, although the underlying factors driving calcification differ. For instance, patients with renal failure tend to

develop medial calcification without the occurrence of atherosclerosis[70]. Main drivers of both medial and intimal calcification are oxidative stress, apoptosis, mitochondrial dysfunction and mechanical stress. However, some calcification drivers are restrained to medial deposition only, while inflammation can only cause intimal calcification[70]. Previous research work demonstrated that apoptotic cell death paves the way for the calcification of VSMCs. The produced apoptotic bodies were shown to collect calcium thus acting as nucleating sites, like MV, for the formation of calcium crystals[74].

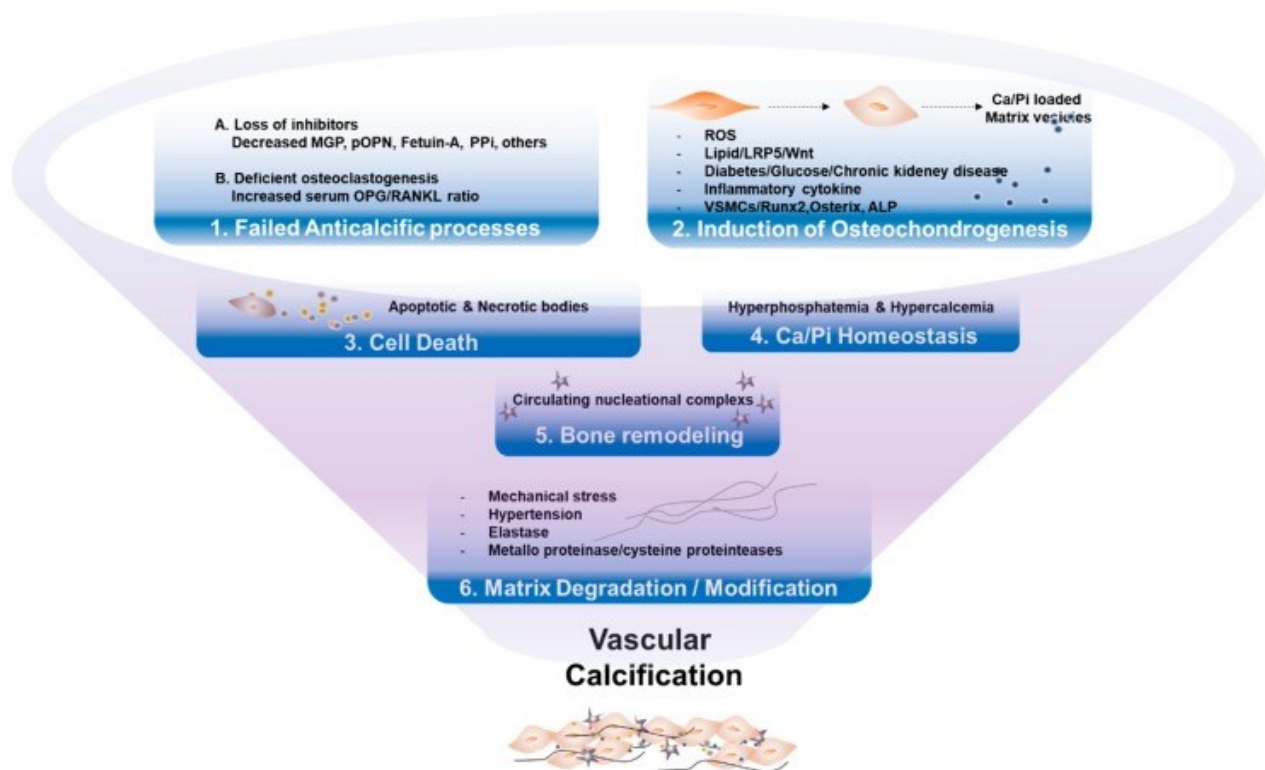


Figure 9: Key mechanisms of vascular calcification. (1) Failure of anti-calcification processes, due to loss of inhibitors and deficiency of constitutively expressed mineralization inhibitors, leads to vascular calcification. (2) Various stressors induce osteogenic trans differentiation of VSMCs, products of matrix vesicles, which act as a nidus of calcium phosphate deposition. (3) Cell death by apoptosis or necrosis leads to release of apoptotic bodies, or necrotic debris, which may act as nucleation of apatite. (4) Abnormal mineral homeostasis causes deposits calcium phosphate hydroxyapatite. (5) Nucleation complexes formed during bone remodeling, promote ectopic mineralization. (6) Matrix degradation/modifications, caused by environmental stressors, are involved in vascular calcification[80].

6.3-Vascular Calcification Inhibitors

Under normal conditions, ectopic mineralization is physiologically prevented by calcification inhibitors, and a deficiency in these inhibitors results in VC. Inorganic pyrophosphate (PPi), composed of two inorganic phosphate molecules linked by a hydrolysable ester bond, prevents the nucleation of amorphous calcium phosphate and the crystallization of hydroxyapatite[81]. Hydrolysis by local phosphatases reduces the circulating levels of PPi leading to VC[82].

Matrix Gla protein (MGP) and Gla-rich protein (GRP), members of the vitamin K-dependent (VKD) protein family, are synthesized by numerous cell types like VSMCs and chondrocytes and require vitamin K stimulation[83]. MGP is a potent VC inhibitor that binds to hydroxyapatite crystals to hinder its nucleation, and to BMP-2 growth factor and downregulates bone-specific transcription factor *cbf1a/Runx2* and the osteogenic protein Osteopontin[84]. GRP exerts its inhibitory actions via SMAD-dependent BMP signaling. Mutations in MGP or deficits in the two posttranslational modifications (γ -glutamate carboxylation and serine phosphorylation) lead to VC[82].

Fetuin-A is a protein produced in the liver and is regarded as the most effective circulating inhibitor. It contains a functional site that allows it to bind to amorphous calcium phosphate. When calcium and phosphate concentrations are elevated, Fetuin-A binding leads to the formation of harmless primary calciprotein particles (CPP), which prevent the precipitation of calcium phosphate. However, through a process known as ripening, these primary CPP can spontaneously transform into harmful secondary CPP, which are internalized by VSMCs, contributing to VC [85].

Klotho is a transmembrane protein acting as a co-receptor for fibroblast growth factor-23 to modulate its inhibitory effects on calcitriol, the vitamin D analogue that increase serum ionized

calcium (sCa^{2+}). Klotho is highly expressed in the Kidneys and modulates renal tubular calcium and phosphate reabsorption[86]. CKD patients demonstrate downregulated Klotho protein, MGP and Fetuin-A inhibitors [87]. Genetic mutations have been identified in humans that attenuate the expression of Klotho[82].

Osteopontin (OPN) is a member of the SIBLING (small integrin- binding ligand, N-linked glycoprotein) protein family that regulates mineralization in teeth and bone. It is expressed by a wide array of cells including macrophages where it acts as a cytokine. OPN was also shown to induce macrophages to dissolve calcification. At sites of calcification, OPN forms bridging proteins linking extracellular matrix to minerals. However, OPN is thought to be an inducible inhibitor as OPN-deficient mice did not develop calcification[82].

Osteoprotegerin (OPG) is a protein endogenously expressed by contractile VSMCs. It acts as a decoy receptor for RANKL and TRAIL that regulates osteoclasts differentiation. OPG was shown to block osteoblastic transformation in VSMCs via endothelial cells specific pathways. However, its function is context dependent as high levels of circulating VC were reported in VC [82].

6.4-Aldosterone and Calcification

A significant amount of evidence has highlighted aldosterone as a potential factor contributing to the development of VC. Studies have shown that VSMCs express functional MR, and when stimulated by aldosterone, these cells increase the expression of genes linked to vascular fibrosis, VC, and inflammation. Moreover, aldosterone encourages osteoblastic differentiation and mineralization through the activation of alkaline phosphatase (ALP) and bone morphogenetic protein 2 (BMP2)[88][89]. It is important to note that the vasculature is responsive to aldosterone, even though cortisol concentrations (1 μ M) are higher than those of aldosterone (1 nM) and both

hormones have the same affinity for the mineralocorticoid receptor (MR). However, VSMCs express the enzyme 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSD), which converts cortisol into its 11-keto derivatives. These derivatives have a lower binding affinity for MR compared to aldosterone [89]. Patients with CKD develop calcified blood vessels as a result of hyperphosphatemia, which is linked to elevated aldosterone levels. In a mouse model of CKD-related vascular calcification, aldosterone was shown to promote vascular calcification via the Allograft Inflammatory Factor-1 (AIF-1) signaling pathway. AIF-1 is recognized as a key inflammatory cytokine that influences the production of other cytokines and the expression of inflammatory mediators [90]. In addition, in vivo studies have shown that aldosterone levels are elevated locally in calcified arteries of uremia patients, but not in peripheral blood. In vitro studies revealed that aldosterone contributes to VC, inflammation, and apoptosis in vascular smooth muscle cells (VSMCs) through the AIF-1/NF- κ B pathway [91]. Calcification of the aortic valve leads to calcific aortic stenosis (AS) which is the most prevalent heart disease. Aldo/MR pathway was shown to play a role in early aortic valve calcification in men and in fibrosis in women through the activation of osteogenic pathways including the transcription osteogenic factors BMP-4 and Runx2. Calcification of the aortic valve leads to calcific aortic stenosis (AS) which is the most prevalent heart disease[92]. Aldosterone has been shown to enhance osteo-inductive signaling by boosting the expression of Pit-1 in human aortic smooth muscle cells. It also triggers oxidative stress by increasing NADPH oxidase activity, a key regulator of vascular oxidative stress, in rat mesenteric arteries. Additionally, aldosterone influences various inflammatory molecules, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and TGF- β . In rat vascular smooth

muscle cells (VSMCs), aldosterone induces apoptosis by inhibiting the phosphorylation of protein kinase B (Akt), a serine/threonine protein kinase involved in cell metabolism and regulation. These effects were reversed by spironolactone[93].

6.5-Vascular calcification in PA: Pathology and Detection

It is classically known that aging, hypertension and pathological atherosclerosis associated with the formation of fat streaks and plaques lead to VC and redistribution of collagen in ECM, thus thickening the intima and media aortic wall[94]. However, aldosterone is considered an additional risk factor for the development of VC in PA patients, who are considered to have higher rate of cardiovascular events compared to essential hypertension (EH) patients[95]. Chronic exposure to elevated aldosterone levels has been shown to cause vascular cell dysfunction and increased collagen production, leading to greater arterial wall stiffness. Studies have demonstrated that patients with PA who were treated with mineralocorticoid receptor antagonists (MRAs) experienced a notable reduction in arterial stiffness[96]. Of note, vitamin D and the parathyroid hormone (PTH) are responsible for regulating extracellular sCa^{2+} levels and bone turnover as mentioned before. Specifically, the decrease in sCa^{2+} levels will activate the Ca^{2+} -sensing receptor, which evokes the release of PTH, the reabsorption of Ca^{2+} by tubular cells and the mobilization of Ca^{2+} from the bones and, in parallel, the vitamin D induces Ca^{2+} absorption in the large intestine[97].

Additionally, a functional interaction between the zona glomerulosa and the parathyroid gland has been shown. In PA, hyperaldosteronism triggers glomerular hyperfiltration, resulting in calcium loss and a decrease in serum calcium levels (sCa^{2+}). This decrease stimulates increased parathyroid hormone (PTH) secretion, particularly in more severe PA patients with an APA. It's

important to note that parathyroid glands express MR and AT1R, while adrenocortical tissue expresses the type 1 PTH receptor[98]. This demonstrates the positive relationship between PA and primary hyper-parathyroidism (PHPT), in which elevated aldosterone levels increase levels of PTH and vice versa[86]. In turn, PTH induces vascular remodeling and vascular stiffness via direct actions on VSMCs and endothelial cells. In addition, PTH increases aldosterone and plasma renin levels[99]. Previous studies demonstrated the increase of PTH levels in PA patients and that was corrected by adrenalectomy[100][101].

Detecting and measuring ectopic calcification is regarded as a difficult task, with a range of both invasive and noninvasive methods employed for this purpose. In vivo, the CT scan is considered the gold standard for diagnosing VC [102]. The computation of calcification score is possible using Coronary Artery Calcium score. Other imaging techniques include MRI, plain radiography, ultrasound, PET scan, ankle–brachial index, and angiograph. Of note, imaging diagnostic tools can not differentiate between medial and intimal calcification[66][103]. PA is clinically associated with high prevalence of abdominal aortic calcification (AAC). Employing CT scans and quantitatively measuring calcification deposition using aortic calcification index (ACI) score, it was demonstrated that prevalence and severity of AAC was higher in PA compared to EH patients with higher AAC in unilateral PA compared to the bilateral subtype[104][105]. Results were consistent using an ultrasound[106]. Moreover, it was shown that patients with KCNJ5 mutated APA showed less atherosclerotic calcification plaques but thicker abdominal aorta [107].

The T50 test is a new in vitro method developed to assess the calcification potential in human serum. It determines the time required for primary calcium-phosphate particles (CPPs) to transform into secondary, more harmful CPPs, providing an indication of the serum's ability to

prevent calcium-phosphorus crystallization. Higher T50 values indicate greater resistance to calcification[\[108\]](#). Lower values of T50 tests represent higher cardiovascular risk propensity. However, this approach is devoid of any cellular component since it is based on a chemical reaction and is highly influenced in samples with a mineral imbalance or decreased circulating fetuin-A [\[108\]](#).

More importantly, as VC is an active process, it is important to integrate the circulating biomarkers in the calcification response. Based on that, a research team developed the biohybrid assay to determine individual's vascular calcification propensity, which is an in vitro cell-based assay for measuring the propensity of combined biomarkers on vascular calcification. It is illustrated by real time measuring of in vitro calcium deposition on VSMCs[\[109\]](#).

Aims of the Research

Hyperaldosteronism, defined as excess aldosterone production inappropriate for salt and volume status, is a hallmark of arterial hypertension including PA. In adrenocortical cells, arranged initially as glomeruli structure in intact adrenal tissues, calcium is the key factor regulating aldosterone biogenesis, and genetic alterations in APA activate cytosolic calcium signalling leading to spontaneous aldosterone synthesis in the mitochondria. Based on that, the first part of this research was aimed to:

- 1- Investigate cytosolic calcium dynamics in CD56⁺ APA and APA adjacent cells (AACs)
- 2- Explore if mechanically and enzymatically dispersed CD56⁺ AAC and APA cells form cell clusters (rosettes) ex vivo and if structure regulates function.
- 3- Investigate mitochondrial signature in terms of reactive oxygen species (ROS) formation in AAC and APA upon Ang II stimulation.

Excessive aldosterone is related to various cardiovascular injuries. As vascular calcification is an active process that occurs within the vessel wall and increases the risk of cardiovascular disease, patients with PA caused by an APA may have a higher vascular calcification due to the high levels of aldosterone and of calcification mediators in the circulation. Therefore, we conducted research to investigate whether plasma of APA patients drive in vitro calcification on vascular smooth muscle cells (VSMCs) using Biohybrid Assay.

Materials and Methods

1-Patients Recruitment

We recruited 15 consenting patients with unilateral APA according to the “five corner criteria”[\[31\]](#). The diagnosis of APA pathology had to be confirmed by correction of biochemical cure at follow according to the PASO criteria [\[110\]](#) at one month and by double immunohistochemistry for CYP11B2 and CYP11B1 of adjacent paraformaldehyde-fixed tumor sections using monoclonal antibodies [\[111\]](#) in all patients. Mutational status was determined by Sanger sequencing and, if necessary, with next generation sequencing protocol as reported[\[112\]](#). The collection of samples, their manipulation and following studies were approved by the local ethics committee (Comitato Etico Territoriale Area Centro-Est Veneto, Padova, Italy, Approval number: 1925P).

2-Cell Preparation and Culture

2.1-Human Adrenocortical Cells

APA and APA adjacent tissues were collected in the operating room and then cleared from surrounding fat and connective tissue. To obtain dispersed cells, we applied enzymatic digestion and mechanical separation. Cells were then immune-magnetically separated with anti-CD56 (neural cell adhesion molecule) antibody pre-coated magnetic beads as reported [\[24\]](#).

Isolated CD56+ cells were checked for viability using trypan blue and seeded on 24 mm poly-lysinated glass coverslips 24 h prior to Ca^{2+} imaging experiment in DMEM/F-12 (Dulbecco's

Modified Eagle Medium/Nutrient Mixture F-12) supplemented with 1% Penicillin/Streptomycin, 1 % Insulin-Transferrin- Selenium (ITS), and 5 % cosmic calf serum (CCS).

2.2-hVSMCs culture and characterization

hVSMCs were collected from surgical biopsies of non-atherosclerotic abdominal aortas in accordance with MUMC+ research and diagnostic procedure. Collection, storage and use of tissue and patient data were performed in agreement with the Dutch Code for Proper Secondary Use of Human Tissue (<https://www.federa.org/codes-conduct> accessed on 30 July 2021) and complies with the Declaration of Helsinki. Human vascular tissue samples were washed in phosphate buffered saline (PBS) then the intima, fat and connective tissue were removed. After tissue was fragmented (approx. 2–5 mm in diameter), it was cultured on laminin coated plates (#L2020, Sigma, Saint Louis, MO, USA) in M199 medium (Gibco, Bleiswijk, the Netherlands) which contains 20% fetal bovine serum (FBS), 1% penicillin/streptomycin (PS, Gibco, Bleiswijk, the Netherlands) and 1% Amphotericin B. After reaching confluency, cells were passaged 1:2 for two further passages. The routine culture of hVSMCs was done in M199 medium with 20% FBS and 1% PS. Cells used for experiments were taken between passages 5–10. All samples were mycoplasma tested [109].

For characterization (Supplemental Figure S2), hVSMCs were fixed in 4% paraformaldehyde then blocked in 2% BSA, 0.1% triton in PBS for one hour. Primary antibodies were incubated overnight at 4 °C: alpha-Smooth Muscle Actin (SMA) 1:200 (DAKO, Glostrup, Glostrup, Denmark, M0851), phosphorylated-Myosin Light Chain (pMLC) 1:200 (Cell Signaling, Danvers, MA, USA, 3675S), Calponin (CNN1) 1:200 (Abcam, Cambridge, UK, ab46794), Smooth Muscle protein 22-alpha (SM22a) 1:200 (Abcam, Cambridge, UK ab14106) and S100 calcium-binding protein A4 (S100A4)

1:200 (DAKO, Glostrup, Glostrup, Denmark, A5114). Incubation with secondary antibodies was done for one hour at room temperature: anti-mouse FITC 1:250 (Hycult Biotech, Uden, The Netherlands, HP2001) or anti-rabbit FITC 1:250 (DAKO, Glostrup, Glostrup, Denmark, F0205). Before imaging, cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) for one minute. Imaging was performed on the Cytation 3 system on 20_ magnification (BioSPX, Abcoude, The Netherlands)[109].

2.3-iVSMCs

Peripheral blood mononuclear cells (PBMCs) were used to create induced pluripotent stem cells (iPSCs), which are embryonic-like cell lines capable of unlimited expansion and differentiation into patient-specific vascular cells. Optimized differentiation protocols were developed to generate patient-specific vascular smooth muscle cells (VSMCs) in unlimited quantities. These protocols were tailored to produce lineage-specific VSMCs, including subpopulations primarily found in the ascending aorta, aortic arch, or descending aorta [113][114]. Cells were seeded at a 1:2 ratio on laminin-coated plates using DMEM containing 1X Glutamax, 10% fetal bovine serum (FBS), and 1% PS. They were utilized once reaching 80% confluency.

3-Ca²⁺ Imaging

The day of experiment, CD56+ cells were washed twice with modified Krebs-Ringer Buffer (KRB) consisting of NaCl (135 mM), KCl (2.5 mM), MgSO₄ x 7H₂O (1 mM), MgCl₂ x 6H₂O (1 mM), K₂HPO₄ (0.4 mM), HEPES (20 mM), glucose (5.5 mM), and CaCl₂ (1 mM) at pH 7.4. Afterwards, cells were incubated with 2 μM Fura-2-AM (Life Technologies, Milan, Italy) for 20 minutes at 37°C in KRB added with 0.02% Pluronic acid and 250 μM sulphinpyrazone. After one wash with KRB, cells were transferred to a metal open-top holder and placed on the microscope stage for image

acquisition. After acquisition of basal resting Fura-2 signal, cells were treated with Ang II at different doses (1 nM, 10 nM), then ATP (100 μ M), and ionomycin (1 μ M) were added to induce IP3-dependent ER Ca^{2+} release and to promote Ca^{2+} entry and saturate the Ca^{2+} dye, respectively. Ca^{2+} - dependent Fura-2 fluorescence levels were monitored using an inverted confocal fluorescence microscope Nikon Eclipse Ti equipped with a Zyla-CMOS 4.2-P camera (Andor, Oxford Instruments, Abingdon, UK) and a 40x objective Nikon S Fluor Oil 1.30 DIC H/N2 WD 0.22). Excitation wavelengths were obtained with a 75 W Xenon Lamp (USHIO, UXLS50A) and a monochromator (Cairn Optoscan Monochromator, Cairn Research Ltd., Faversham, UK) controlled by NIS-ELEMENTS AR (Nikon) software provided alternating excitation wavelengths (340/380 nm). A neutral density filter, ND4 (Nikon, USA) and a FF-409-DiO3 Dichroic (Semrock) were used in the excitation pathway. Collection of emitted fluorescence was done using a 510/84 nm filter (Semrock). Images were acquired every 1 s with 100 ms exposure time, by a Zyla-CMOS 4.2-P (Andor, Oxford Instruments) controlled by the same software.

4-Cell Treatment

To inhibit AT1R, CD56⁺ AAC and APA cells were treated with AT1R antagonist Irbesartan 10 μ M (sigma Aldrich) for 1 hour.

For TASK-2 inhibition experiments, cells were incubated with quinidine 20 μ M (22600-10G-F, Merck) for 25 minutes prior to Ca^{2+} imaging.

For Ca^{2+} depleted extracellular medium, after Fura-2 incubation cells were washed for 4 minutes with EGTA (200 μ M) Ca^{2+} ion chelator and then imaged in Ca^{2+} free KRB.

5- Data Analysis

5.1- Ca^{2+} Data Processing and Workflow

A custom-made pipeline was developed to analyze the variations in individual cell Ca^{2+} signals assessed by Fura-2 (Fig. 10). The input was a confocal microscope image and the resulting output was distinct individual peaks with peak parameters associated to each. The pipeline enclosed 3 main steps: 1) data extraction and construction, 2) trace plotting and peak identification and 3) peak filtering. We used ImageJ-based Fiji software, Microsoft Excel 2019 MSO (Version 2304 Build 16.0.16327.20200), and OriginPro 2023 software (10.0.0.154 academic version, OriginLab Corporation, Northampton, MA, USA-). Data extraction was executed manually on ImageJ based Fiji image analysis software, (available at <https://imagej.net/Fiji>), by assigning individual cell as a region of interest (ROI) on raw confocal images (Figure 11). Fura-2 is a dual excitation ratiometric Ca^{2+} indicator commonly used for the measurements of intracellular $[\text{Ca}^{2+}]$. The Ca^{2+} free form of Fura-2 has a peak excitation wavelength at ~ 380 nm, whereas the Ca^{2+} bound form has a peak excitation wavelength at ~ 340 nm. An elevation of Ca^{2+} concentration induces an increase in Fura-2 emission fluorescence when the indicator is excited at 340 nm, with a corresponding decrease in fluorescence at 380-nm excitation. The ratio between emission after excitation at 340 nm and 380 nm (340/380 ratio) represents a good estimation of the level of Ca^{2+} in the sample [25]. The fluctuations of Fura-2 fluorescence emission at the 2 different exciting wavelengths (340 nm and 380 nm) were recorded and extracted separately, each normalized to the relative background noise (a region of the coverslip devoid of cells), then expressed as 340/380 ratio.

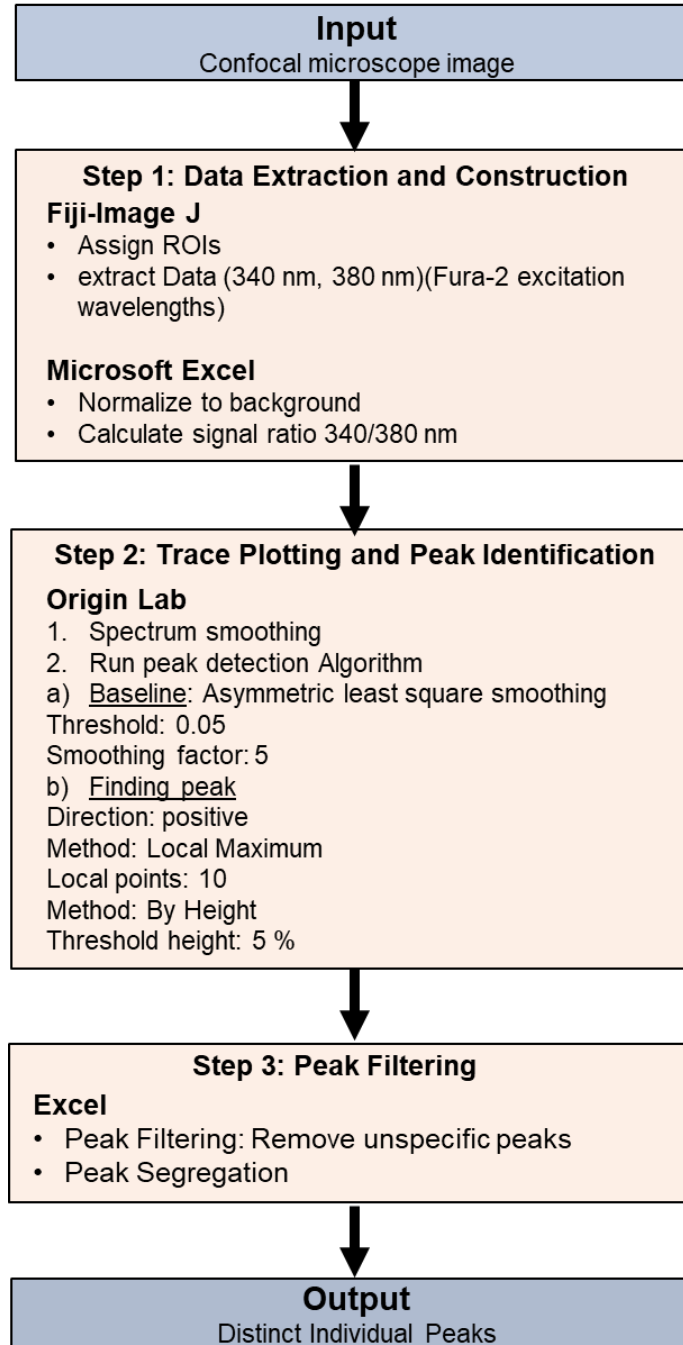


Figure 10: Workflow of custom-made peak detection pipeline. Input data are the raw confocal microscope images of the Ca^{2+} - sensitive probe fluorescence and the output results are dynamics parameters (number, frequency and shape) of individual cell Ca^{2+} peaks. The pipeline comprises 3 data processing steps. Step 1 (Data Extraction and Construction): assignment of ROIs, extraction of Fura-2 signal value for the two excitation wavelengths (Fiji-Image J), normalization of data to the background, and calculation of Fura-2 340/380 nm signal ratios (Microsoft Excel). Step 2 (Trace Plotting and Peak Finding): data smoothing followed by peak detection through the application of the OriginPro software algorithm. Step 3 (Peak Filtering): implementation of Microsoft Excel's equations to identify spontaneous vs Ang II evoked Ca^{2+} peaks. For Ang II-evoked peaks, first and subsequent oscillations are considered separately.

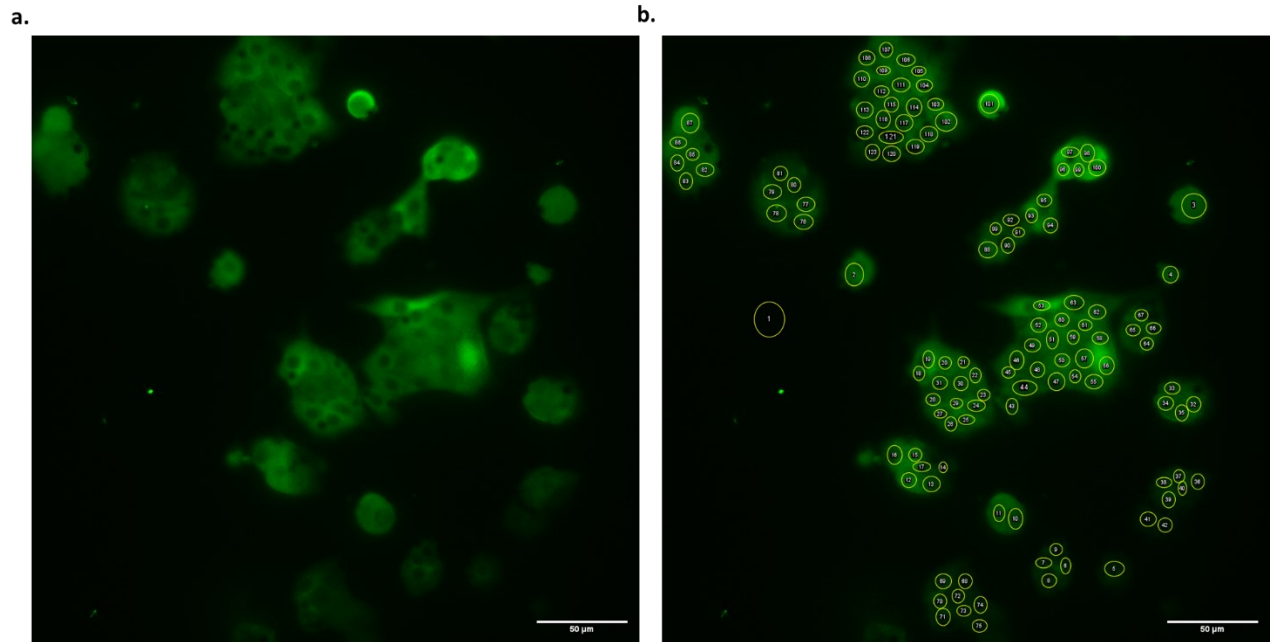


Figure 11: Representative confocal image of CD56⁺ APA cells after 24 hours in culture. a Raw image, b same as A after ROIs assignment. Black dots represent anti-CD56+ magnetic beads used for cell isolation. ImageJ based Fiji software was utilized to assigned ROIs. Scale bar = 50 µM.

5.2- Cluster Analysis and linear mixed effects models

5.2.1-Clustering and Feature Extraction

A custom Python class was developed to perform clustering analysis on 9 different patients. The initial step involved calculating the correlation matrix for the cells, followed by the conversion to a dissimilarity matrix using the formula $1 - r_{xy}$, where r_{xy} represents the Pearson correlation coefficient between cells x and y. Hierarchical agglomerative clustering was then performed using the scipy library's linkage function with complete linkage. The cluster function was used to form clusters by cutting the dendrogram at a specified cophenetic distance. For example, cutting the dendrogram at a distance of 0.1 ensured that all clusters contained cells with a correlation greater

than 0.9. Predicted clusters were then analyzed to identify subclusters within labeled clusters, focusing on their synchronicity in response to Ang II-induced Ca^{2+} oscillations.

Feature extraction involved determining the number of predicted subclusters within labeled clusters, the number of cells in these subclusters, and the total number of cells in labeled clusters. Clustering was performed at various distances (0.05, 0.1, 0.15, 0.2, and 0.25) to explore the optimal number of clusters at different correlation strengths. Single-cell labeled clusters were excluded from the analysis to avoid redundant information. The cross-tabulation of predicted and labeled clusters was performed using the pandas crosstab function, facilitating the extraction of significant features which were subsequently stored in data frames and exported to CSV files.

5.2.2- Implementation of Linear Mixed Effects Models

Given the inherent dependency and non-normal distribution of the extracted features, linear mixed effects models were employed to perform the analysis. The models were implemented in RStudio using the lme4 package. Three different models were constructed to analyze features such as the number of predicted subclusters and the number of cells in both predicted and labeled clusters, considering the origin of the samples (AAC or APA) and the patient identifier as random effects.

The models investigated various relationships, such as the influence of cluster size and sample origin on the number of predicted subclusters and the size of these subclusters. The general form of the models included fixed effects for the number of cells in labeled clusters (NCL) and sample origin (AACAPA), along with a random effect for patient ID to account for inter-subject variability. Model comparisons were performed using the ANOVA function to assess the significance of different predictors.

The three different models were constructed utilizing the extracted clustering features that are shown below:

- NPS: Number of predicted subclusters found in a labeled cluster.
- NCL: Number of cells in a labeled cluster
- NCPS: Number of cells found in the predicted subclusters found in a labeled cluster.
- AACAPA: 0 = AAC, 1 = APA (categorical value representing the sample's origin)
- Patient-id: patient number 1, ... $m = 9$

Model 1: The objective of this model was to investigate if the size of a labeled cluster as well as its origin influence the number of predicted sub-clusters were contained in the labeled cluster.

$$NPS = \beta_0 + NCL\beta_1 + AACAPA\beta_2 + Z_i\alpha_i + \epsilon_i \text{ with } i=1, \dots, m$$

$$\alpha_i \sim N(0, \sigma_\alpha^2 I), \epsilon_i \sim N(0, \sigma^2 I)$$

Model 2: This model positions NCL as the response variable rather than a predictor, with AACAPA as the predictor. This was done to investigate if the size of a labeled cluster was influenced by the sample's origin.

$$NCL = \beta_0 + AACAPA\beta_1 + Z_i\alpha_i + \epsilon_i \text{ with } i=1, \dots, m$$

$$\alpha_i \sim N(0, \sigma_\alpha^2 I), \epsilon_i \sim N(0, \sigma^2 I)$$

Model 3: Similar to *model 1* this model investigates if the size of a labeled cluster and its origin impact the size of the predicted cluster.

$$NCPS = \beta_0 + NCL\beta_1 + AACAPA\beta_2 + Z_i\alpha_i + \epsilon_i \text{ with } i=1, \dots, m$$

$$\alpha_i \sim N(0, \sigma_\alpha^2 I), \epsilon_i \sim N(0, \sigma^2 I)$$

All models were created treating *patient-id* as the sole random effect, this accommodates the random inter-subject variation, which is a hallmark of patient-level data. Additionally, by

accounting for the variability at the patient level, the accuracy of the fixed-effects estimates is often improved. The models are better able to isolate the impact of the fixed effects from the random noise associated with individual patients.

5.2- Functional ANOVA

The objective was to investigate the existence of 'functional' clusters showing high levels of synchronicity in Ca^{2+} signaling patterns. ANOVA was used to determine if the variability among labeled clusters was greater than the variability within each cluster.

A non-parametric bootstrap F-type test suitable for functional data, which is scale-invariant, proposed in previous literature [115] was used. The functional ANOVA tested the equality of mean functions across the labeled clusters over time.

The non-parametric bootstrap method, was used to perform ANOVA on Ca^{2+} fluctuations, chosen for its robustness to non-normality and heteroscedasticity. Bootstrap samples $y_{ij}^*(t)$ were generated from the original data $y_{ij}(t)$ for each group $i = 1, \dots, k$. These bootstrap samples are used to establish k sets of bootstrap samples mean functions $\bar{y}_i^*(t)$ and bootstrap sample covariance functions $\hat{\gamma}_i^*(s, t)$. Subsequently, the vector $z^*(t)$ is computed in the following manner:

$$z_i^*(t) = \sqrt{n_i}[\bar{y}_i^*(t) - \bar{y}_i(t)], \text{ for } i = 1, 2, \dots, k$$

Where $\bar{y}_i(t)$ is the original i^{th} sample mean. The bootstrap F-type test statistic was calculated as:

$$F_n^* = \frac{T_n^*}{S_n^*}, \quad T^* = \int z^*(t)^T A z^*(t) dt, \quad S_n^* = \sum_{i=1}^k a_{n,ii} \text{tr}(\hat{\gamma}_i^*) [1].$$

Where the scaling factor $a_{n,ii}$ represent the diagonal elements of the matrix A_n shown below:

$$A_n = I_k - \frac{b_n b_n^T}{n},$$

And where $b_n = [n_1^{1/2}, n_2^{1/2}, \dots, n_k^{1/2}]^T$, Thus the (i^{th}, j^{th}) entry of A_n can be calculated as shown below:

$$a_{n,ij} = \begin{cases} 1 - n_i/N & \text{if } i = j, \\ -\sqrt{n_j n_i}/N & \text{if } i \neq j \end{cases}$$

The estimated covariance functions are calculated as below:

$$\hat{\gamma}_i(s, t) = \frac{1}{n_i - 1} \sum_{j=1}^{n_i} [y_{ij}(s) - \bar{y}_i(s)][y_{ij}(t) - \bar{y}_i(t)], \quad i = 1, 2, \dots, k$$

Where T_n^* represents between-group variability and S_n^* is the sum of adjusted covariance functions.

The analysis was implemented in R, and the bootstrap procedure was iteratively applied to determine the empirical distribution of the F-type test statistic.

This methodology allows for reproducible analysis of functional data, accommodating the challenges posed by temporal dependence and non-normality in time series data.

6-Mitochondria Function Assay

6.1-MITOSOX Assay

To monitor mitochondrial superoxide production levels, we used MitoSOX Red (Thermo Fisher, Milan, Italy) live cell fluorescent probe on cells obtained from 3 different patients. AAC and APA cells were seeded on 24 mm coverslips for 24 hours, and then incubated with MitoSOX Red (6 μ M, Thermo Fisher, Milan, Italy) in 1 mL of KREBs ringer buffer (KRB) at 37 °C for 40 min in the dark. Before imaging, cells were extensively washed four times in KRB and placed on an Olympus IX73 microscope with PlanFluor40 x/1.3 N.A. objective for image acquisition. Excitation was

performed with a Delta RAM V high-speed monochromator (Photon Technology International, Birmingham, NJ, USA) equipped with a 75 W xenon arc lamp. Images were captured with a high sensitivity Evolve 512 Delta EMCCD (Photometrics, Tucson, AZ, USA). The system was assembled by Crisel Instruments and is controlled by Meta Fluor 7.5 (Molecular Devices, San Jose, CA, USA). Cells were thus illuminated at 396 nm and fluorescence was collected through a 390-band emission filter (Chroma Technology Corp, Bellows Falls, VT, USA). Acquisitions were performed at 600 ms exposure time. Cells were stimulated with Ang II (1 nM) for 10 mins and Antimycin A (AA) (10 μ M) for 10 mins consecutively. AA is used as a positive control to elicit mitochondrial superoxide. All the acquisition parameters were set according to Meta Fluor 7.5 system and MitoSOX Red dye. Multiple fields were collected per coverslip. Analysis was performed with the Fiji distribution of ImageJ v1.52 [116]. Background noise was subtracted by removing the signal of a cell-free region of interest in the chosen field. Cell's mitochondria are presented as the mean intensity of the selected region of interest.

6.2-TMRM Assay

To measure mitochondrial membrane potential on cells obtained from 3 different patients, we used with the Fluorescent Dye Tetramethyl rhodamine Methyl Ester (TMRM) dye (Thermo fisher). AAC and APA cells were seeded on 24 mm coverslips for 24 hours, and then incubated with TMRM solution (20 nM) in KRB at 37 °C for 60 min in the dark. Cells were placed on an Olympus IX73 microscope with PlanFluor40 x/1.3 N.A. objective for image acquisition. Excitation was performed with a Delta RAM V high-speed monochromator (Photon Technology International, Birmingham, NJ, USA) equipped with a 75 W xenon arc lamp. Images were captured with a high sensitivity Evolve 512 Delta EMCCD (Photometrics, Tucson, AZ, USA). The system was assembled by Crisel

Instruments and is controlled by Meta Fluor 7.5 (Molecular Devices, San Jose, CA, USA). Cells were thus illuminated at 561/d3 and fluorescence was collected through 390-band emission filter (Chroma Technology Corp, Bellows Falls, VT, USA). Acquisitions were performed at 200 ms exposure time. Cells were incubated with CCCP (6 μ M), a protonophore that rapidly disrupt mitochondrial membrane, for 10 mins, and then TMRM signal was recorded. Analysis was performed with the Fiji distribution of ImageJ v1.52 [116]. Background noise was subtracted from each ROI by removing pixel values of a cell-free region. Mitochondria's signal is presented as the mean intensity of the selected region of interest.

7-Immunofluorescence

Double immunofluorescence staining was performed on Paraffin embedded APA slices using two sets of antibodies. CYP11B2/CYP11B1 or CYP11B2/SDHA of 4 μ m thickness. For deparaffinization, tissue slices were incubated for 2 hours at 63°C followed by sequential incubation in the following solutions correspondingly (Xyline; 2X 10 mins, isopropanol; 5 mins, ethanol 96%; 5 mins, ethanol 70%; 5 mins, ethanol 10%; 5 mins, distilled H₂O 2X; 5 mins). Slices were then autoclaved for a duration of 1 cycle (120°C for 20 mins) immersed in the antigen retrieval buffer (EDTA; 10mM, SDS; 0.5%, Ph=9, 10 X), then washed once with distilled H₂O. Non-specific binding of primary antibodies was blocked using 5% Bovine Serum Albumin (BSA) serum in the following blocking buffer (TRIS;0.1M, SDS;0.5%, Ph=7.4). Primary antibodies diluted in PBS +0.1% Triton were incubated overnight at 4°C (CYP11B2 41-17B mouse monoclonal, dilution 1:200) (CYP11B1-807-5 rat monoclonal, dilution 1:50) (CYP11B2 and CYP11B1 are kindly provided as gifts from Dr. Celso E. Gomez-Sanchez [111]) (SDHA (D-4): sc-166947, dilution 1:200). The following day, slices were washed 3X 5 mins in PBS+ 0.1 % Triton followed by incubation of secondary antibodies for 1 hour

at room temperature. For CYP11B2/CYP11B1 the following secondary antibodies were used (Anti-mouse IgG Rhodamine, Invitrogen, dilution 1:200) (Alexa Fluor® 488-AffiniPure F(ab')₂, Fragment Donkey Anti-Rat IgG, Jackson immune research Europe ltd, dilution 1:100). For CYP11B2/SDHA the following secondary antibodies were employed (Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488, Life Technologies Italia, dilution 1:200) (Goat anti-Mouse IgG2a Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568, Life technologies Italia, dilution 1:200).

The confocal microscope used was a 3i Marianas-SDC inverted microscope system based on a Zeiss Axio Observer microscope frame with 10x 0.3NA, 20x 0.8NA and 63x 1.4NA objectives with a Yokogawa CSU-W1 spinning disk confocal with 50um pinhole disk and SoRa disk, and Teledyne Prime95B22 camera for imaging. The lasers used were 405nm 100mW, 488nm 150mW, 561nm 100mW and 640nm 140mW combined in the 3i Laser Stack v4 laser launch. The software used was Slide Book 2024.1 from Intelligent Imaging Innovations.

8-Biohybrid Assay

To assess calcification deposition, cells were seeded in culture well plates at a density of $10\text{--}15 \times 10^3$ cells/cm². After 24 h, cells were cultured in calcification medium; for hVSMCs (M199, 5% human plasma, 1% PS and 3.6 mM Ca²⁺), and for iVSMCs (DMEM-1 X Glutamax, 5% human plasma, 1% PS and 3.6 mM Ca²⁺) for up to 12 days. fetuin-A-Alexa Fluor®-546 (1–3 g/mL; prepared in house) and Hoechst 33,342 (1 g/mL, Invitrogen, Waltham, MA, USA) were supplemented at the start of the experiment. At various time points after calcification induction, red fluorescent protein (RFP) and DAPI channel (cell count) were imaged up to 12 days. Imaging was done every 3 days to follow calcification progression employing Citation 3 system (BioSPX, Abcoude, the Netherlands) and analyzed using Gen5 software v.2.9 (Biotech, Abcoude, the Netherlands).

Plasma controls were made up of pool of 8 healthy volunteers. As readout RFP confluence per well was normalized against cell count[\[109\]](#).

9-Statistical Analysis

Data were analyzed using GraphPad software (Prism 10) (version 10.1.2). Normal distribution of the data was assessed using Shapiro Wilk test. Data were plotted as mean and standard error (SEM). Comparison of data was done using non-parametric Mann-Whitney test between two groups, or one-way analysis of variance (ANOVA: Kruskal- Wallis). Data were plotted as mean and standard deviation (SD) when Gaussian distribution was detected, and as median and interquartile ranges when no Gaussian distribution was detected. The scheme of figure 1 was created using bio-Render.

Results

1-Patients Recruitment

In total, 15 patients underwent adrenalectomy after AVS guided diagnosis. Due to insufficient sample material, 1 patient provided only APA cells, while another patient provided only AAC. Overall, the basal Ca^{2+} levels in 14 AAC and APA tissues were investigated. Due to insufficient sample material, 1 patient provided only APA cells, while another patient provided only AACs. The adrenal cells from 5 patients were used only for basal Ca^{2+} measurements.

To study the spontaneous and Ang II-evoked $[\text{Ca}^{2+}]_i$ dynamics, in APA cells and the corresponding AAC cells, 10 APA patients were analyzed .

The study of TASK-2 inhibition with quinidine was done on AACs from 4 different patients.

2- Ca^{2+} Analysis Pipeline Development

2.1-Trace Plotting and Peak Identification

OriginPro lab software was used to plot Ca^{2+} traces. A noise reduction algorithm was applied to reduce undesired background fluctuations, that was observed in some samples, based on the Savitzky-Golay (SG) method to all sample traces[\[117\]](#)[\[118\]](#)[\[119\]](#). The SG method fits a polynomial regression to successive data points of a moving window (local least-squares polynomial approximation) to recover the trace shape. The degree of smoothness was optimized at a window size = 20 resembling the number of data points used in each local regression. The mentioned data processing tool achieves data smoothing while preserving the distinct features and properties of various data patterns and frequencies. The mentioned settings have been determined for few

traces selected as templates and then used in the following batch analysis for the required large data set.

As shown in (Figure 12) in both AAC and APA cells, Ang II-evoked Ca^{2+} signals do not always return to the baseline level. Thus, establishing of a straight-line baseline functional to the event detection is rather inefficient. Therefore, a critical step towards processing dynamic live cell recordings is to adopt a baseline calculation approach capable to drift and adapt to the peculiarity and specificity of the signal of each cell. Accordingly, we implemented the “Asymmetric least square smoothing baseline setting” (ALS) in the peak detection algorithm of OriginPro software. To run the algorithm, the following parameters were implemented; a threshold at 0.05, smoothing factor at 5, with a number of iterations set at 10. These settings correct the baseline by asymmetrically weighting the trace deviations and the estimated baseline adjusts to the highly dynamic $[\text{Ca}^{2+}]_i$ oscillations, allowing to adapt to the drift in signals (Figure 13, panel a). The settings of the peak finding algorithm were set as follows: Direction = positive; Method = Local Maximum; Local points=10. In addition to that, the algorithm promoted detection of peaks following the indicated peak filtering settings: Method = By Height; Threshold height= 5 %. We focused our analysis on selected peak features, defined as: (i) amplitude from baseline (height of the peak), (ii) area under the curve (AUC, which represents an estimation of the total amount of Ca^{2+} entering the cell) and (iii) full width at half maximum (FWHM, which describes the extension/duration of the peak (Figure 13, panel b). The results of the optimized algorithm described are identified individual peaks (Figure 14, panel a–d).

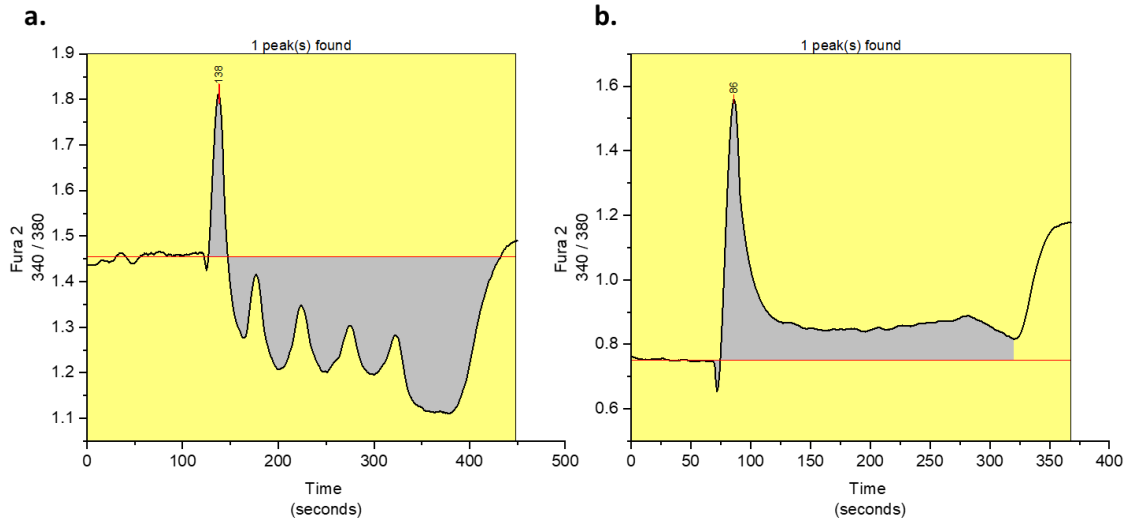


Figure 12: Baseline detection. **a, b** Representative Ca²⁺ signal traces of 2 different AAC showing the inadequacy of setting a straight-line (red line) as baseline when performing dynamic Ca²⁺ recordings and peak detection. In these samples, the set baseline vanishes the detection of oscillations (panel a) and fails to accurately detect AUC (panel b).

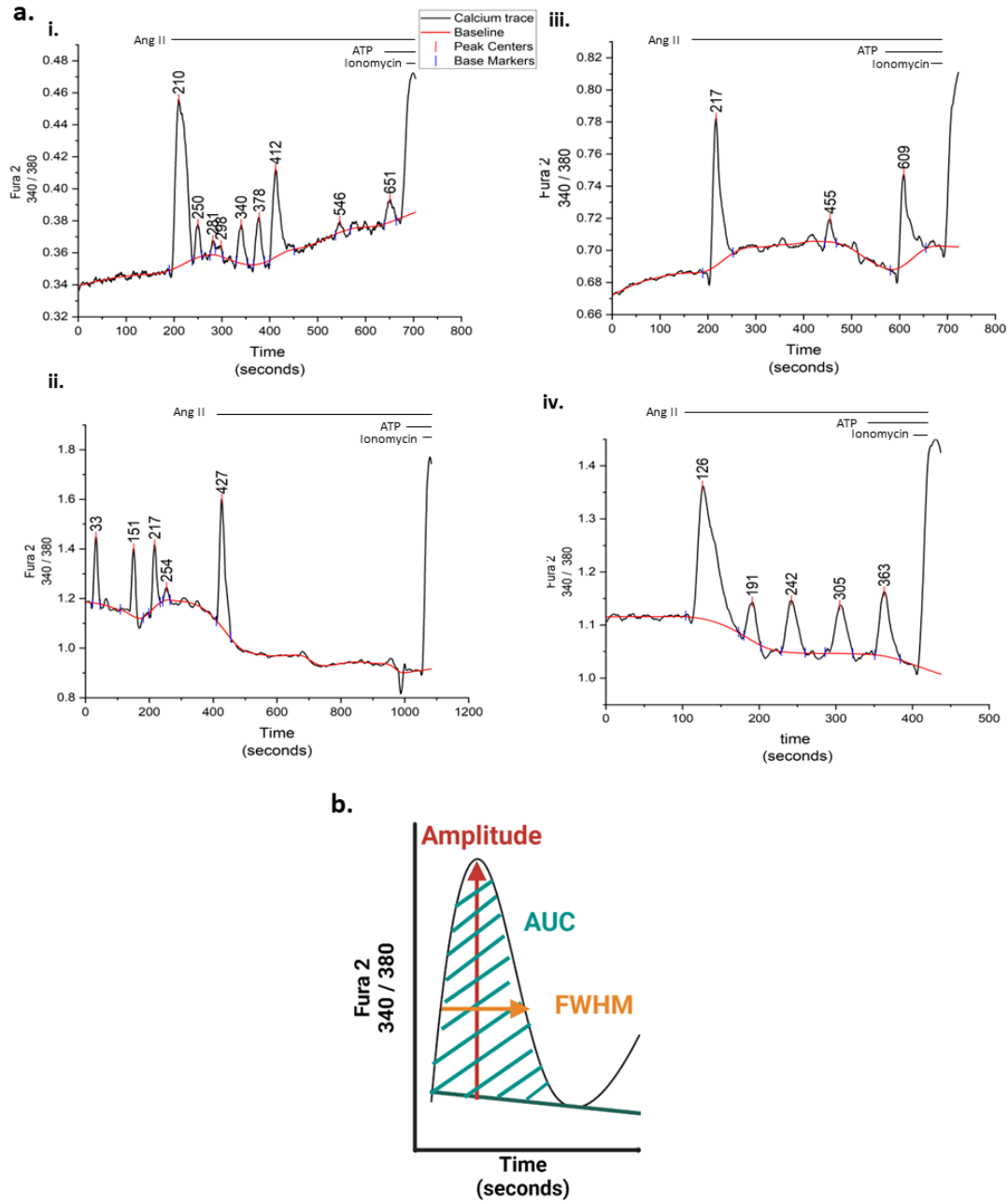


Figure 13: Modeling human CD56⁺ AAC and APA cells intracellular Ca²⁺ dynamics by standardized peak detection and characterization. **a.** Baseline setting (red line) was adapted to the dynamic nature of the Ca²⁺ signals using the OriginPro 2023 OriginLab Corporation lab software as described in the peak detection algorithm settings. 10 nM Ang II was used to induce the Ca²⁺ response. ATP was positive control for IP₃-mediated increase of [Ca²⁺]_i. To saturate the probe, 1 μ M ionomycin was used in the presence of excess of Ca²⁺. Numbers on peaks represent the time in seconds at which each event has occurred. i + ii: AAC, iii + iv: APA. **b** Peak characterization was performed by calculating different features of each individual Ca²⁺ peak: amplitude (red line); full width at half maximum (FWHM, orange line); area under the curve (AUC, green area).

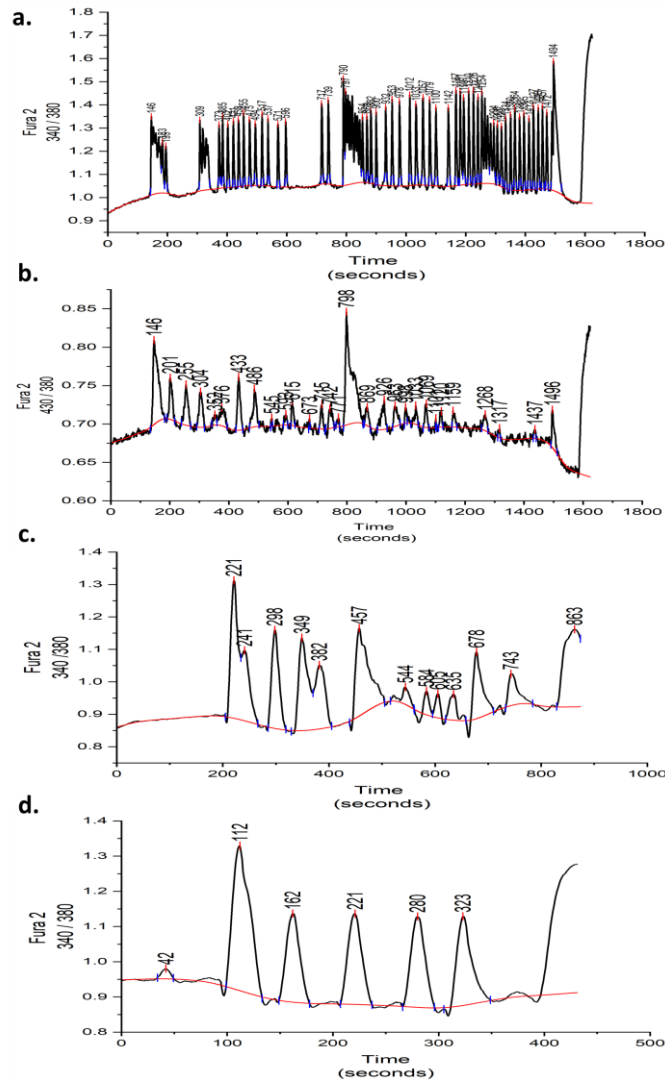


Figure 14: Representative Ca^{2+} traces of AAC (a, b) and APA (c, d) cells with detection of Ca^{2+} peaks according to our pipeline. Algorithm developed shows flexibility and adjustability to different types of behaviors. Numbers on peaks represent the time in seconds at which each event has occurred.

2.2-Peak Filtering

The strategy here developed allowed flexible application of the pipeline to variable experimental conditions, such as the addition of multiple stimuli and the time of addition of the stimulus itself.

We could filter out residual noise and nonspecific signals, through simplified equations designed on Microsoft Excel 2019 MSO, thus performing a second step of data cleaning, where peaks

posing an area less than 0.5 combined with a height less than 0.016 were removed, using the following the equation:

$$= \text{If} ((\text{Area column} < 0.5) * (\text{height column} < 0.016, \text{"Delete"}))$$

The Ca^{2+} traces could be subdivided in various regions for subsequent detailed analysis according to the experimental procedure applied. Here, we defined a region where spontaneous intracellular Ca^{2+} activity occurs (i.e. before the addition of the stimulus) and a region where Ca^{2+} fluctuation occurs in response to the stimulus (Ang II-evoked peaks). The time of Ang II addition discriminate the two regions in the Ca^{2+} trace plot.

In case the addition of Ang II was provided at sec 130, the equation used is:

$$= \text{If} (\text{time cell} < 130, \text{"spon"}, \text{If} (\text{AND} (\text{time cell} \geq 130, \text{time cell} < 260), \text{"10 nM dose"}, \text{"none"}))$$

However, Ca^{2+} traces could adopt either a unique single Ca^{2+} transient behavior upon stimulus addition or an oscillatory behavior as depicted before in [\(Figure 12 and 13\) \[120\]](#). In case of a multi peak behavior, it is applicable to segregate detected individual Ca^{2+} peaks in a time manner to allow the differentiation between the first response transient and the subsequent peaks following this equation (with A3 and A2 demonstrating the number code of the cell);

$$= \text{If} (A3 = A2, \text{OS (oscillating)}, \text{"First Peak"})$$

To be able to quantify the number of cells showing unique or a multi peak Ca^{2+} behavior, we employed the subtotal count function on Excel. As individual ROIs are indicated by different ROI-ID number, we sum all the detected peaks originating from 1 ROI using the distinct ID numbers and then we apply the following equation on the subtotal function results which resembles the

number of ROIs showing oscillation activity with column I representing the subtotal count function results (fig 6)

= COUNTIF(I4:I172," >= 2")

Frequency of oscillations was also characterized when 2 or more peaks were detected ($F = 1/T$) (period= T represents time for one oscillation) (Figure 15). To calculate the period (T), copy the Id index (ROI name) and index (Time) with the subtotal to a new sheet, then subtract each 2 consecutive row indexes ($= B4 - B3$). Remove nonspecific values resulting from data ID count by conditional formatting, highlighting cells, then pick the Text that contain (count). Filter out Column A (data Id ROIs) by color then Column C (T): by number, greater than 0. Continue to calculate the frequency in Hz ($= 1/T$).

Ca²⁺ signals plots were divided into resting conditions, in which spontaneous activity could be detected, and Ang II-stimulated conditions.

In our experiment, we used ATP and ionomycin as controls to determine the responsiveness and viability of the cells only and thus the corresponding peaks were eliminated from detection by the algorithm (Figure 15).

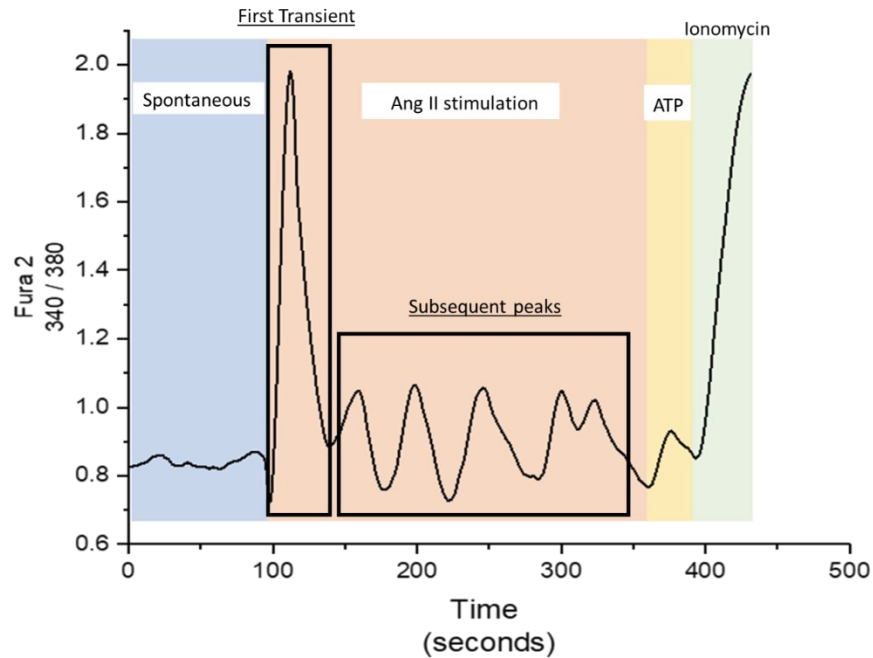


Figure 15: Time defined segments of a single Ca^{2+} trace in CD56^{+} aldosterone-producing cell. All Ca^{2+} recordings followed the sequence shown: spontaneous phase (unstimulated conditions), Ang II-stimulated phase divided into first transient and subsequent peaks, ATP and ionomycin stimulated phases. Different segments are marked with different colors. Period of each phase differs for each slide of cells.

3-Resting Ca^{2+} levels in human CD56^{+} adrenocortical Cells

We sought to evaluate the unstimulated Ca^{2+} dynamics, so we measured the level of $[\text{Ca}^{2+}]_i$ in AAC and APA CD56^{+} cells at resting conditions of Ang II-responsive cells. The general IP_3 -generating stimulus ATP and the ionophore ionomycin were used to challenge cell ER Ca^{2+} release and to normalize the Fura2 signal at saturating Ca^{2+} concentration, respectively. Cells that did not show Ca^{2+} response after Ang II stimulation were not considered.

we quantified $[\text{Ca}^{2+}]_i$ levels by averaging signals over 20 seconds prior to Ang II addition (Figure 16, panel a). As shown in (Figure 16, panel b), AAC (0.80 AU (25% percentile=0.65; 75% percentile=0.90)) displayed significant and consistent higher basal $[\text{Ca}^{2+}]_i$ levels compared to APA cells (0.53 AU (25% percentile=0.33; 75% percentile=0.79) ($p=0.0212$)).

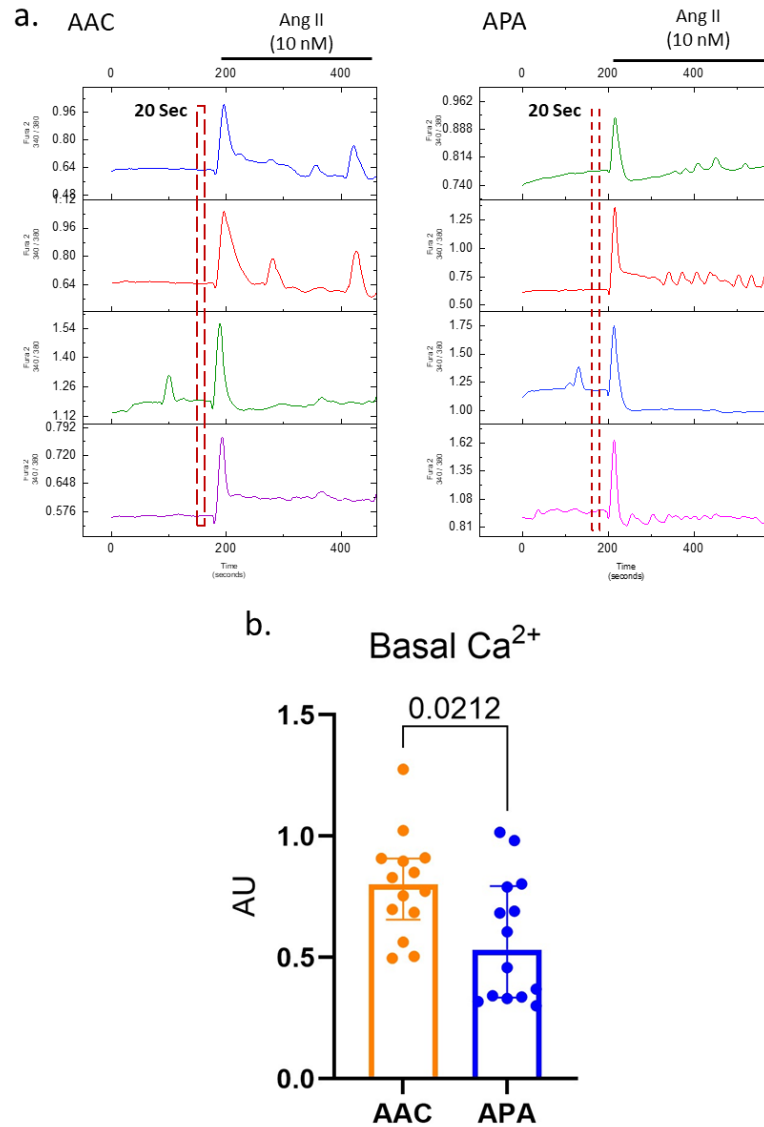


Figure 16: a. Representative traces of basal Fura2 Ca^{2+} signal used for the quantification of basal cytosolic $[\text{Ca}^{2+}]_i$ levels in AAC and APA cells. Red square represents the 20 seconds averaged period of calcium trace used to quantify the average basal $[\text{Ca}^{2+}]_i$. b. The histogram represents the average resting $[\text{Ca}^{2+}]_i$ levels in human CD56+ adrenocortical cells estimated as the Fura2 340/380 nm signal ratio from data obtained from AAC and APA cells treated as in a and b. Data is presented as median with interquartile range. Each dot represents the average basal Ca^{2+} level of manually assigned ROIs (each ROI approximately corresponds to one cell). 10 to 150 cells per coverslip were analyzed, from 15 patients. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

4- Spontaneous intracellular Ca^{2+} transients in human CD56⁺ adrenocortical cells

During the recording of basal $[\text{Ca}^{2+}]_i$ and before the addition of any stimuli, we noticed that some cells showed spontaneous intracellular Ca^{2+} transients. We therefore quantified the spontaneous activity appearing in both CD56⁺ AAC and APA by applying our custom-made pipeline already described [121].

The average time window for the detection of spontaneous peaks was set at 177 seconds. The assessment of spontaneous activity demonstrated the presence of either discrete and separate Ca^{2+} spikes or a burst of spikes regardless of the cell type (Figure 17, panel a and b).

The percentage of cells showing spontaneous Ca^{2+} activity was calculated by dividing the number of cells demonstrating spontaneous activity over the total number of cells in the coverslip. No significant difference was found in the cell percentage between AAC and APA; AAC (3% (25% percentile=1.25; 75% percentile=4)) APA (3.5% (25% percentile=1.5; 75% percentile=12.50)) $p>0.05$. Also, the other peak parameters analyzed revealed no difference between the two cell types (Figure 18).

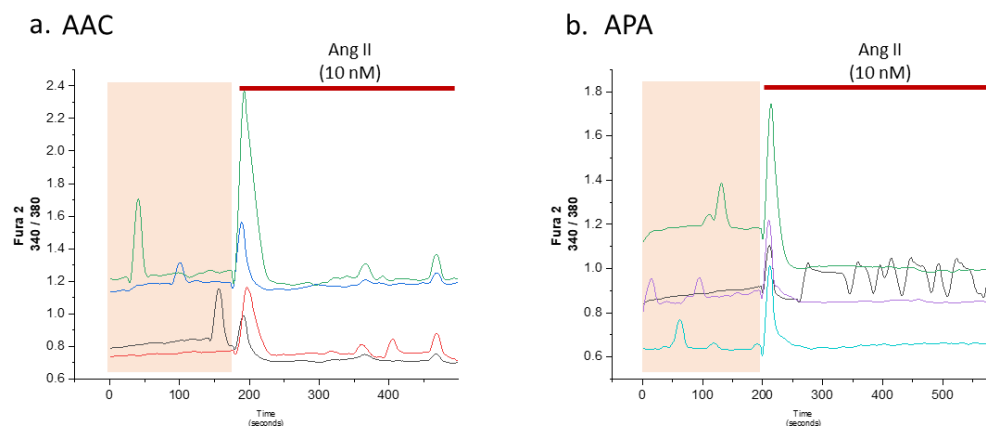


Figure 17: Representative traces of $[Ca^{2+}]_i$ levels measured as Fura2 340/380 nm signal ratio in AAC (a) and APA (b) cells, before (pink-shaded area) and after Ang II stimulation. Each trace represents a single cell ROI. (AAC= 1037 cells) (APA= 1166 cell). Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

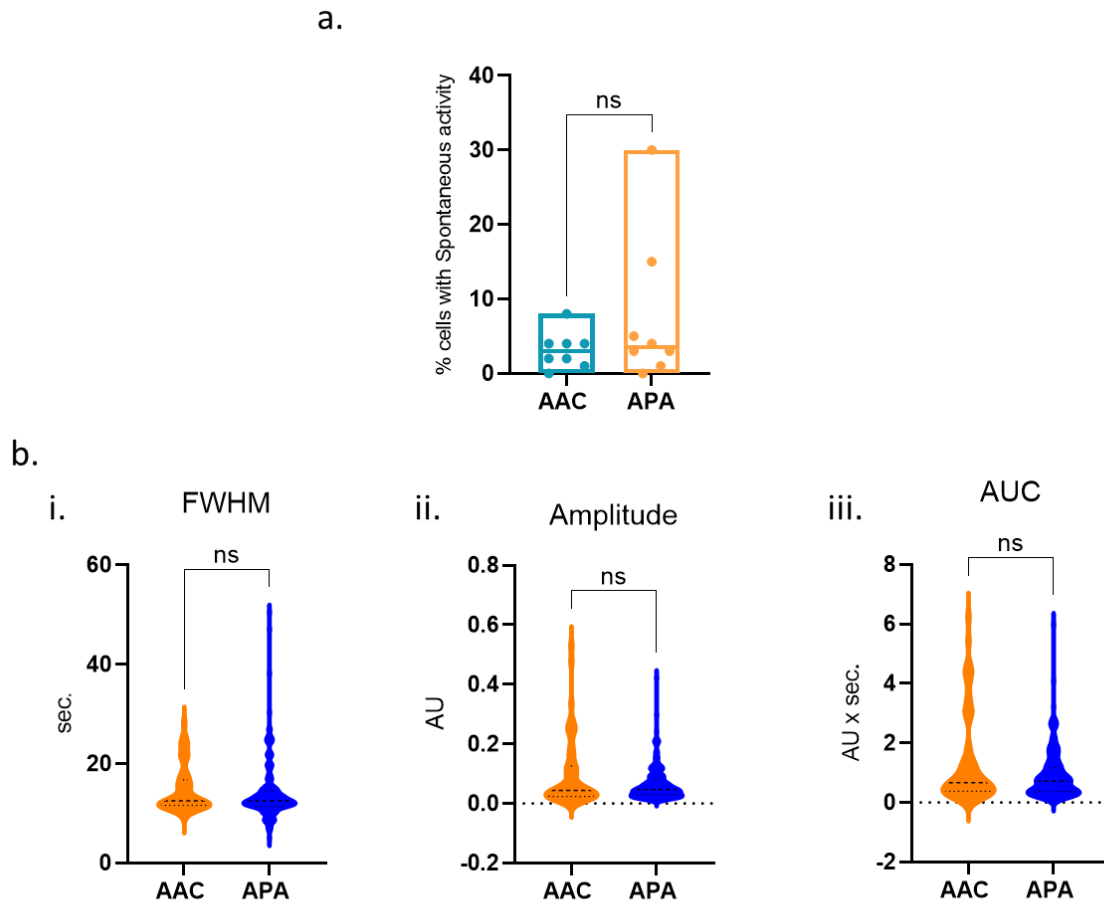


Figure 18: Detection of Spontaneous $(Ca^{2+})_i$ peaks in AAC and APA cells during unstimulated conditions. a. Floating bars graph depicts the % of cells showing detected spontaneous Ca^{2+} activity. AAC 3 (25% percentile=1.25; 75% percentile=4) APA 3.5 (25% percentile=1.5; 75% percentile=12.50) $p > 0.05$. b. Peak parameters of detected spontaneous Ca^{2+} activity in both AAC and APA cells. FWHM (full width at half maximum) (i), Amplitude (ii) and AUC (area under the curve) (iii) from AAC and APA cells. Data of violin plots are represented as medians and interquartile ranges. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

5-Ang II-evoked $[Ca^{2+}]_i$ oscillations in CD56⁺ AAC and APA cells

5.1- Ca^{2+} first and subsequent events features

Ang II binding to AT1R evoked ER Ca^{2+} release with consequent induced $[Ca^{2+}]_i$ elevation, which we called first peak, and which was eventually followed by additional $[Ca^{2+}]_i$ fluctuations, which we named subsequent oscillations. We aimed to evaluate the first Ang II-dependent peaks according to our custom-made pipeline: transient duration (measured as FWHM), amplitude and AUC were recorded, see (Figure 19, panel a). All three parameters were significantly higher in AAC compared to APA cells (Figure 19, panel b).

However, many cells showed additional Ca^{2+} transients after the first Ang II-evoked peak. The quantitative analysis of these subsequent oscillations revealed that APA cells displayed only higher amplitude peak parameter compared to AAC cells while AA showed elevated FWHM (Figure 19, panel c).

To further validate our results, we stimulated our cell model with 10 nM Ang II dose. Quantification of Ang II evoked First and subsequent transient's parameters confirmed our results obtained with 1 nM Ang II in AACs with the first peak compared to APA cells (Figure 20 panel a). Quantification of subsequent peaks revealed a significant increase in peak amplitude and AUC parameters in APA cells compared to AAC (Figure 20, panel b), whereas, subsequent peak duration was not significantly different in AAC compared to APA.

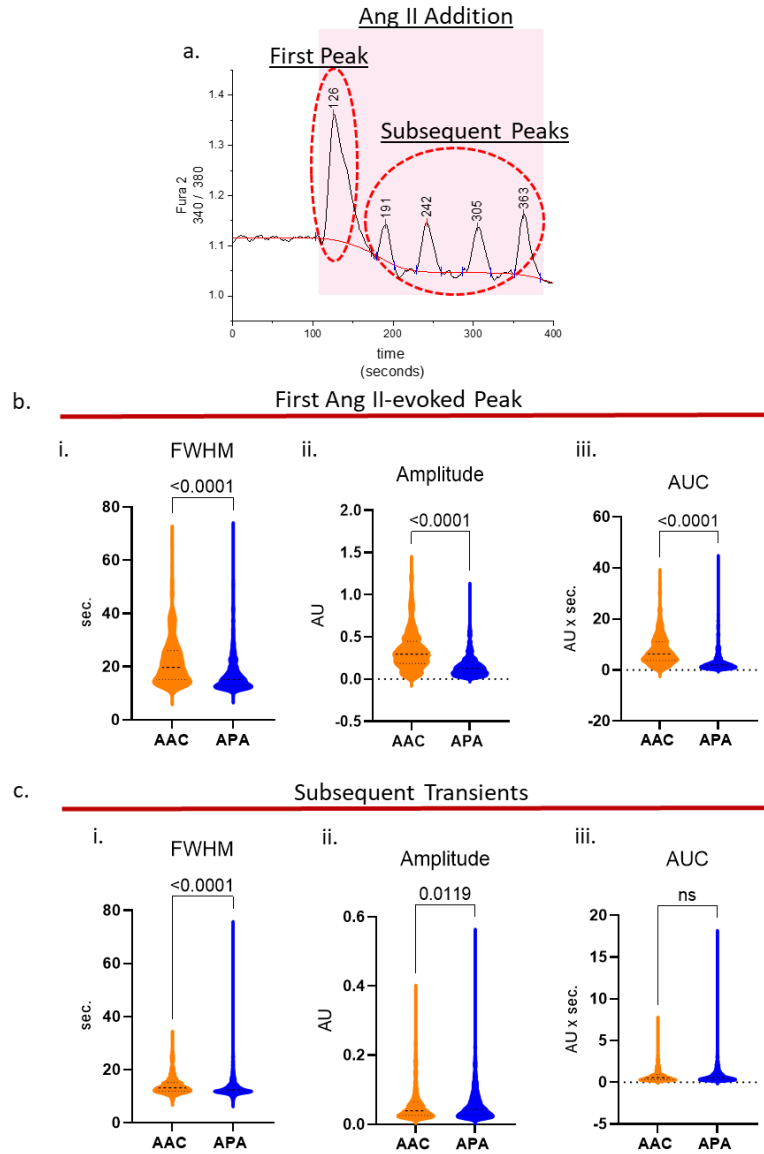


Figure 19: Quantification of 1 nM Ang II-evoked $[Ca^{2+}]_i$ spikes of CD56+ AAC and APA cells employing the custom made pipeline. a. Representative Ca^{2+} trace with indication of the applied approach: the first transient (Ang II-evoked peak) and the following transients (oscillations) have been distinct and analyzed separately in b and c. b. Violin plots show a significant increase in FWHM, Amplitude from baseline and AUC of the first Ang II-evoked peak in CD56+ AAC compared to APA cells. c. Quantification of subsequent oscillation peak parameters demonstrates increased Amplitude from baseline in APA cells compared to AAC. FWHM of events detected showed to be elevated in AACs; Patients = 6 for both AAC and APA. Data of violin plots are represented as medians and interquartile ranges. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

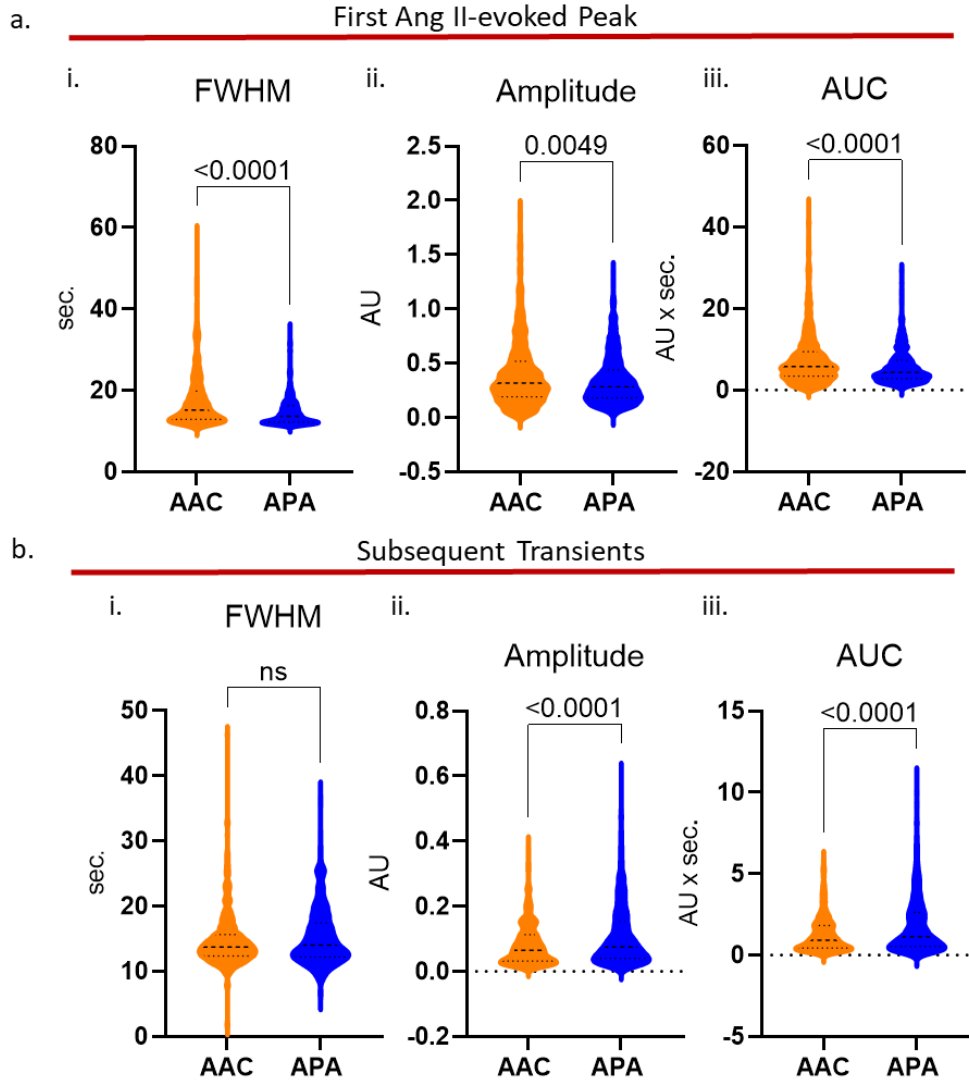


Figure 20: Peak parameters of first and subsequent peaks of 10 nM Ang II-induced $[Ca^{2+}]_i$ peaks in CD56+ AAC and APA cells. a. Violin plots report a significant elevation in first transients Ca^{2+} peak parameters in AAC compared to APA. b. Subsequent peaks quantification report similar peak duration quantifications in AAC and APA, but with an increase in amplitude and AUC parameters in APA compared to AAC. AAC and APA (patients =5). Data of violin plots are represented as medians and interquartile ranges. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

5.2- Number of oscillatory cells and frequency of oscillation

Based on our observations, the Ang II evoked Ca^{2+} peak transient was not necessarily accompanied by oscillations in each cell in our samples. To quantify the extent of cells showing

subsequent oscillations, we calculated the % of oscillating cells of each coverslip over the total number of cells that responded to Ang II. 1 nM Ang II stimulation promoted significantly increased number of oscillating APA cells (Figure 21, panel a).

We then calculated the frequency of the secondary oscillation using our custom-made pipeline [121]. The average frequency of oscillations after the first Ang II peak was significantly higher in APA vs AAC cells. This suggests that APA cells, despite a lower first peak, have a higher subsequent oscillatory activity compared to AAC cells and it persists over time (Figure 21, panel b).

Further confirmation of our data was obtained with 10 nM Ang II stimulation. Indeed, with this dose, the average frequency of oscillations was significantly higher in APA, compared to AAC. Also, the percentage of oscillating cells tended to be higher in APA compared to AAC despite not reaching statistical significance (Figure 22, panel a and b).

Together, these data indicate that APA cells demonstrate a more intense oscillatory behavior respect to AAC cells after stimulation regardless the Ang II dose used [1 nM or 10 nM].

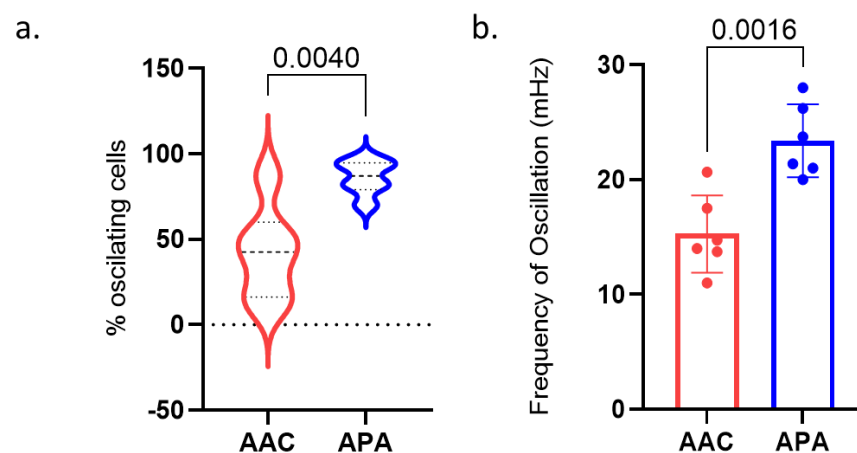


Figure 21: Analysis of 1 nM Ang II-induced $[Ca^{2+}]_i$ subsequent oscillations a. Violin plot representing the percentage of oscillating cells over the total cells responsive to Ang II stimulation b. Bar graph indicating the frequency of oscillating Ca^{2+} transients recorded after the first Ang II Ca^{2+} peak. (patients = 6). Data of violin plots are presented as medians and interquartile ranges. Frequency of oscillation's values are indicated as means $\pm$ SD. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

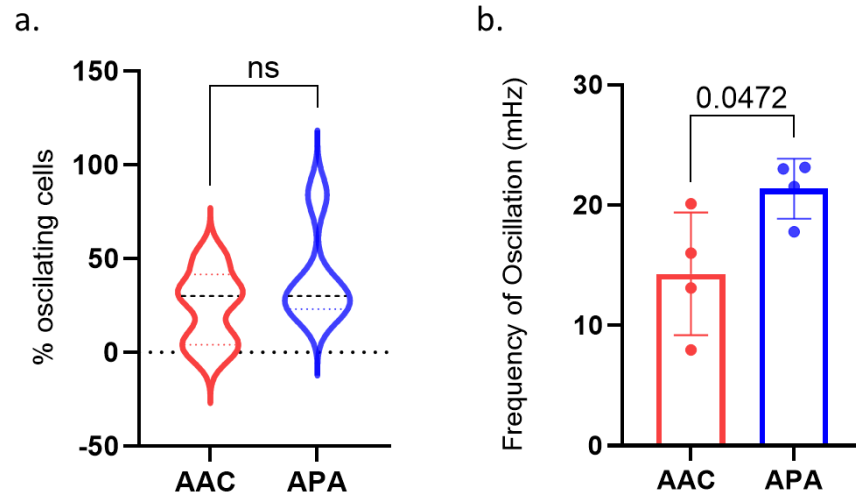


Figure 22: Assessment of 10 nM Ang II-induced $[Ca^{2+}]_i$ subsequent oscillations Data reports a trend towards a higher % oscillating cell in APA compared to AAC. b. bar graphs of average frequency of oscillation identify significant elevation in subsequent oscillating peak's frequency in APA cells compared to AAC. Data of violin plots are presented as medians and interquartile ranges. Frequency of oscillation's values are indicated as means $\pm$ SD. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

6-Impact of Irbesartan on Ca^{2+} activity

To validate the Ca^{2+} transients are Ang II mediated, we treated AAC and APA cells with Ang II receptor antagonist (AIIRA) Irbesartan [10 μ M]. Ca^{2+} activity was monitored after Ang II addition [10 nM] was added. Ca^{2+} traces recordings show no response upon Ang II stimulation (Figure 23). ATP and Ionomycin ionophore were used as positive controls for cells viability and responsiveness respectively.

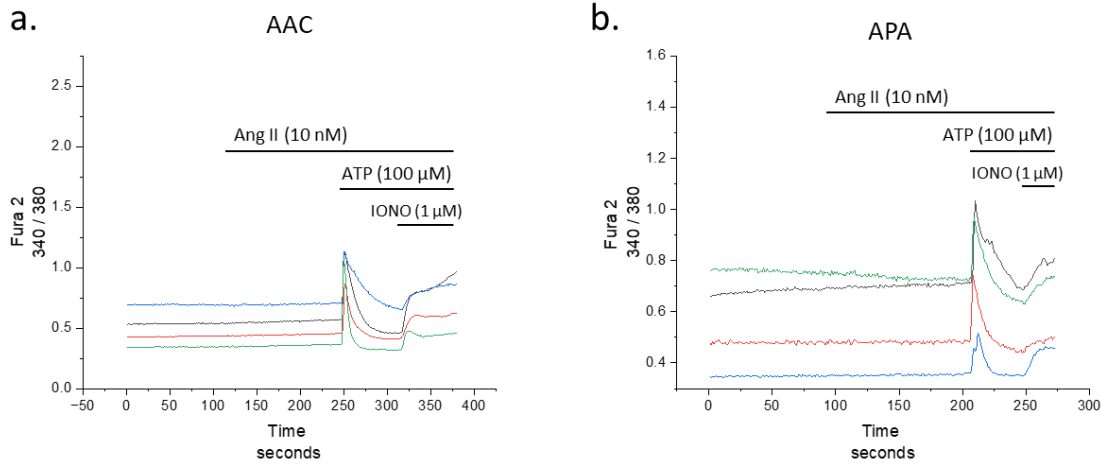


Figure 23: Ca^{2+} traces of 4 (a) AACs and 4 (b) APA cells showing ablated Ang II response upon Irbesartan treatment.

7-Modulation of Ca^{2+} activity of CD56^+ AAC cells upon TASK-2 pharmacological inhibition

In light of the our previous findings of a consistent under-expression of TASK-2 in APA tissue compared to normal adjacent tissue, we applied our pipeline to further investigated the regulation of TASK-2 channels on $[\text{Ca}^{2+}]_i$ dynamics in normal APA adrenocortical adjacent cells. We pharmacologically inhibited TASK-2 in AAC using the specific inhibitor quinidine (20 μM). TASK-2 inhibition ($0.62 \text{ AU} \pm 0.16$) did not significantly alter its corresponding AAC basal calcium levels ($0.68 \text{ AU} \pm 0.18$) (Figure 24, panel a).

However, quinidine induced robust and periodic spontaneous Ca^{2+} oscillations in AAC at resting conditions, which suggests a role of TASK-2 in the regulation of the spontaneous $[\text{Ca}^{2+}]_i$ activity of these cells (Figure 24, panel b). Moreover, TASK-2 inhibition augmented the number of cells displaying spontaneous activity ($25.25 \% \pm 24.78$) compared to non-treated AAC cells AAC (2.5 %

± 1.9) ($p > 0.05$) (mean $\pm$ SD) (Figure 24, panel c). Assessment of detected spontaneous Ca^{2+} activity revealed no significant difference upon Task-2 inhibition (Figure 24, panel d).

Ang II-evoked first peak's parameters were not altered by quinidine. The quantification of subsequent oscillating transient parameters showed a significant elevation in transient duration (FWHM) and total Ca^{2+} entry in AAC cells treated with quinidine compared to vehicle treated cells (AUC). Despite that, there was no impact of Task-2 inhibition on the amplitude of oscillating transient amplitude (Figure 25, panel a and b).

Upon 1 nM Ang II stimulation, Task-2 inhibition of AAC did not significantly alter the % of oscillating cells nor the frequency of oscillation (Figure 25, panel a and b).

Our results show that TASK-2 is involved in shaping the duration and amplitude of the Ca^{2+} transients, as well as the total calcium influx, while it seemed not to influence the frequency of oscillations nor their extent (i.e. the number of cells that oscillate).

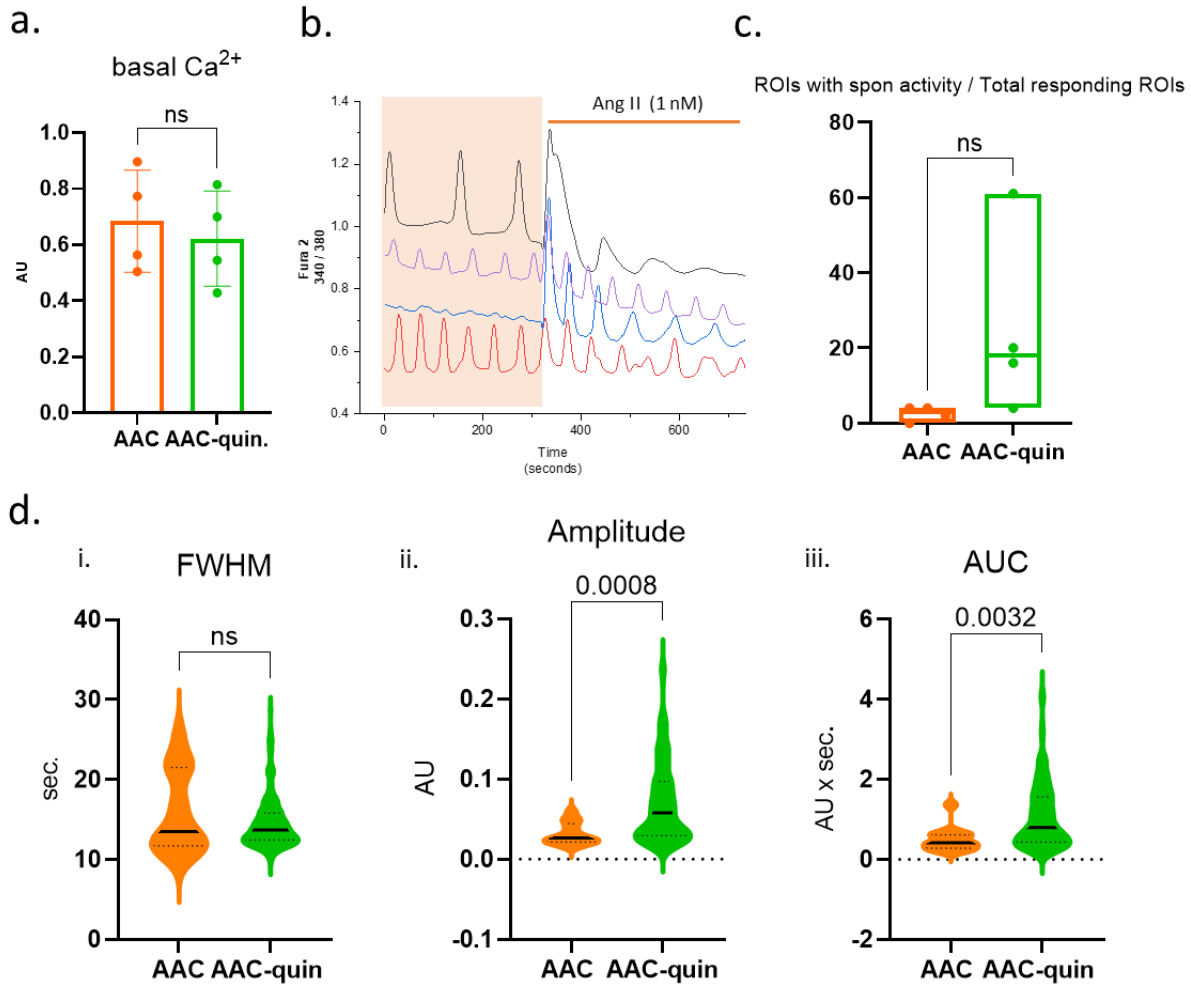


Figure 24: a. Bar graph representing basal calcium levels of AAC and AAC-quinidine treated; AAC (0.68 AU $\pm$ 0.18), TASK-2 inhibited AACs (0.62 AU $\pm$ 0.16) (Mean $\pm$ SD). b. Representative traces of quinidine treated-AAC cells $[\text{Ca}^{2+}]_i$ levels before (pink region) and after Ang II addition. c. Floating bars represent the % of cells harboring spontaneous Ca^{2+} activity during resting states; AAC (2.5 % $\pm$ 1.9) and TASK-2 inhibited AAC (25.25 % $\pm$ 24.78) (mean $\pm$ SD). D. Peak parameters of spontaneous activity detected. AAC and AAC -quinidine (patients = 4). Dots represent individual patients. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

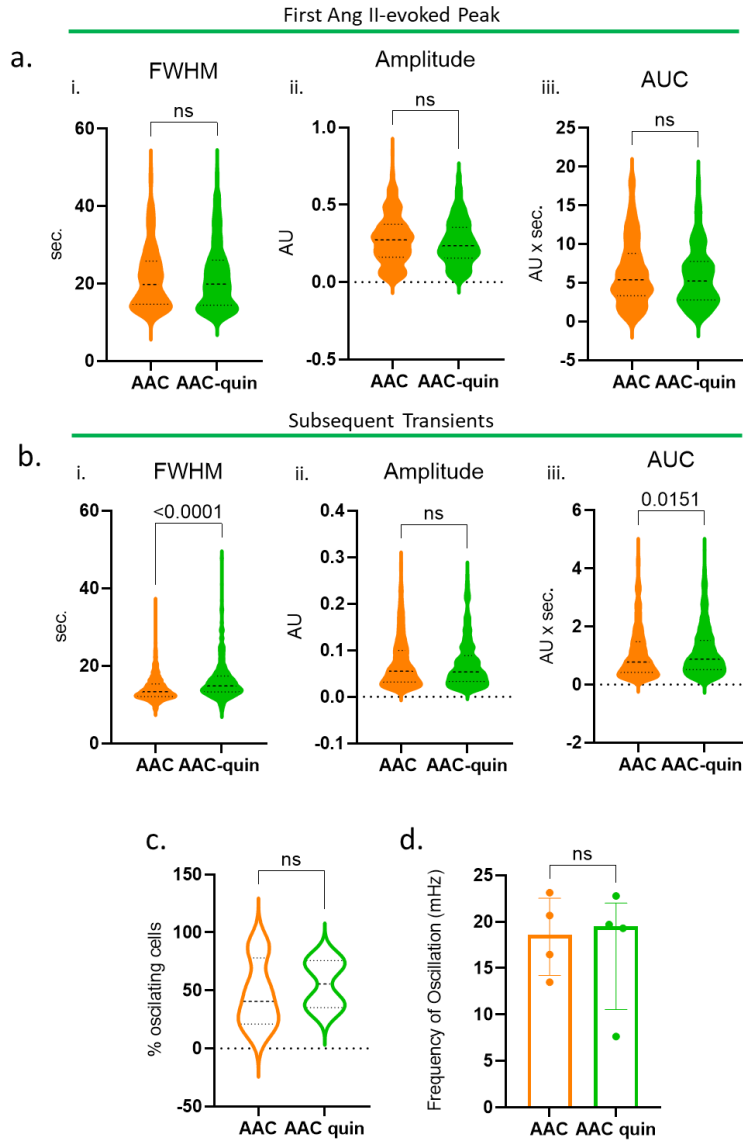


Figure 25: 1 nM Ang II-evoked Ca^{2+} peak parameters after TASK-2 inhibition. Peak parameters of (a) first Ang II-evoked Ca^{2+} transients and (b) oscillating transients following the first Ang II-evoked peak. (i) FWHM (full width at half maximum), (ii) Amplitude from baseline and (iii) AUC (area under the curve). c. Violin plots of the % of oscillating cells after evoked with 1 nM Ang II of TASK2 inhibited AAC cells. d. bar graph representing frequency of oscillation of TASK-2 inhibited cells after 1 nM Ang II stimulation. (patients =3). Data is represented as median with interquartile ranges. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

8-Ca²⁺ transients upon extracellular Calcium depletion

To answer the speculations of the source of Ca²⁺ leading to oscillatory activity seen in AAC and APA cells, extracellular Ca²⁺ was depleted using Ca²⁺ ion chelator EGTA. Intracellular Ca²⁺ dynamics were monitored using our pipeline mentioned above. Depletion of the extracellular source of Ca²⁺ did not alter the basal Ca²⁺ levels nor the number of ROIs (cells) demonstrating spontaneous activity in Both AAC and APA cells (Figure 26, panel A (a, b), B (a, b)). Spontaneous Peak Features (amplitude and AUC) were only altered in APA cells upon extracellular Ca²⁺ depletion compare to AAC (Figure 26, panel A (c), B (c)). These findings demonstrate possible higher dependency of APA cells on the extracellular Ca²⁺ source for excessive aldosterone production.

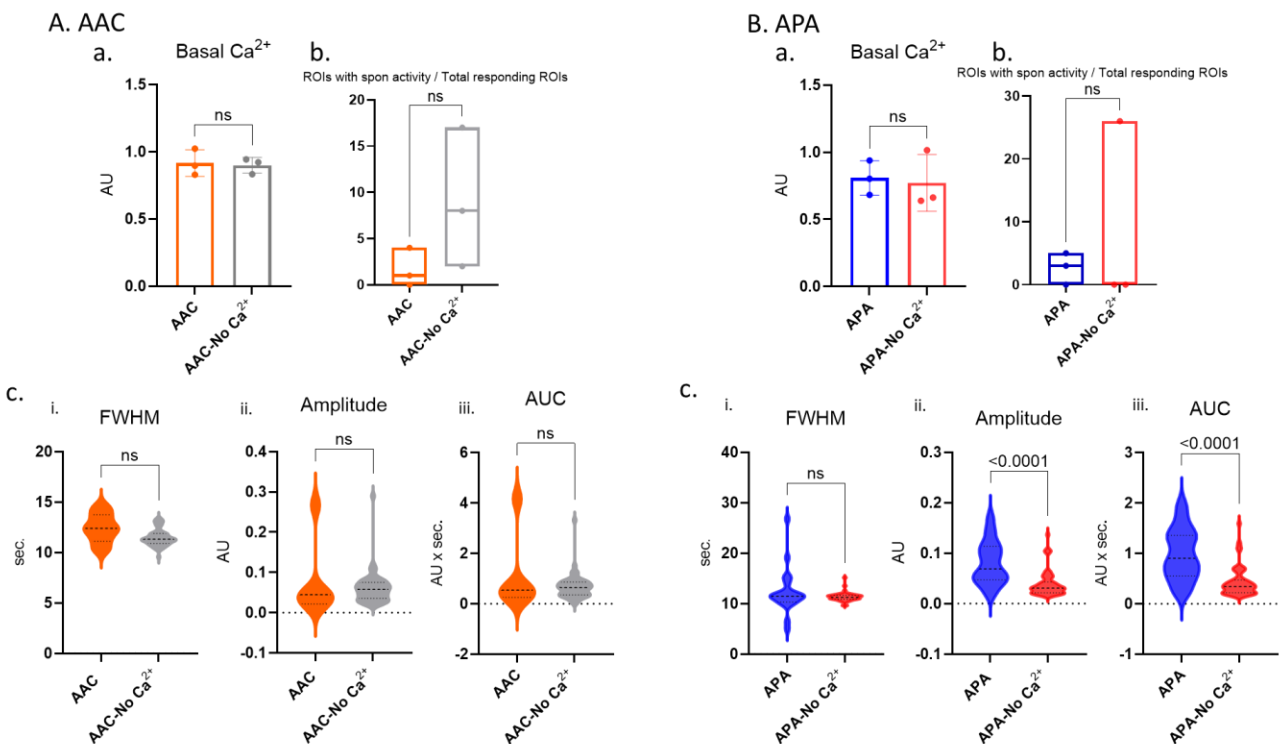


Figure 26: Basal levels and detected spontaneous Ca²⁺ activity upon extracellular Ca²⁺ depletion using Ca²⁺ ion chelator EGTA. a. basal Ca²⁺ levels, b. % of cells showing spontaneous Ca²⁺ activity, c. Features of spontaneous Ca²⁺ activity detected (i) FWHM (full width at half maximum), (ii) Amplitude from baseline

and (iii) AUC (area under the curve) of both (A) AAC and (B) APA. (patients =3). Data is represented as median with interquartile ranges. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

Assessment of 1 nM Ang II-evoked Ca^{2+} transients in both AAC and APA, revealed a decrease of First Ang II-evoked peak's amplitude and AUC however only in APA cells FWHM was also lowered (Figure 27, A and B panel a and b). In contrast, lack of extracellular Ca^{2+} promoted elevated subsequent peak parameters in AAC (FWHM, amplitude, AUC) and APA (only FWHM). These findings unveiled partial dependency of both first and subsequent oscillations on extracellular Ca^{2+} in which none of the peaks were diminished.

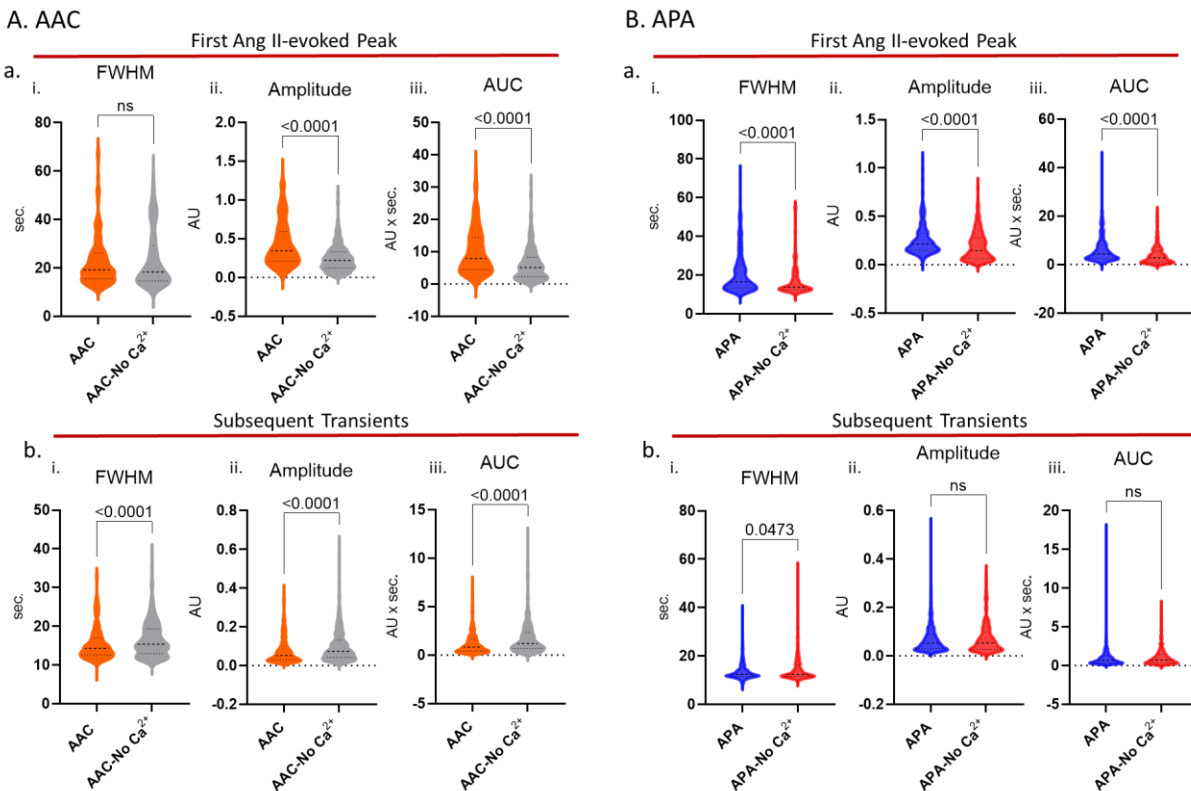


Figure 27: 1 nM Ang II-evoked Ca^{2+} peak parameters upon extracellular Ca^{2+} depletion using Ca^{2+} ion chelator EGTA. Violin plots representing (a) First Ang II- evoked peaks and (b) subsequent transients of both A. AAC and B. APA cells upon extracellular Ca^{2+} depletion (i) FWHM (full width at half maximum), (ii) Amplitude from baseline and (iii) AUC (area under the curve) of both (A) AAC and (B) APA. (patients =3).

Data is represented as median with interquartile ranges. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

Calculations of % of cells showing oscillations demonstrated no impact of the extracellular Ca^{2+} depletion on the oscillatory activity in both AAC and APA (Figure 28 panel a (A and B)). Frequency of oscillation was decreased upon Ca^{2+} depletion only in APA cells (Figure 28 panel b (A and B)). These findings reveal the exquisite reliance of APA cells on extracellular Ca^{2+} source for the maintenance of frequency of oscillation needed excessive steroidogenesis.

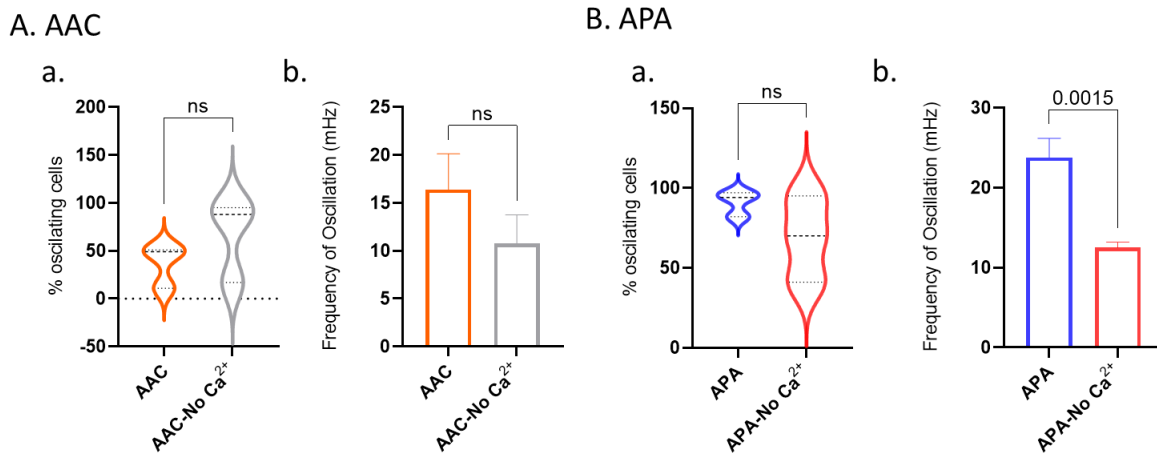


Figure 28: Impact of extracellular Ca^{2+} depletion on oscillatory activity of AAC and APA cells. a. violin plots representing % of oscillating cells and b. bar graphs representing frequency of oscillation upon 1 nM Ang II stimulation in both (A) AAC and (B) APA cells. (patients =3). Data is represented as median with interquartile ranges. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

9. CD56⁺ cell cluster formation and coordinated activity

9.1- Clusters assembly of dissociated CD56⁺ AAC and APA cells

Enzymatically and mechanically single dissociated CD56⁺ AAC and APA cells when put in culture promoted the formation of glomeruli-like clusters. Clusters appeared at 24 hours in culture and demonstrated variant number of cells with a contact point in the middle. Monitoring clusters at 96 hours in culture revealed well-formed clusters with higher number of cells and aggregates of cells in the center of the cluster (Figure 29, panel a and b).

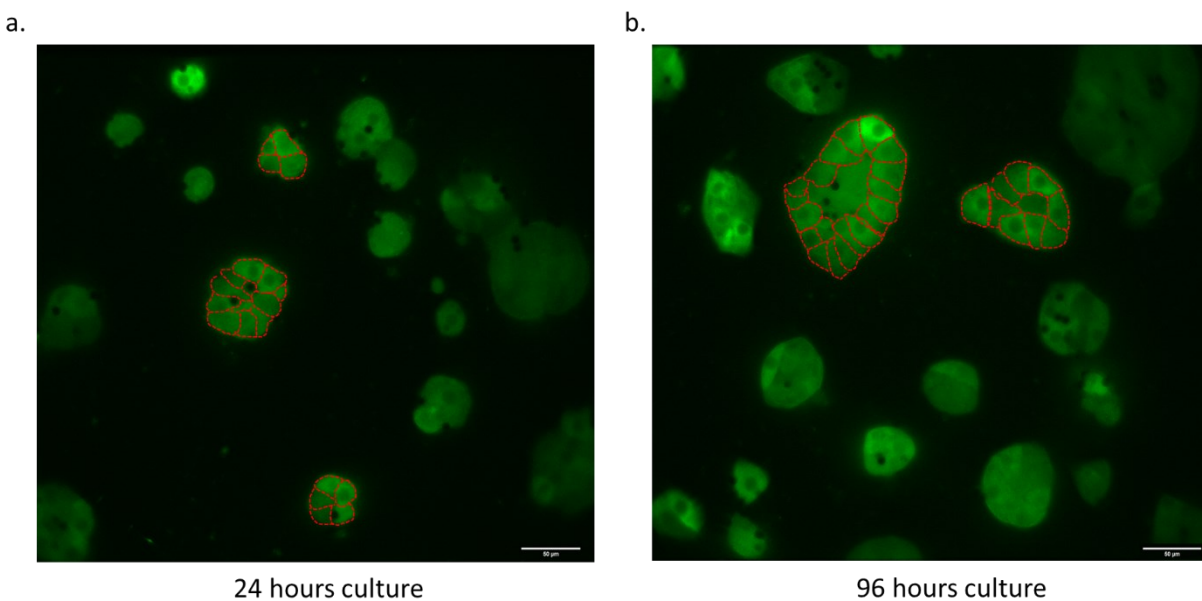


Figure 29: glomeruli-like clusters formation of APA cells in 24 and 96 hours in culture. Red dots demonstrate clusters formed and align single cells. Black dots represent immunomagnetic beads. Scale bar = 50 μm.

9.2- Coordinated Ca²⁺ activity of cells within 1 cluster of AAC and APA

Cluster analysis and linear mixed effects models

Model 1: Number of Predicted Subclusters (NPS)

The analysis reveals that the number of cells in labeled clusters (NCL) is a significant predictor of NPS, with a positive coefficient (0.27, $p < 0.001$). This suggests that an increase in NCL is associated with a higher number of predicted subclusters, indicating a direct relationship between the density of cells in labeled clusters and the formation of subclusters. However, the covariate AAC_APA, distinguishing between AAC and APA, does not significantly impact NPS (coefficient = -0.12, $p = 0.39$), implying that the type of cell used does not influence the number of predicted subclusters.

Model 2: Number of Cells in Labeled Clusters (NCL)

The non-significant effect of AAC_APA (coefficient = 0.3, $p = 0.61$) supports the notion that the AAC/APA distinction does not affect the number of cells in labeled clusters.

Model 3: Number of Cells in Predicted Subclusters (NCPS)

Similarly, NCL is a significant predictor of NCPS, with a positive coefficient (0.07, $p < 0.001$). This result indicates that an increase in NCL leads to a corresponding increase in the number of cells in predicted subclusters. The AAC_APA covariate again shows no significant effect (coefficient = -0.01, $p = 0.97$), reinforcing the consistency of its no-influence across different models.

Variability and Random Effects

The random effects analysis highlights significant patient-specific variability in all models. The conditional modes of random effects provide further insight into individual patient deviations from the population mean. Given the inherent nature of clustering features, where predicted clusters are composed of highly correlated cells, it is evident that both AAC and APA cells exhibit similar clustering behavior. This is reflected in the non-significant impact of the AAC_APA covariate across all models, suggesting that the switch between AAC and APA does not influence the number of predicted subclusters or the number of cells in these subclusters.

Functional ANOVA

The results of the functional ANOVA statistical testing procedure indicate that out of the 31 different samples spanning the nine patients, twenty-four resulted significant (p-value less the 0.05) when their real (assigned) labels were considered, resulting in 77% of the samples indicating a larger between cluster variance compared to the variance of cells found within the same cluster. Every sample resulted non-significant when clusters were randomly labeled.

The insignificance of the ANOVA test results for sample AAC 2 from patient 7 and sample AAC 5 from patient 8 could be attributed to the low number of cells in these samples, 4 and 13 respectively; their small sample sizes could lead to a lack of statistical power. Instead, samples AAC 1, APA 3 and AAC 4 from patients 3, 4 and 8 respectively display only one main peak and are otherwise flat. This uniformity in the cell traces could be a reason why the ANOVA might not yield significant results. If the traces within and between clusters are too similar, as is the case with a single peak and flat traces, the test predictably finds little to no difference in variance leading to a non-significant result. If the samples with low number of cells and uninformative cell traces are excluded from the analysis, 92% of the samples would be significant

The mostly significant test results, observed when utilizing the true labels, underscore a substantial level of synchronization and coordination among the cells within the same cluster, reflecting a more profound functional connection that leads to similar responses to Ang II stimulus.

	Real labels			Random Labels			
Patient	Sample	Test statistic	P value	Sample	Test statistic	P value	Number of cells (excluding single cell clusters)
Patient 1	AAC 3	18.02	0	AAC 3	1.26	0.35	93
	AAC 5	16.02	0	AAC 5	0.87	1.00	93
	APA 4	8.96	0	APA 4	0.91	0.98	106
	APA 6	13.34	0	APA 6	1.04	0.96	86
Patient 2	CD_5	7.79	0.02	CD_5	0.99	0.46	119
	CD_4	34.65	0	CD_4	1.10	0.89	128
Patient 3	AAC 1	0.93	1	AAC 1	1.11	0.59	79
	AAC 3	8.50	0	AAC 3	0.93	0.96	82

	AAC 5	8.45	0	AAC 5	1.07	0.49	72
	APA 2	15.29	0	APA 2	1.02	0.96	136
	APA 4	55.65	0	APA 4	0.66	0.89	150
Patient 4	AAC 1	9.49	0	AAC 1	2.59	0.14	35
	AAC 4	2.78	0.33	AAC 4	1.09	0.67	61
	AAC 6	8.26	0	AAC 6	0.83	1	83
	AAC 7	8.50	0	AAC 7	1.25	0.97	81
	APA 2	10.24	0	APA 2	0.82	0.83	45
	APA 3	5.41	0.99	APA 3	1.01	1	37
	APA 5	16.60	0	APA 5	0.46	1	56
Patient 5	AAC 2	10.34	0	AAC 2	1.68	1	42
	AAC 4	10.39	0	AAC 4	0.853	0.98	32
Patient 6	AAC 1	7.17	0.01	AAC 1	0.98	0.97	60
	AAC 2	4.1	0.14	AAC 2	1.7	0.52	47
Patient 7	AAC 2	15.94	1	AAC 2	0.17	1	4
	APA 4	14.98	0	APA 4	1.24	0.49	67
Patient 8	AAC 4	2.09	0.85	AAC 4	0.93	0.94	50
	AAC 5	3.70	0.12	AAC 5	1.19	0.38	13
	APA 2	7.33	0.01	APA 2	1.19	0.72	25
Patient 9	AAC 1	6.67	0	AAC 1	0.64	0.98	59
	AAC 3	12.50	0	AAC 3	0.76	1	100
	APA 2	10.01	0	APA 2	1.19	0.64	99
	APA 4	43.90	0	APA 4	0.87	0.56	112

Table 1 : Functional ANOVA results. The table illustrates the test statistics and p-values for all patients' samples. The rows shaded in blue signify the samples labeled with their assigned labels that were determined as insignificant (p-values larger than 0.05).

10. Mitochondrial status in CD56⁺ AAC and APA cells

10.1- Mitochondrial ROS production in AAC and APA cells

To assess mitochondrial ROS production, we used the Mito Sox assay to quantitate ROS produced by CD56⁺ AAC and APA dissociated cells after 24 hours in culture at both resting levels and upon Ang II stimulation [1 nM]. Mito Sox dye is oxidized specifically by Superoxide ion, a member of ROS. The dye intensity was evaluated by dividing the reported ROI intensity over the background

to remove interfering noise. Quantification of basal ROS shows significant lower levels of APA cells compared to AAC (Figure 30, panel a). To assess the influence of ANG II on ROS production, cells were incubated with 1 nM Ang II then ROS levels were measured. To quantify the produced ROS, we divided ROS levels reported after 10 mins of ANG II stimulation over basal ROS levels. Data demonstrates significant lower ROS produced in APA compared to AAC (Figure 30, panel b).

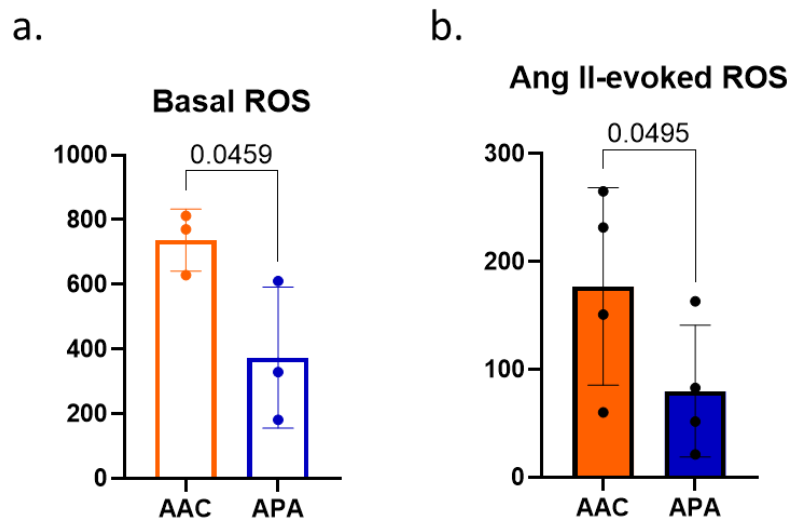


Figure 30: Measurements of ROS of CD56⁺ AAC and APA cells. a. Basal ROS levels shows significant lower levels of APA cells compared to AAC. b. 1 nM Ang II-evoked ROS production, calculated by dividing ROS levels reported after 10 mins of ANG II stimulation over basal ROS levels, is significantly lower in APA cells compared to AAC. Patients= 3. Data is reported as mean + SD. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

10.2- Mitochondrial membrane potential of AAC and APA cells

To further examine mitochondrial function, we aimed to assess mitochondrial membrane potential ($\Delta\Psi_m$), the key marker of cellular health, in AAC and APA cells using the fluorescent dye TMRM. TMRM dye is picked up by mitochondria through the $\Delta\Psi_m$ which allows the assess the $\Delta\Psi_m$ by the intensity of TMRM detected. Due to the non-quenching mode of TMRM, depolarized

mitochondria exhibit Lower TMRM intensity. Exposure to CCCP, the protonophore that prevents mitochondrial permeability transition pore opening is known to increase $\Delta\Psi_m$, was utilized for 10 mins as a positive control. TMRM dye intensity was evaluated by dividing the monitored TMRM ROI intensity over the background intensity to remove interfering noise. Quantification of Basal TMRM revealed no significant difference in mitochondrial $\Delta\Psi_m$ between AAC and APA cells (Figure 31, panel a). TMRM intensity trajectories displayed a decrease in dye intensity upon CCCP incubation in al cells (Figure 31, panel b).

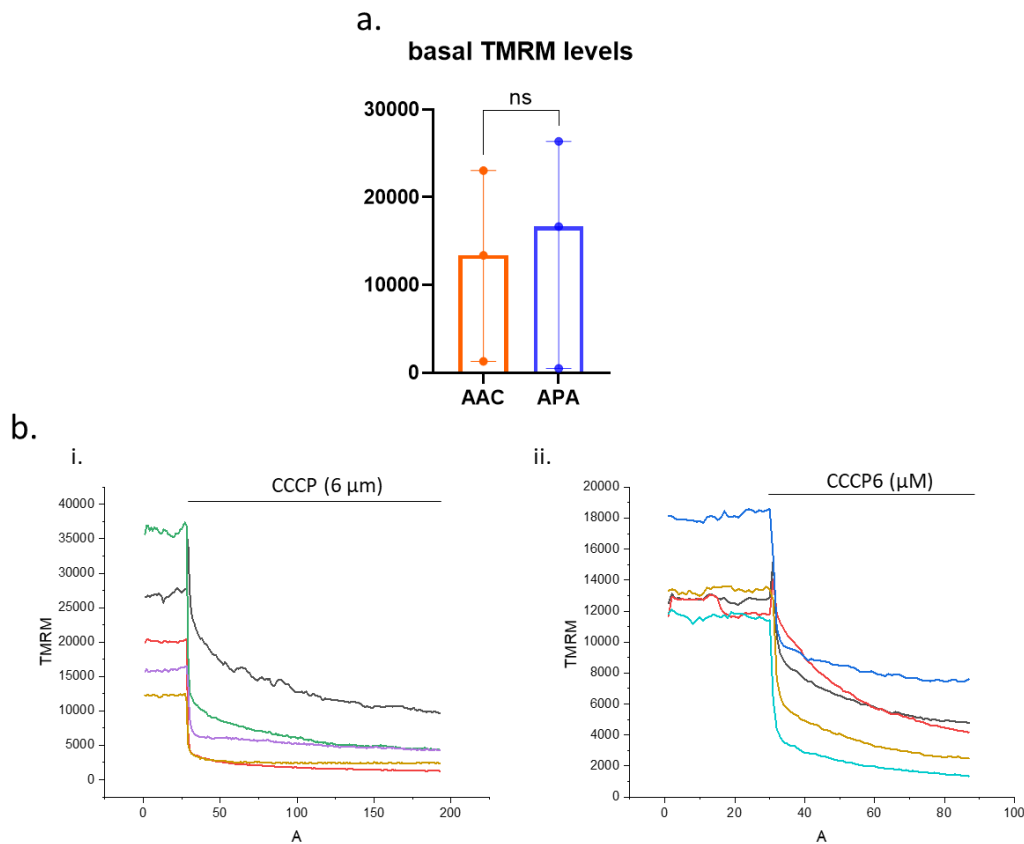


Figure 31: Measurements of mitochondrial membrane potential ($\Delta\Psi_m$) of CD56⁺ AAC and APA cells. a. No difference detected in basal TMRM levels between AAC and APA cells. b. TMRM dye traces of (i) AAC and (ii) APA reveals a dim in TMRM signal detected upon (6 μM) CCCP incubation for 10 mins. CCCP was used as a positive control. Patients= 3. Data is reported as mean + SD. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

11. Double SDHA/CYP11B2 Immunofluorescence

To expand our knowledge on the pattern of expression of the functional enzyme CYP11B2 in the mitochondria of intact adrenocortical tissue, we double stained Paraffin embedded APA slices with SDHA antibodies, a major catalytic subunit of succinate-ubiquinone oxidoreductase which is a complex of the mitochondrial respiratory chain, used to mark mitochondria, and CYP11B2 antibodies. High resolution confocal microscopy was employed to assess the coexistence of SDHA and CYP11B2. (Figure 32, panel A-C) demonstrate the expression pattern of CYP11B2 in the APA. Interestingly, coimmunostaining reveals that CYP11B2 is not expressed in in all the mitochondria detected in the APA cells as shown by the confocal microscopy (Figure 32, panel E-F), and high-resolution imaging confocal microscopy (Figure 32, panel G-I).

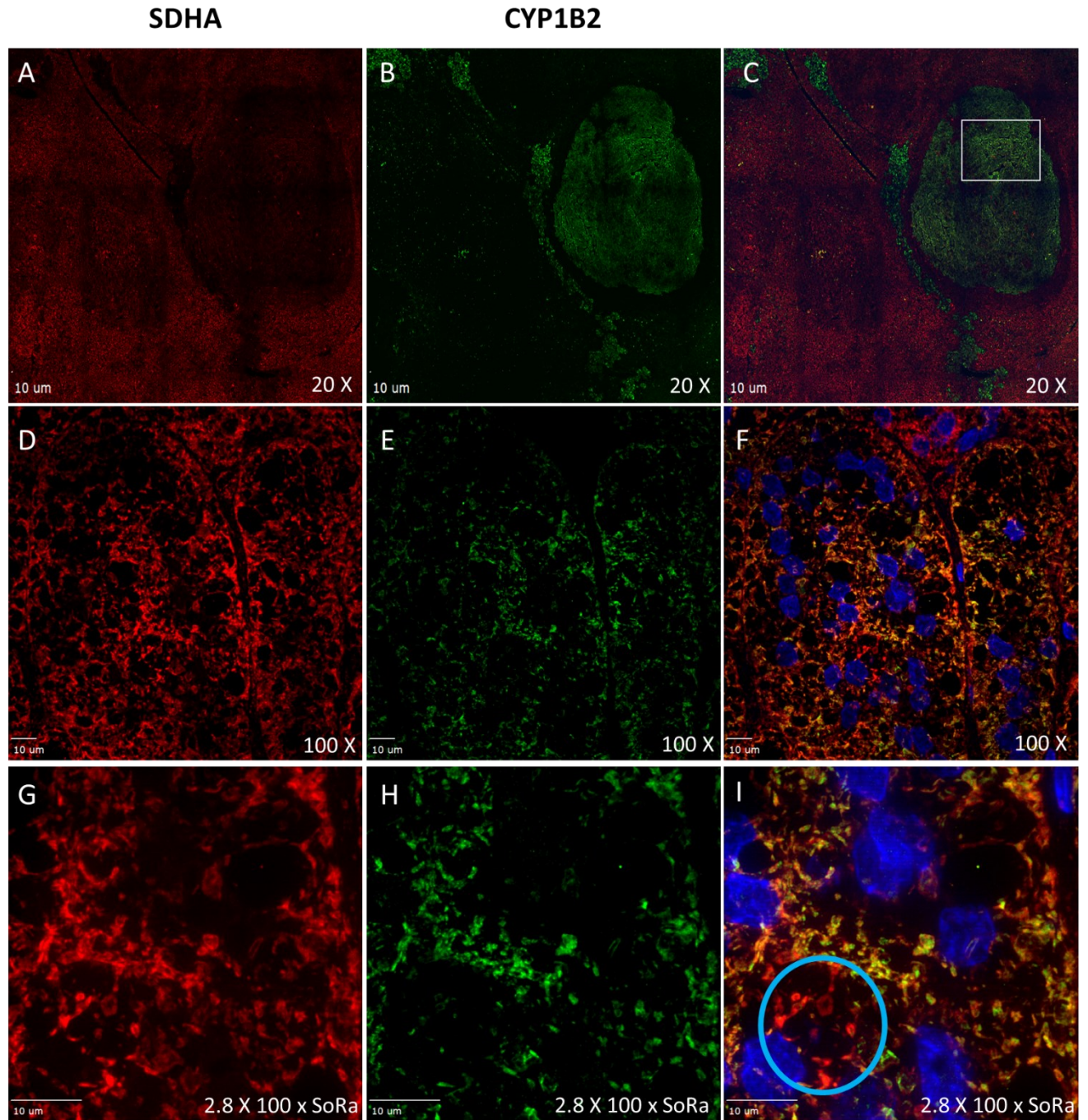


Figure 32: SDHA/CYP11B2 double immunofluorescence of Patient APA slices at different magnification. Panels A-C demonstrate 20X APA magnification. White box represents the region higher that as assessed with higher magnification. Panel D-E represent APA slice with 100 X magnification. Panel G-H represent 100X magnification using 2.8X SoRa tool utilized for super resolution imaging. The adenoma is immune-stained with monoclonal antibodies for both SDHA (in red), CYP11B2 (in green), and DAPI (in blue).

12. Double CYP11B1/CYP11B2 Immunofluorescence

We aimed to assess the possible colocalization of CYP11B1 and CYP11B2 in the same cell. Coimmunostaining was performed using monoclonal antibodies for CYP11B1 and CYP11B2. Confocal images using 20 x magnification showed differential distribution of the 2 mitochondrial enzymes (Figure 33, panel A-C). However, high resolution imaging demonstrates the intertwined cells expressing either CYP11B1 or CYP11B2 and the expression of both enzymes in the same mitochondria (Figure 33, panel D-F).

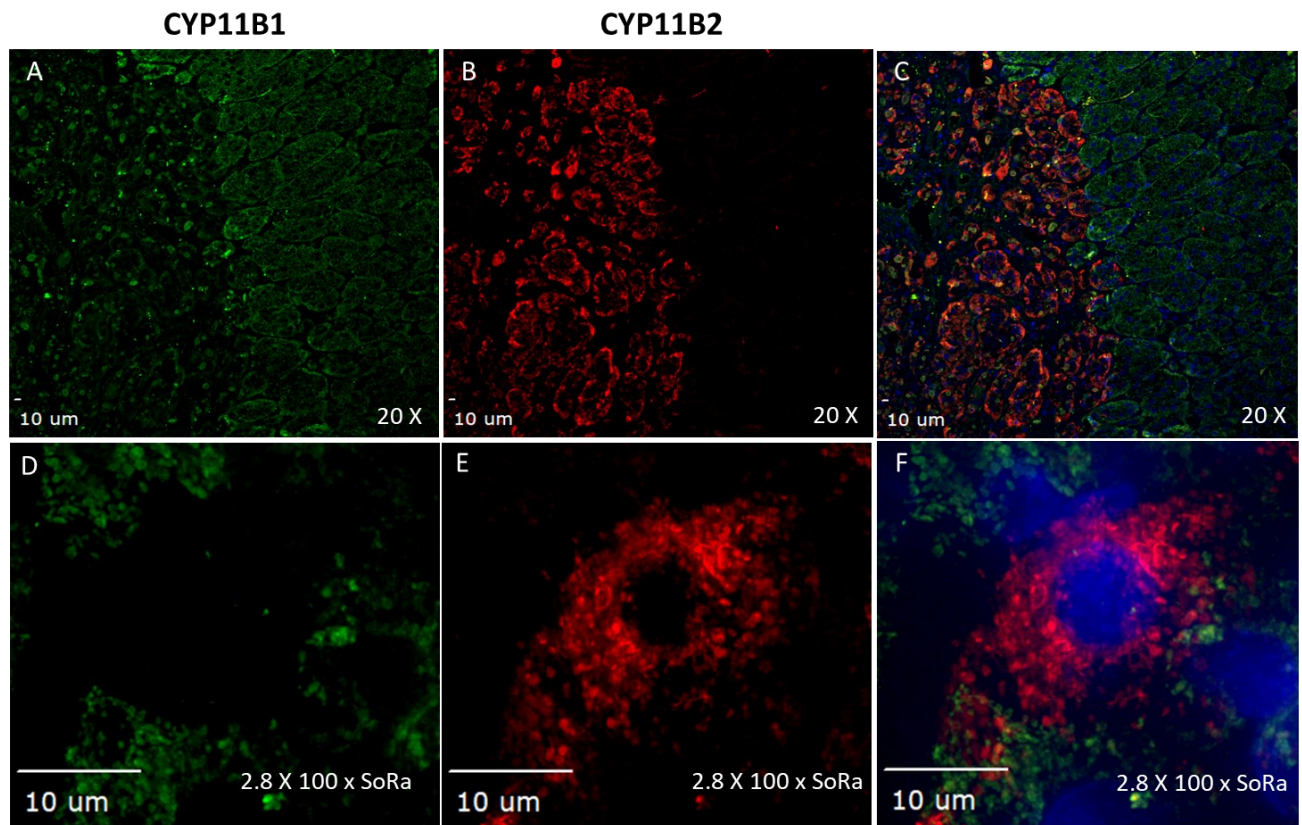


Figure 33: CYP11B1/CYP11B2 double immunofluorescence of Patient APA slices at different magnification. Panels A-C demonstrate 20X APA magnification. Panel D-E represent APA slice with 100X magnification using 2.8X SoRa tool utilized for super resolution imaging. The adenoma is immune-stained with monoclonal antibodies for both SDHA (in red), CYP11B2 (in green), and DAPI (in blue).

13. Calcification Propensity of PA and EH plasma

We investigated the propensity of in vitro calcification of plasma of PA patients and compare it to plasma of EH patients. We utilized the biohybrid system to follow up development of calcification deposits in real time. We implemented 2 cell models, iVSMCs and hVSMCs. Calcification deposition is visualized as depicted on the representative images (Figure 34 and 35, panel a). Calcification trajectories of both PA and EH plasma demonstrate increased calcification over time compared to apparently healthy controls (Figure 34, panel b (i) and c (i)). Quantification of mineral deposits at day 6 of the experiment reveal a significant increase in calcification deposition of PA plasma compared to control (Figure 34, panel b (ii)). However, EH plasma did not show significant differences in calcification deposition compared to apparently healthy volunteers (Figure 34, panel c (ii)).

The same experiments were performed on hVSMCs. Calcification trajectories demonstrate increase of calcification deposition of patients compared to healthy volunteers (Figure 35, panel b (i) and c (i)). Quantification of deposits at day 6 reveals a significant increase in calcification of PA and EH plasma compared to plasma of apparently healthy controls (Figure 35, panel b (ii) and c (ii)).

Comparing calcification deposition at day 6 of incubation with PA and EH plasma, a significant increase in calcification of PA plasma compared to EH is seen using the iVSMCs cell model (Figure 36 panel a and b).

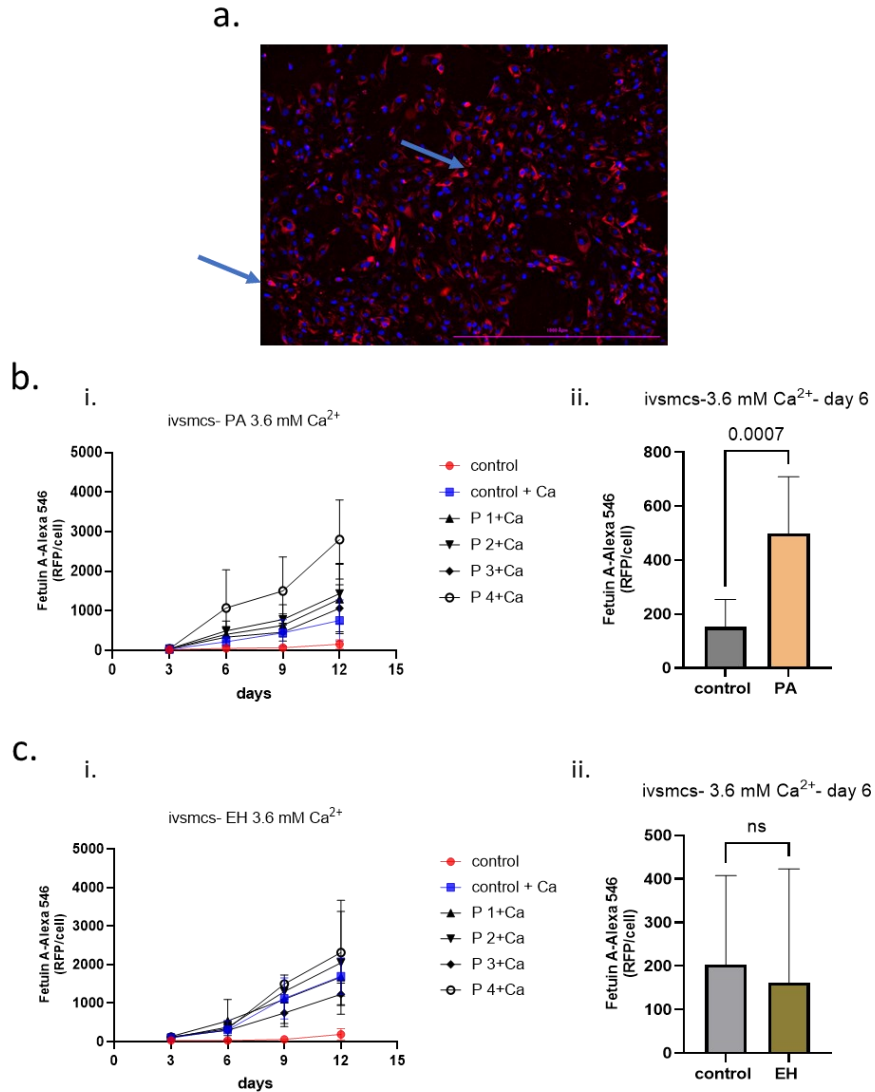


Figure 34: Biohybrid system for real time calcification development monitoring of PA and EH patient's plasma on iVSMCs cell model. a. Representative image (4x magnification) of the calcification deposits at day 6 of the experiment. Arrows point to the mineral deposition. (b) (i) Time trajectory of calcification deposition over 12 days of PA patient's plasma. (ii). Quantification of calcium deposition on day 6 of the experiment. (c) (i) Time trajectory of calcification deposition over 12 days of EH patient's plasma. (ii). Quantification of calcium deposition on day 6 of the experiment. (patients =4). Plasma controls were made up of pool of 8 healthy volunteers. Red trajectories demonstrate controls with no Calcium added. Data is represented as median with interquartile ranges. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

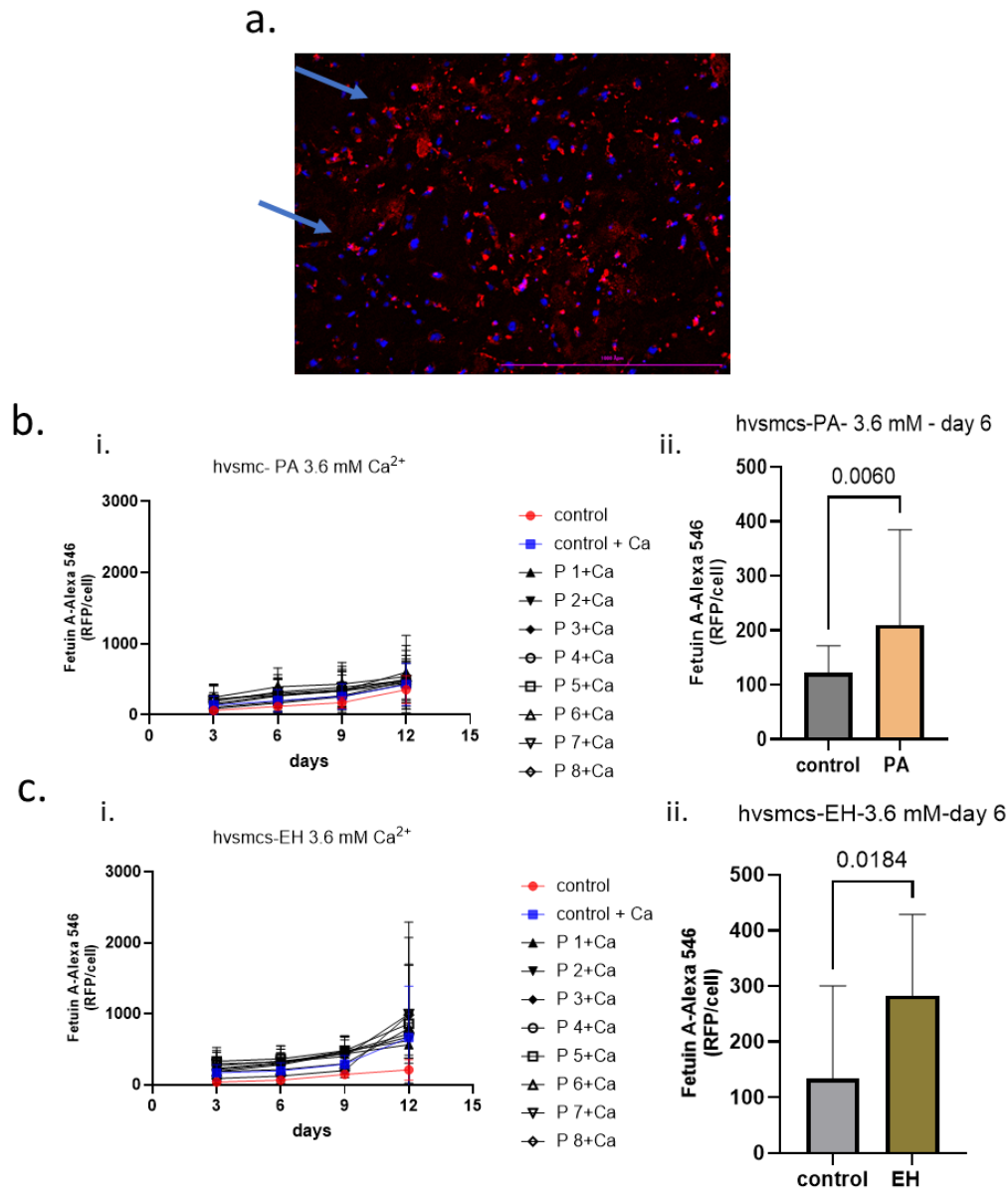


Figure 35: Biohybrid system for real time calcification development monitoring of PA and EH patient's plasma on hvs mcs cell model. a. Representative image (4x magnification) of the calcification deposits at day 6 of the experiment. Arrows point to the mineral deposition. (b) (i) Time trajectory of calcification deposition over 12 days of PA patient's plasma. (ii). Quantification of calcium deposition on day 6 of the experiment. (c) (i) Time trajectory of calcification deposition over 12 days of EH patient's plasma. (ii). Quantification of calcium deposition on day 6 of the experiment. (patients =8). Plasma controls were made up of pool of 8 healthy volunteers. Red trajectories demonstrate controls with no Calcium added. Data is represented as median with interquartile ranges. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

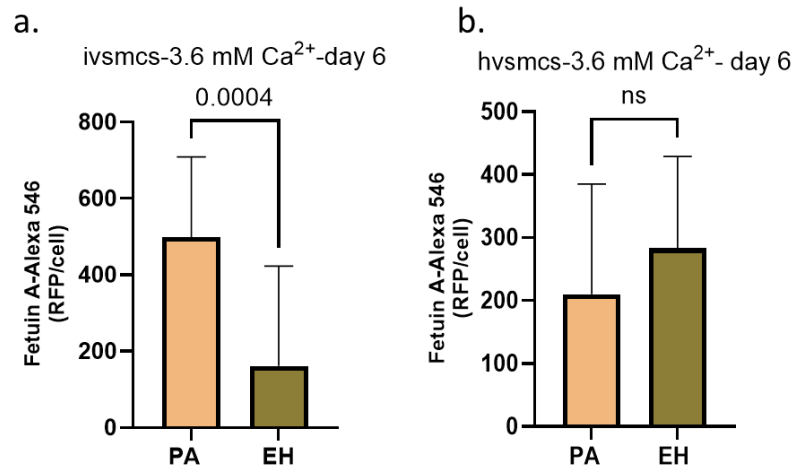


Figure 36: Biohybrid system for calcification monitoring of PA and EH patient's plasma on iVSMCs and hVSMCs cell models. a. quantification of calcification deposition between PA and EH patient's plasma on iVSMCs cell model. b. quantification of calcification deposition between PA and EH patient's plasma on hVSMCs cell model. Data is represented as median with interquartile ranges. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

Discussion

The regulation of intracellular calcium ($[Ca^{2+}]_i$) dynamics plays a vital role in the function of adrenocortical cells. It is unsurprising that many efforts have been dedicated to understanding this process. However, so far, the study and quantification of $[Ca^{2+}]_i$ dynamics have only been conducted in adrenocortical cells from bovine or murine samples, with no such studies in humans. This is likely due to the challenges associated with collecting and culturing primary human adrenal cells.

Through our study, we present the first characterization of $[Ca^{2+}]_i$ levels in a pure population of immune-isolated CD56⁺ primary human adrenocortical cells, by monitoring the variation of cytosolic Ca^{2+} concentration in individual cells from both AAC and APA tissues.

To perform this analysis, we developed a novel pipeline methodology to analyze in vitro Ca^{2+} imaging data of individual adrenocortical cells in a simple and reliable way. Thus far, many computational tools for analyzing intracellular Ca^{2+} changes that have been used require programming skills and knowledge of software languages and scripts [65][122][64][50] that are not familiar to a significant part of the scientists conducting experimental work [123][124].

The workflow outlined here offers a practical method for generating reproducible data and promotes independence for researchers with little to no prior bioinformatics experience.

It is worth noting that the OriginPro 2023 software already provides an App to analyze Ca^{2+} signals of patient-derived induced pluripotent stem cell cardiomyocytes (iPSC-CMs). However, as it stands it requires an embedded Python and NumPy, SciPy libraries, and furnishes only an average

information of all peaks per each assigned cell and not an assessment of individual peaks. Therefore, this approach is not reliable to provide a detailed detected-peaks analysis in aldosterone producing cells.

Importantly, the pipeline's flexibility and robustness can be standardized across various studies, producing objective results that are suitable for large data sets while minimizing human error. The main drawback of our methodology, which requires careful execution, is the unavoidable need for manual intervention, particularly in step 3, where peak filtering involves Excel-based equations that require meticulous handling, though this process is still considered time-efficient.

As an initial observation, we noted that human adrenocortical cells displayed variable responses to Ang II. Specifically, we either recorded a single acute transient response or a transient followed by oscillations. In most cases, regardless of the response type, the Ca^{2+} concentration did not fully return to the baseline level prior to stimulation. This finding is consistent with previous reports of cell-to-cell variability observed in murine and bovine adrenocortical cells [125][126][127][120][128][129].

APA has recently been identified as a channelopathy, as mutations in ion channels or pumps within the hyperpolarized membrane of zona glomerulosa (ZG) cells can elevate intracellular Ca^{2+} levels. This occurs either through increased Ca^{2+} influx from the extracellular environment or release from internal stores, ultimately enhancing aldosterone production [122]. Supporting this, our quantitative analysis of Ca^{2+} signaling in CD56+ human adrenocortical cells has provided valuable insights into the intricate mechanisms of $[\text{Ca}^{2+}]_i$ modulation in primary aldosteronism. Interestingly, we observed a reduction in basal cytoplasmic Ca^{2+} levels in APA cells compared to APA adjacent normal controls (AACs), regardless of the specific APA mutation. These findings suggest that APA cells may rapidly sequester cytosolic Ca^{2+} into the endoplasmic

reticulum (ER) or mitochondria via the mitochondrial calcium uniporter (MCU) [130]. Notably, these organelles are key sites for aldosterone biosynthesis, and their enhanced Ca^{2+} uptake may underlie the mechanisms driving excessive aldosterone production.

Analysis of peak parameters showed that Ang II, a primary aldosterone secretagogue, triggered a rapid Ca^{2+} transient, which was more pronounced in AAC cells compared to APA cells. However, APA cells exhibited higher amplitude in the subsequent Ca^{2+} oscillations. Importantly, the broad variability in data across patients highlights significant interpatient differences in peak parameters.

Notably, we observed that APA cells displayed a significantly higher and more consistent percentage of cells exhibiting oscillatory behavior and an increased frequency of oscillations following Ang II stimulation compared to AAC cells. This suggests that the activation of Ca^{2+} signaling in APA cells, associated with excessive aldosterone production, leads to enhanced Ca^{2+} oscillatory activity and frequency.

The variations in the expression of ion channels and pumps between species could be a key factor contributing to the observed differences in Ca^{2+} behavior [58][54]. In murine cell models, the majority of evidence indicates the differential expression of only TASK-1 and TASK-3. In contrast, normal human ZG cells exhibit a broader expression of all three channel subtypes. Specifically, TASK-1 and TASK-2 are the most highly expressed in human ZG cells, while TASK-3 is only minimally detectable[58].

Through whole transcriptome analysis, we previously observed that TASK-2 is notably under expressed in APA compared to normal surrounding ZG cells, a condition linked to increased

aldosterone production and thus serving as a key characteristic of PA [58]. To explore the potential role of TASK-2 channels in regulating downstream Ca^{2+} signaling, we inhibited TASK-2 channels in human AACs using quinidine, a known potent TASK-2 inhibitor [131][132]. We found that baseline levels remained unaffected, but there was a significant increase in the amplitude and area under the curve (AUC) of spontaneous Ca^{2+} oscillations following TASK-2 inhibition. After Ang II stimulation, the initial peak parameters remained unchanged, while subsequent peaks showed longer duration and higher AUC.

Blunting the activity of TASK-1 and TASK-3 in mice adrenal slices generated spontaneous Ca^{2+} spikes that were non-responsive to Ang II [65]. TASK-2 is essential for regulating membrane potential by maintaining K^+ homeostasis across the cell membrane. Inhibition of TASK-2 leads to a more depolarized membrane potential, which facilitates the opening of T-type calcium channels and triggers cytosolic Ca^{2+} peaks. This suggests a mild increase in excitability and a higher frequency of oscillating peaks in APA cells. However, our data show that blocking TASK-2 did not affect the number of cells exhibiting Ca^{2+} oscillations or the frequency of these oscillations in treated AAC cells. This indicates that other potassium channels, in addition to TASK-2, contribute to regulating Ca^{2+} dynamics in human adrenocortical cells.

We showed that isolated zona glomerulosa cells remain active in culture rather than quiescent. Furthermore, irbesartan suppressed all calcium responses to Ang II stimulation, including the initial transients and subsequent oscillations, indicating that these responses were all mediated by the AT1 receptor. Depleting extracellular calcium did not affect the transients but altered the peak parameters of calcium levels and reduced the frequency of APA oscillations. These findings

demonstrate that Ang II, through AT1R activation, triggers calcium release from the endoplasmic reticulum, driving both the initial and subsequent calcium activities.

It is important to emphasize that our primary cells in culture exhibit a very slow proliferation rate in vitro. However, after 24 hours in culture, we observed the formation of multicellular structures resembling the cytoarchitecture of intact human adrenocortical glomeruli, known as "rosettes," originating from dispersed CD56⁺ adrenocortical cells. Therefore, the patterns observed in our experimental setup likely mirror in vivo behavior. The functional activity of cell assemblies in intact adrenocortical tissues has been extensively studied and shown to coordinate calcium spikes induced by Ang II in mouse models[\[64\]](#)[\[63\]](#)[\[61\]](#). In our study we demonstrated the synchronicity of human CD56⁺ cell clusters formed from invitro dispersed cells. These data indicate the importance of rosette assemblies for the normal function of adrenocortical tissue.

Ang II is known to provoke higher production of ROS upon binding to its AT1 receptor [\[133\]](#). However, surprisingly, mitochondrial ROS was depicted to be significantly lower in APA compared to AAC. As ROS cause cellular damage [\[134\]](#), our findings propose either a defense mechanism that APA cells adapt to enhance the proliferation of aldosterone tumor or an elevated ROS production is required in APA to meet the increased need for aldosterone production in these cells.

Mitochondria are the site of aldosterone synthesis in ZG cells. The mitochondrial membrane potential controls the extent of Ca²⁺ influx from the cytoplasm, which is a crucial regulator of steroidogenesis. Previous studies using HEK 293T cells have shown that mitochondrial TASK-3 channels play a role in regulating the mitochondrial membrane potential [\[135\]](#). However, upon

assessing mitochondrial membrane potential of CD56⁺ AAC and APA cells, results did not show any difference between both conditions because of a huge variability among subjects that affects mitochondrial membrane potential.

Our double immunofluorescence analysis using SDHA/CYP11B2 reveals that CYP11B2 is not present in all mitochondria within APA cells, implying that those mitochondria lacking CYP11B2 may perform other functions. Additionally, CYP11B1 and CYP11B2 are known to exhibit a closely intertwined expression pattern in the intact adrenal cortex[\[136\]](#). However, it has not yet been demonstrated whether both enzymes are expressed in the same cell. Our high-resolution imaging revealed the expression of both enzymes, but further analysis is needed.

Vascular calcification is associated with cardiovascular diseases morbidity and mortality[\[137\]](#). Traditional methods for measuring calcification involve endpoint assays such as alizarin red staining and o-Cresol phthalein quantification. These techniques, however, do not account for the cellular components or circulating biomarkers that could contribute to calcification deposition[\[109\]](#). Our study is the first to demonstrate that PA plasma can induce calcification deposition in vitro on human vascular smooth muscle cells and in induced vascular smooth muscle cells. Notably, some patients served as controls, highlighting the importance of evaluating biomarkers, such as PTH, that are known to influence calcification and contribute to patient variability. In conclusion, we introduce a stem cell model for researching in vitro calcification, which offers a valuable diagnostic tool for future personalized cardiovascular risk assessments.

Conclusions

In summary, our established custom-made pipeline provides a novel tool for the detection and quantification of Ca^{2+} dynamics in human aldosterone producing adrenocortical cells that could be used by researchers with no prior knowledge in coding skills. The emerging description of Ca^{2+} dynamics in CD56^+ APA cells reveal that excessive steroidogenesis is due to elevated oscillatory activity and frequency of oscillation. Moreover, our findings unveiled significant lowered production of ROS in APA cells compared to AAC and the strong tendency of adrenocortical cells towards forming glomeruli-like clusters that are coordinated in Ca^{2+} signaling. Collectively, these data provide new approaches for target therapy for PA pathology.

In closing, as it is clinically manifested that PA is associated with arterial stiffness and arterial calcification due to excessive aldosterone production[\[138\]](#), it was essential to test calcification tendency of PA patients invitro. we discovered, through the novel biohybrid assay, that PA is capable of inducing calcification deposits significantly higher than healthy volunteers and EH patients when iVSMCs were utilized thus providing a non-invasive personalized cardiovascular assessment tool.

Societal Impact

Primary aldosteronism constitutes 11% of hypertensive subjects. It can be corrected if timely diagnosed, otherwise it might lead to deleterious consequences like excess damage to the heart, vessels and kidney leading eventually to cardiovascular diseases [3]. Aldosterone production stimulated by Ang II, and mediated by AT1R, leads to the activation of Ca^{2+} signaling [31]. Moreover, it is well established that mutations of ion channels and pumps detected in APA are responsible for the Ang II-independent excessive aldosterone production via the increase of intracellular Ca^{2+} [55].

In my thesis, we investigated Ca^{2+} dynamics for the first time in human CD56^+ AAC and APA cells. We demonstrated a mechanism of Ca^{2+} regulation which APA cells rely on for excessive aldosterone production depicted by increased oscillatory activity and frequency of oscillation. In addition, we assessed in AAC and APA cells the status of mitochondria, where aldosterone production occurs, by quantifying mitochondrial ROS that revealed a lower ROS level in APA proposing either a defense mechanism for sustaining tumor growth or a requirement to sustain excessive hormone production. In addition, we demonstrated the importance of glomeruli structure on the function of aldosterone producing cells that is maintained in APA cells. Through our findings, we provided novel insights on Ca^{2+} regulation in APA using human cell models taking into consideration the key function of Ca^{2+} in provoking aldosterone production.

Moreover, we aimed to investigate vascular calcification in PA beyond the clinical approach that is already established using CT abdominal scans [104][138]. Our approach aimed to monitor the real time invitro calcification development of PA patient's plasma by integrating blood circulating

molecules with cell models like iVSMCs and hVSMCs. Our study provides a tool for scientists to in vitro test possible therapeutic approaches towards curing vascular calcification using a personalized non-invasive diagnostic tool.

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Publications

- H. Ajjour, G. Pallafacchina, L. Lenzini, B. Caroccia, and G. P. Rossi, “Intracellular Calcium Dynamics in Primary Human Adrenocortical Cells Deciphered with a Novel Pipeline,” *High Blood Press. Cardiovasc. Prev.*, no. 0123456789, pp. 15–22, 2024, doi: 10.1007/s40292-024-00641-5.
- B. Caroccia *et al.*, “Double CYP11B1/CYP11B2 Immunohistochemistry and Detection of KCNJ5 Mutations in Primary Aldosteronism,” *J. Clin. Endocrinol. Metab.*, vol. 00, no. 0, pp. 1–11, 2024, doi: 10.1210/clinem/dgae411.
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Thesis Summary in Italian

L'aldosteronismo primario (PA), la forma più comune di ipertensione secondaria, è caratterizzato da un aumento delle concentrazioni intracellulari di Ca^{2+} citosolico $[\text{Ca}^{2+}]_i$ e da un'eccessiva produzione autonoma di aldosterone.

Il nostro scopo è quello di studiare i transitori e le oscillazioni del $[\text{Ca}^{2+}]_i$ e di valutare la funzione mitocondriale in un modello umano di cellule CD56+ isolate da adenoma produttore di aldosterone (APA) e normale corteccia surrenale circostante (AAC). Inoltre, poiché la PA è associata alla calcificazione dell'aorta addominale, ci concentreremo sulla misurazione in vitro della propensione alla calcificazione dei pazienti con PA e plasma iperteso essenziale (EH).

Abbiamo valutato il $[\text{Ca}^{2+}]_i$ in CD56+ APA e AAC in condizioni basali e dopo stimolazione con angiotensina II (Ang II) utilizzando il colorante Fura-2, la microscopia confocale in vivo e una pipeline sviluppata su misura per analizzare i segnali $[\text{Ca}^{2+}]_i$ in termini di parametri di frequenza e di picco come ampiezza, larghezza intera a metà massimo (FWHM) e area sotto la curva (AUC). Poiché i canali K^+ TASK-2 del dominio a due pori sono sottoespressi nell'APA, abbiamo utilizzato la chinidina (20 μM), 20 secondi prima dell'acquisizione dell'imaging $[\text{Ca}^{2+}]_i$ per esaminare l'effetto dell'inibizione farmacologica di questo canale. Inoltre, abbiamo impoverito il Ca^{2+} extracellulare per identificare la fonte dei transitori di Ca^{2+} .

Inoltre, abbiamo utilizzato il test Biohybrid per valutare la propensione alla calcificazione plasmatica dei pazienti PA ed EH in vitro utilizzando 2 modelli cellulari, vale a dire cellule muscolari lisce vascolari indotte (iVSMC) e cellule muscolari lisce vascolari umane (hVSMC).

I livelli di $[\text{Ca}^{2+}]_i$ a riposo erano più alti nelle cellule AAC (0,80 AU (25% percentile=0,65; 75% percentile=0,90)) rispetto alle cellule APA (0,53 AU (25% percentile=0,33; 75% percentile=0,79)) ($p=0,0212$). Abbiamo rilevato l'attività spontanea del Ca^{2+} , durante condizioni di riposo, in entrambi i tipi

cellulari senza differenze significative nei parametri di picco. Dopo la simulazione dell'Ang II, l'AAC ha mostrato elevati parametri di FWHM, ampiezza e picco AUC dei primi transitori di Ca^{2+} . I parametri di picco delle successive oscillazioni del Ca^{2+} erano variabili tra le cellule AAC e APA. La frequenza delle oscillazioni del Ca^{2+} e il grado di oscillazione delle cellule erano significativamente più alti nelle cellule APA che nelle AAC. Nella AAC, l'inibizione di TASK-2 con la chinidina tendeva ad aumentare l'attività spontanea e ad alterare i parametri del picco, ma non influenzava la frequenza di oscillazione. La deplezione extracellulare di Ca^{2+} non ha diminuito i transitori di Ca^{2+} .

Dopo 24 ore di coltura, sia le cellule AAC che quelle APA hanno sviluppato una struttura cellulare simile a una rosetta che ricorda i "glomeruli" dei cluster intatti della zona glomerulosa. Le statistiche funzionali e l'analisi dei cluster hanno mostrato un comportamento sincrono delle cellule appartenenti allo stesso cluster senza differenze significative tra AAC e APA.

Inoltre, i risultati del test bioibrido hanno dimostrato un'elevata deposizione di calcificazioni extracellulari dopo la stimolazione di entrambi i modelli VSMC con plasma PA rispetto ai volontari sani. Inoltre, utilizzando iVSMC abbiamo osservato un aumento significativo della deposizione di calcificazioni rispetto al plasma EH.

La valutazione quantitativa dei segnali $[\text{Ca}^{2+}]_i$ ha rivelato differenze significative nella dinamica del $[\text{Ca}^{2+}]_i$ tra le cellule AAC e APA umane. Questi risultati suggeriscono la modulazione simultanea della frequenza e dell'ampiezza delle successive oscillazioni di $[\text{Ca}^{2+}]_i$ come potenziale fattore di aumento del tasso di produzione di aldosterone. Inoltre, abbiamo identificato una propensione in vitro del plasma dei pazienti con PA a favorire la deposizione di mineralizzazione, fornendo uno strumento diagnostico non invasivo da utilizzare per la futura medicina personalizzata per la valutazione del rischio cardiovascolare.