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HYPERTENSION, HYPERCHOLESTEROLEMIA, HYPERALDOSTERONISM: A GENETIC AND PROTEOMIC PERSPECTIVE FOR PERSONALIZED THERAPY

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Abstract

Cardiovascular disease (CVD) accounted for 30% of the estimated 58 million deaths globally. This proportion is equal to that due to infectious diseases, nutritional deficiencies, and maternal and perinatal conditions combined. It is noteworthy that a substantial proportion of these deaths (46%) occurred in individuals under 70 years of age, in the most productive period of life. In addition, 79% of the disease burden attributed to cardiovascular disease is in the same age group.

High blood pressure is one of the most important preventable causes of premature illness and death. It is the major risk factor for stroke, heart attack, heart failure, chronic kidney disease and cognitive decline. The risk associated with increasing blood pressure is continuous, with each 2 mmHg rise in systolic blood pressure associated with a 7% and 10% increased risk of mortality from ischaemic heart disease and stroke respectively. According to the international guidelines of the World Health Organization (WHO) and the International Society of Hypertension (ISH) individuals may be divided into different groups according to the blood pressure value: patients with optimum (<120/80 mm Hg), normal (120–129/80–84 mm Hg), high (130/139 - 85/89 mm Hg) blood pressure (BP) and hypertensive (140/90 mm Hg). Periodic screening of BP in adults is recommended to detect the onset of hypertension[1], so that appropriate measures can be instituted to prevent morbidity and mortality associated with raised BP[2]. Due to the absence of definitive data regarding the time course of hypertension evolution from lower BP values, current international guidelines vary widely in their recommendations for the clinical monitoring of individuals without hypertension. For instance, the sixth report of the Joint National Committee on the Prevention, Detection, Evaluation and Treatment of High Blood Pressure in the United States (JNC VI) recommends that people with high-normal BP should undergo yearly monitoring, whereas those with normal or optimum BP should be screened every 2 years. By contrast, the European Task Force on Prevention of Coronary Disease proposes that all individuals without hypertension should be screened at least once every 5 years. The British Hypertension Society advocates an intermediate position: patients with a systolic BP of 135–139 mmHg or a diastolic BP of 85–89 mmHg should be reassessed yearly, whereas those with lower BP should be assessed at 5–year intervals.[3,4]

Essential, primary, or idiopathic hypertension is defined as high BP in which secondary causes such as renovascular disease, renal failure, pheochromocytoma, hyperaldosteronism,

or other causes of secondary hypertension are not present. Essential hypertension accounts for 80-90% of all cases of hypertension; it is a heterogeneous disorder, with different patients having different causal factors that may lead to high BP. Life-style, diet, race, physical activity, smoke, cultural level, environmental factors, age, sex and genetic characteristics play a key role in the increasing risk.

Conversely to the essential hypertension, secondary hypertension is often associated with the presence of other pathological conditions such as dyslipidaemia, hypercholesterolemia, diabetes mellitus, obesity and primary aldosteronism. Amongst them, primary aldosteronism represents one of the most common cause of secondary hypertension, with a prevalence of 5-15% depending on the severity of blood pressure. Besides high blood pressure values, a principal feature of primary aldosteronism is the hypersecretion of mineralcorticoid hormone, aldosterone, in a manner that is fairly autonomous of the renin-angiotensin system. Primary aldosteronism is a heterogeneous pathology that may be divided essentially in two groups, idiopathic and familial form.

Despite all this knowledge, there are so many hypertensive cases that cannot be explained. These individuals apparently seem to be healthy, but they have a great risk to develop CVD. The lack of known risk factors makes difficult their classification in a scale of risk. Over the last three decades a good help has been given by the pharmacogenetics/pharmacogenomics, a new area of the traditional pharmacology that try to explain and find correlations between genetic variation, (rare variations, SNPs, mutations), and the risk to develop a particular disease.

This study was realized with the aim to add new informations on the hypertensive susceptibility starting from two different populations: an unselected Italian population, the Brisighella population, used for the Brisighella Heart Study, and a selected Australian cohort of 251 patients affected by primary aldosteronism.

1.0 Cardiovascular system

1.1 Heart, vessels and blood pressure

Cardiovascular apparatus is one of the most fascinating and intriguing, for complexity and importance, among all systems that regulate our life. It is constituted by an important central organ, the heart, and by a wide number of blood vessels, all differing in function and structure. The heart and blood vessels form a transportation system that delivers to all cell nutrients and oxygen, needed for their proper function, and moves away the products of their metabolism, providing a sort of communication between the cells and the environment.

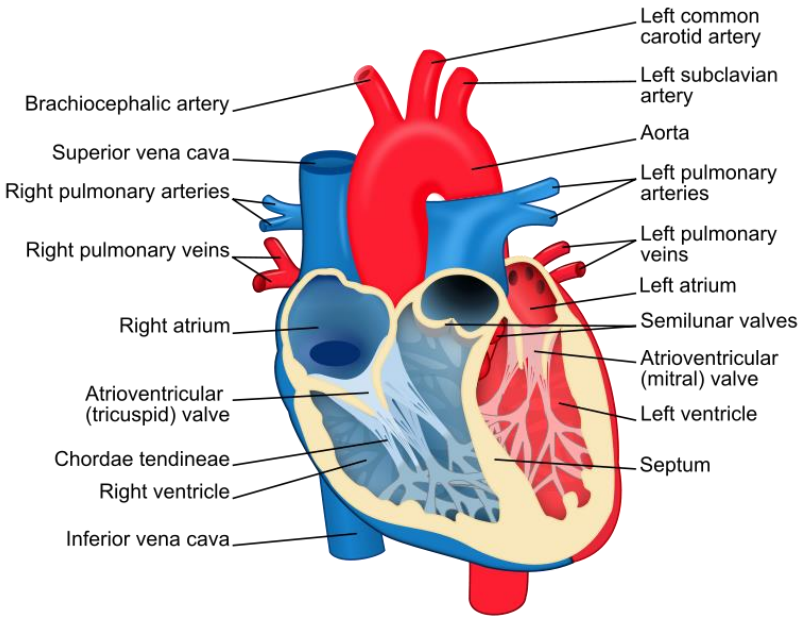

Fig.1 *Frontal view of the heart structure and the most important arteries and veins*

The heart is an involuntary muscle, able to contract an relax autonomously with regular rhythm, pumping the blood in the pulmonary and systemic circulations. This organ, located anteriorly to the vertebral column and posteriorly to the sternum, is surrounded by a double wall sac, called pericardium, with a pericardic fluid between them. Outside the parietal pericardium there is a fibrous layer called fibrous pericardium. The human heart can be divided in three layers: the outer layer called *epicardium or visceral*, the middle layer, called

myocardium, constituted prevalently by contractile cardiac muscle, and the inner *endocardium,* in contact with the blood. The heart (Fig. 1) may be divided in four chambers, **two atria** left (sx) and right (dx) on top, that constitute the base of the organ and similarly **two ventricles** dx and sx in the bottom of the heart, named apex. The two sides of the organ dx and sx are separated by a wall, called *septum*. The two atrium dx and sx communicate with the corresponding ventricle below, allowing the blood to flow downwards. However, the heart needs a set of valves, located across each atrium and ventricle, to keep the fluid flowing in one direction; the dx atrial-ventricular valve is called *tricuspid valve* and the sx atrial-ventricular valve is the *bicuspid or mitral*. There are other two important *semilunar valves,* housed between right and left ventricle and pulmonary and aortic artery, respectively with the same function of the others.

Blood circulation is divided in small and big circulation; the first that starts from the dx atrium and receives deoxygenated blood from the systemic circulation through the superior vein cava; once the dx atrium is completely filled, blood starts to flow down in the corresponding ventricle below, from which deoxygenated blood through pulmonary artery is driven in the lug to be oxygenated. From the lug, this blood reach of oxygen and poor of anhydride carbonic flows back in the sx atrium by the pulmonary vein. Thus, the big circulation starts with the oxygenated blood that has completely filled the sx atrium, from which, in the similar way to the right part, it flows down in the ventricle below and get off by aortic artery. In both, sx and dx atrium blood is able to flow down in the ventricle located below for the 90% of the total volume thanks to the force of gravity. The remaining 10% of volume is driven down by the atrial myocytes contraction, *atrial systole*, reducing the atrial volume and in the same time increasing the pressure inside that overcomes the ventricle pressure, according to the ideal gas law: **P**= nRT/**V**.

At the end of atrial systole all the blood volume is located in the ventricle below; in the same time the semilunar valves, located between the ventricle orifice and aortic artery, are still closed, keeping all the volume inside. At the beginning of left ventricle systole, as aforementioned, the two physical parameters, volume and pressure change in an inversely-related manner, volume decreases while the pressure starts to increase overcoming the aortic pressure; this leads to the opening of semilunar valves, allowing the blood to flow out in the aortic artery. The left ventricle is empty now and ready to receive again blood from the overlying atrium. (Fig. 2)

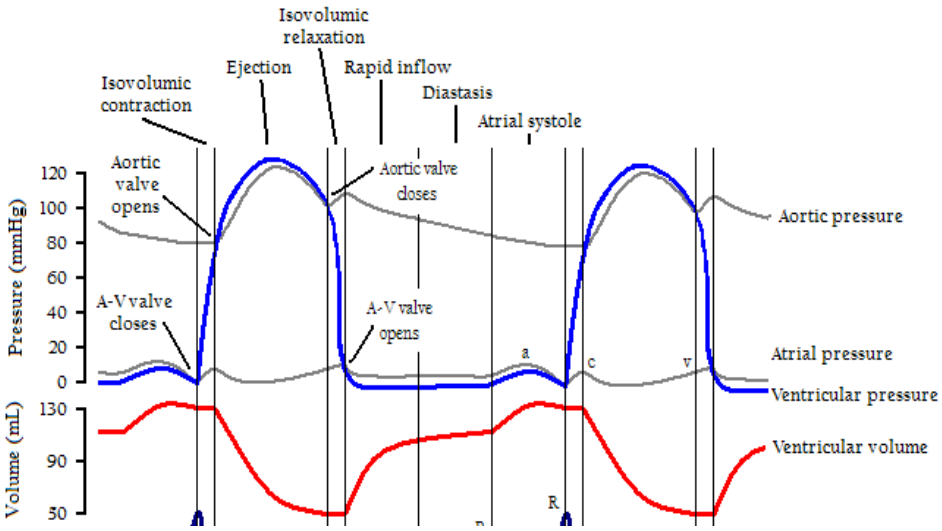

Fig.2 *Schematic representation of the volume and pressure values changes during a complete cycle of contraction-relaxation of the heart.*

The heart is an involuntary muscle able to contract autonomously with a regular rhythm thanks to own electrical system, constituted by three important structures such as **sinus atrial node (SAN)**, **atrio-ventricular node (AVN)** and **His-Purkinjie fibres.** SA node is located in the right atrium near the entrance of the superior vein cava; it is the most important of this system because of presence of particular type of cells, named pacemaker cells, responsible of the normal heartbeat, which ranges between 60 and 80 beats for minute. As in all other cells, the resting potential membrane of pacemaker cells range between -60 and -70mV that is kept by a continuous outflow of K^+ ions in the extracellular environment. However, this outflow decreases during the time leading to a small depolarization of the resting potential membrane; in the same time there is a slow inward flow of Na^+ as well as of Ca^{2+} ions, increasing the potential membrane until the threshold potential is reached. At a this moment, the true depolarization can start and differently from other exciting cells, in which this phase is characterized by Na^+ channel opening, it is caused by a slow influx of Ca^{2+} ions through *L-type calcium channel*; for this reason the depolarization is slower than in neurons. At the end of depolarization, Ca^{2+} and Na^+ channels result to be closed, while the K^+ ions flow out rapidly repolarizing the resting membrane potential. Under normal conditions the depolarization wave will be propagated throughout the right atrium, and through the *Bachmann's bundle* to the left atrium, stimulating the atrial myocardium to contract, *atrial systole*. During the atria relaxation, called *diastole*, since atria are electrically isolated from the ventricles and the AV node is the only connection between the two areas, the electrical conduction from the AV node to the underlying ventricles is realized with a small delay

through the *Bundle of His* along the septum to the *Purkinjie fibres*. The conduction delay between atria and ventricles is very important for the heart functionality in order to avoid a simultaneously contraction. This phenomenon gives the possibility to the atria to contract together, allowing the blood flow toward the ventricles. An important characteristic of the myocardium cells is the ability to contract immediately and simultaneously with the depolarization wave passage. Myocytes of the heart can be considered as a *functional syncytium* because of the electrical impulses propagate freely between cells in every direction, so that the myocardium can contract as a single contractile muscle. The free and rapid propagation of the electrical impulses can happen thanks to the presence of particular junctions, called *gap-junctions*, between myocytes. Changes of depolarization/repolarization frequency of pacemaker cells and contraction strength of myocytes as well as alterations of the peripheral vascular resistance and alteration of volemia, including water and Na^+, K^+, Cl^- ions levels, have wide effects on the systolic blood pressure values according to the physiological definition of blood pressure:

SBP = Q x TPR

SBP = systolic blood pressure

Q = cardiac output

TPR = total peripheral resistance

Therefore, SBP is directly proportional to TPR and Q. The latter is defined as a volume of blood being pumped by the heart, in particular by the right and left ventricle and represents the effective volume that comes back with the vein determining how much blood the heart pumps out.

The principal types of vessels, different in composition and function, include *arteries, capillaries and veins.* Arteries are characterized by three different layers: the innermost layer, called *tunica intima or internal*, which show an elastic membrane and a thin layer of endothelial cells directly exposed to the blood; the *tunica media*, the middle layer, containing concentric sheets of smooth muscle tissue in a framework of loose connective tissue, and *tunica external, or adventitia*, the outermost layer of a blood vessel that forms a connective tissue sheath. The connective tissue fibres of the tunica external typically blend into those of adjacent tissues, stabilizing and anchoring the blood vessel.

Arteries may be divided into elastic and muscular arteries; the first, which include the pulmonary trunk, aorta, and their major arterial branches, transport large volume of the blood away from the heart; the second group, also known as *medium-size arteries*, distribute blood to the body's skeletal muscle and internal organ, and it includes the carotid, brachial,

mesenteric, and femoral arteries. The tunica media of the muscular arteries contains scattered smooth muscle cells, although they do not form a proper layer, and the contraction or relaxation of this muscle can produce a great changes in the diameters of the lumen. Since resistance to blood flow depends in part on this diameter, the activity of smooth muscle cells is very important on blood pressure regulation.

Capillaries, that show a singular thin layer of endothelial cells, are the only responsible of the exchange between the blood and the surrounding extracellular fluid. There are two major types of capillaries: *continuous and fenestrated.* Continuous capillaries allow the diffusion of water, small solutes and lipid-soluble material, and avoid the passage of blood cells and peptide. Fenestrated capillaries, as the name implies, are not completely attached each other, but show some fenestration that allow the passage of small peptide. Capillaries do not function as individual units but as part of an interconnected network called capillary bed, or capillary plexus, which is characterized by a lot of connections between capillaries and venules.

Veins transport de-oxygenated blood from periphery to the heart with a blood flows at very low pressure. Veins are classified on the basis of their size in three different types: venules, medium-size veins and large veins. Venules, which collect blood from capillary beds, are the smallest venous vessels and show three layers: an inner endothelium composed by endothelial cells, a middle layer of muscle and elastic tissue and an outer layers of fibrous connective tissue. Medium-size veins, comparable in size to the muscular arteries, characterized by a thin tunica media containing relatively few smooth muscle cells, and a thick layer, the tunica external, which contains longitudinal bundles of elastic and collagen fibres. The blood pressure in venules and medium-size veins is so low that it cannot oppose the force of gravity. Large veins include superior and inferior *venae cavae*. One of the most important characteristic of the large veins is the presence of valves, at more or less regular intervals, that prevents the retrograde flow of blood. These valves act like the valves of the heart, allowing blood flow in only one direction. In particular, when the individuals is in the standing position, these valves are closed, and only the compression of deeper veins, by contractions of the skeletal muscle, allows to the blood to open these valves, which in turn will be closed by the flowing back of blood itself. This force, called *muscle pumping*, works through the thoracic force giving to blood the needed energy to overcome the force of gravity.

1.2 Nervous system and its role in the blood pressure modulation

It has been well established over the years that modification of one of aforementioned parameters, heart rate, peripheral vascular resistance, volemia, directly modulates the arteriolar blood pressure. For this reason, blood pressure regulation, likes other parameters of our body, is strictly settled by numerous and complex mechanisms such as endocrine and autonomic nervous system (ANS), which often work synergically.

ANS is constituted by sympathetic and parasympathetic pathways, which have opposite activities on the same organs and tissues, and use two different types of neurotransmitter activating several receptors. The most abundant sympathetic fibres use **noradrenaline (NA)** as neurotransmitter and are named adrenergic or noradrenergic fibres while parasympathetic fibres use **acetylcholine (Ach)** thus called cholinergic. NA and ACh neurotransmitters are able to exert different activities and evocate different responses thanks to the activation of several receptors. Among the cholinergic receptors it is possible to distinguish two families: muscarinic receptors (M), M_1, M_2, M_3, and nicotinic (N) receptors, N_n, N_m. Noradrenergic receptors are divided in alpha (α) and beta (β), which in turn are classified in α_1 and α_2, and β_1, β_2, and β_3. To date, it is well understood the localization and the mechanism by which these receptors are activated and the effects that their activation lead to. Muscarinic and β adrenergic receptors are G-protein-coupled-receptor, and their different action depends on the sub-type of G-protein with which they are coupled. It is well known that G-protein can exist as a Gs (stimulatory), Gi (inhibitory) and Gq proteins, that work activating or inhibiting respectively an important effector, adenylate cyclase, that converts ATP in cAMP, which in turn stimulates a kinase cAMP-dependent such as PKC. Activation of Gq protein, leads to an increased activity of phospholipase C, that converts inositol-bisphosphate-membrane-phospholipid (IP2) in inositol-triphosphate (IP3) and dyacilglycerole (DAG) increasing cytoplasmatic calcium (Ca^{2+}) concentration.

All β receptors, β_1, β_2, and β_3 are associated with a Gs protein even if in some case β_2 has been seen coupled with a Gi protein. Alpha receptors, α_1 and α_2, are coupled with a Gq and a Gi protein respectively.

M_1, M_3 receptors are associated with a Gq protein while M_2 is associated with an Gi protein. Nicotinic N_n and N_m receptors are classical potassium and sodium channels, with a pentameric structure, 2α, β, γ, δ subunits. These receptors are widely distributed, and the most part of organs and tissues receive sympathetic and parasympathetic fibres. Heart, vessels,

kidneys, endocrine glands, are the principal organs and tissues, strictly involved in the blood pressure regulation, that undergo sympathetic and parasympathetic modulation.

Heart is modulated by autonomic nervous system; indeed, muscarinic and adrenergic receptors have been isolated from this organ. Pacemakers cells, whose role has been already described, show muscarinic M_2 and adrenergic β_1 receptors. M_2 receptors, coupled to a G_i protein, once activated by ACh, vary ions flow, increasing the K^+ conductance and reducing the Ca^{2+} inflow in both pacemaker and myocytes cells. The final effect of the M_2 receptor activation is heart rate reduction and decreased electrical conductibility. Furthermore, there is a reduction of the contraction strength. All together these effects wok reducing the blood pressure regulation. Similarly, activation of adrenergic β_1 receptors is associated with electrical and mechanical effects. It has been observed an increased Ca^{2+} flow across the cell membrane of pacemaker and myocytes, that leads to an increase in the discharge frequency as well as electrical conduction and ventricle strength contraction. These effects have hypertensive implication.

The cardiovascular system maintains an adequate circulation of blood to all part of the body due to the blood vessels regulation, realized through the branches of the autonomic nervous system that reach almost 100% of them. Density and function of this innervation vary widely according to different types of vessels, modulating the vasculature tone. Vascular tone is the result of smooth muscle cells contraction, which may be modulated by different neuronal neurotransmitters, different receptors, cell-to-cell spread excitation, chemical events at the cell membrane. The vascular tone reflects directly the resistance to the blood flow of small vessels, in particular arterioles and pre-capillary arterioles. The increased vascular tone results in reduction of blood vessels diameter and consequently in blood flow, causing inadequate nourishment and oxygenation of the organ or tissue with a decreased activity. For all these reasons vascular tone or vascular resistance regulation is very important in the blood pressure control, and for this reason it is strictly controlled by autonomic nervous system. Vascular tone undergoes influences of adrenergic fibres, through NA release and β_2 and α_1 adrenergic receptors activation. β_2 receptors, localized on top of the membrane of endothelial cells, are coupled with a Gs protein; its activation lead to an increased adenylate cyclase activity and cAMP production; raised cAMP concentration activates a cAMP kinase which in turn phosphorylates and activates a myosin light chain kinase, inducing relaxation of the endothelial cells. On the other hand, activation of adrenergic α_1 receptors, coupled to a Gq protein, leads to an increased activity of phospholipase C, that up-regulates the IP3 and DAG production with a resulting increase of Ca^{2+} concentration. The Ca^{2+}-calmoduline complex

activates myosin light chain kinase, which in turn phosphorylates the myosin light chain. It is this phosphorylation that promotes the interaction of myosin ATPase and actin and the cross-bridge formation, leading to the initiation of a transient increase in vascular smooth muscle contraction. These effects mediate vasoconstriction and increase blood pressure. Muscarinic receptors that have been discovered across the membrane of endothelial cells are principally M_3. Activation of these Gq-coupled receptors leads to an up-regulation of phospholipase C, which in turn, releasing DAG and IP_3, increase the cytoplasmatic Ca^{2+} concentration. This effect seems to be important for activating the endothelial NO synthase (eNOS), that produces NO. This substance is able to spread easily in the smooth muscle cells, where activates the guanylyl cyclase increasing the conversion of GTP to cGMP; furthermore cGMP activates a cGMP-dependent protein kinase that most probably dephosphorylates the MLC, leading to a relaxation of these cells. Even if the relaxation effect of NO is well known, its mechanism to date is not completely clarified.

It is well established that several substances of endothelial cells metabolism, such as CO_2 and O_2 are involved in the vascular tone and in the blood pressure regulation.

It has been seen that reduction of O_2 concentration is associated with diminished contractility of vascular smooth muscle cells as well as increased CO2 concentration is associated with a vasodilatation. Similarly a wide number of substances with a vasoactive properties have been isolated and characterized, such as kallidin, bradykinin etc.... Some of these have important functions, like *angiotensin-II* (AG-II): which is an extremely powerful vasoconstrictor; another peptide is the *vasopressin* also called antidiuretic hormone, that besides the renal effects on Na^+ and K^+, has been shown to exert a cutaneous vasoconstriction in the renal vascular bed; *histamine* that is released from cells of the skin in response to injury or antibody-antigen reactions, not only dilates arterioles and venules but also increases their permeability; *prostaglandins* are a group of biologically active lipids that show a vasodilator effects.

1.3 Kidney involvement in the blood pressure regulation

Kidneys are the principal organs of regulation of the volume and solute composition of the body fluid by reabsorption and excretion of water and electrolytes. The composition of blood plasma varies only slightly from individual to individual in a healthy condition. In the table

below are reported the average normal values for the most important ions that come into play in the composition of these two compartments, blood plasma and cytoplasm.

Ions	Blood plasma	Cytoplasm
Na^+	145 mM	139 mM
K^+	4 mM	12 mM
Ca^{2+}	1-2 mM	<0.0002
Mg^{2+}	1.5 mM	0.8 mM
Cl^-	116 mM	4 mM
HCO^{3-}	29 mM	12 mM
Amino acids	9 mM	138 mM

Table n.1 *Electrolytes composition of blood plasma and cellular cytoplasm in a normal condition.*

Therefore, kidney exerts this important function but is not the only one, because other organs participate in this process, notably the lungs in respect to acid-base regulation, autonomic nervous system and endocrine glands. However, the kidney through its excretory activity, provides the major mechanism for maintaining homeostasis. In order to keep a constant internal and external environment the kidney must respond appropriately to variations in dietary intake and in extra-renal losses of solutes and water. It is well known that ion concentration, measured in terms of *osmolarity*, is one of the most important factor able to influence the volemia. In case of increased osmolarity our body through autonomic nervous system, endocrine and kidney systems induce a reabsorption of water in order to dilute these ions, increasing the volemia. Otherwise, if there is a wide loss of ions with a consequent decrease of osmolarity our kidneys eliminate the right volume of water to concentrate the solution within physiological range. All of this is possible because our systems always work trying to keep our body in a homeostasis condition. Since the structure and the function of the kidney are so closely related, it is necessary to have some knowledge of its structure before looking at its function. The basic unit of structure and function of the kidney is the nephron, that includes the glomerulus and its attached tubules; in succession there are: *proximal convoluted tubule*, *loop of Henle* and *distal convoluted tubule* and *collecting duct*. Glomerulus is completed surrounded by a Bowman's capsule, an anastomotic network of freely branching capillaries. These capillaries originate from an *afferent arteriole*, and leave the glomerulus as a single *efferent arteriole*. The afferent arteriole in turn breaks up to form peritubular

capillaries surrounding the cortical tubules reaching also the deepest zone of the nephron, medulla, where they are called *vasa recta*. The presence of these blood vessels all around the renal tubules is very important because in this way water and electrolytes exchanges between them are possible. Our blood is filtered almost 26.7 times during the day and considering that the blood volume ranges between 5-7 L it results that total volume of 186 L is filtered for day. Of these 186 L of glomerular ultra-filtered liquid the 99% is reabsorbed and only the 1% is eliminated as urine, i.e. 1.8 L per day.

The rate of filtration across the glomerular filtration membrane of the Bowman's space is determined by physical forces. The hydrostatic pressure in the glomerular capillaries (Pgc) is the driving force of the filtration; opposing filtration are the hydrostatic pressure in the Bowman's capsule (Pbs) and the colloid osmotic pressure (COP) exerted by the proteins of the capillary plasma. Because the filtrate in Bowman's space is protein free the COP may be considered negligible. The glomerular filtration rate (GFR) is directly proportional to the net balance of the these forces according to the mathematical equation:

$$GFR = Kf\,[Pgc - (Pbs + COP)]$$

Kf is considered constant and is defined as the product of the hydraulic conductivity and the surface area of glomerular capillaries. According to this definition, it's possible to say that at the Bowman's space the net difference between these forces is almost 8 mmHg, driving the ultra-filtered from the lumen of capillaries to the Bowman's capsule. Along all the distal tubes the difference between these forces becomes positive in the peritubular capillaries with a consequent reabsorption of the fluid that was lost before.

Two thirds of the filtered volume is reabsorbed immediately in the proximal tube, while the remaining part starts to flow down along the descending trait of the Henle's loop. Along all the descending trait of Henle's loop there is a great reabsorption of water and the filtered solution reach a concentration of 1200mOsm, that is comparable with that of the external medulla zone; along the ascending trait of Henle's loop, ions such as Na^+, K^+ and Cl^- but not water, are widely reabsorbed reducing the concentration of filtered liquid. The filtered liquid, that is not yet called urine, continues its trip along the distal tubule, which similarly to the ascending trait is responsible of Na^+ reabsorption but in the same time is impermeable to the water. The distal tube is the physiological target of an important hypertensive hormone, *aldosterone*, released from the glomerulosa cells of the adrenal gland (see chapter 2.0), responsible Na^+ and consequently water reabsorption, increasing the volemia and the blood pressure. The last tube of nephron, the *collecting duct*, exerts an important function of

changing the urine concentration, regulating the water reabsorption. In this way, this tube is able to produce *hypertonic urine* in case of excess of ions that have to be eliminated or *hypotonic urine* in case of excess of water. This function is realized through specific channels called *aquaporin*, whose expression is under the control of a particular type of hormone, *antidiuretic hormone (ADH) or vasopressin*, released from anterior pituitary gland, whose activity is important for maintaining the osmolarity (see parag.1.4). Kidney is able to bring about these adaptive responses because of its interaction with the autonomic nervous system, which constantly receives informations about the ion content, volemia and blood pressure. The major level of regulation lies in a microscopic structure, *juxtaglomerular apparatus (JGA)*, so-called for its proximity to the glomerulus. This apparatus is morphologically a highly specialized structure with both vascular and tubular components. It probably has an important role in a feedback mechanism controlling glomerular filtration rate and/or renal blood flow. It consists of several parts: granulated cells in the afferent and efferent arterioles, the macula densa, and the extra glomerular mesangium. It has been demonstrated only recently the presence of gap-junctions among these cells, whose role seems to be very important in the exchanges of informations. Some of the cells in the media of the afferent and probably the efferent arteriole close to the glomerulus are specialized myoepithelioid cells and contain granules that appear to be composed of the renin enzyme. The macula densa cells position is useful to monitor some aspect of distal tubular function possibly hydrostatic pressure and the concentration of one or more solutes especially Na^+ concentration. In case of increased Na^+ concentration in the filtered liquid that lies in the distal tubule, this cells start to work, releasing paracrine substances that seem to have double effects: on the one hand act on the afferent arteriole increasing the vascular tone and resistance while on the other hand transfer these informations to the epithelioid cells of juxtaglomerular apparatus, thereby regulating the release of renin.

Renin is a protolithic enzyme that once released and entered in the systemic circulation converts the angiotensinogen, that is produced and released constitutively by the liver, to release the inactive decapeptide angiotensin-I. Angiotensin converting enzyme (ACE) synthesized by several tissues, split off the last two amino acids of the angiotensin I to form the octapeptide AG-II, which has important hypertensive activities. This mechanism represents the key response at the kidney level toward a Na^+ reduction. However, other regulation mechanisms, as aforementioned, are involved in this complicated process. Afferent and efferent arterioles show particular type of receptors, called baroceotor, that are able to monitor constantly the pressure with which blood enters and leaves from the kidney, and in

case of reduced blood pressure these receptors through afferent fibres induce a sympathetic response. Furthermore, cellular membrane of juxtaglomerular cell has adrenergic β_1 receptors that response to noradrenaline/adrenaline increasing the renin release.

These observations show as the blood pressure regulation is a key mechanism realized in a cooperation manner between cardiovascular, autonomic nervous system, renal and endocrine glands.

1.4 Endocrine involvement in the blood pressure regulation

It is possible to imagine and assume that the connection point between the nervous and the endocrine system is represented by the *hypothalamus* through the *pituitary gland or hypophysis*. Hypothalamus is unique in this function and in the same time it is involved in several important processes. The hypothalamus is a relatively small area of the brain located beneath the thalamus nuclei and extends from the mesencephalon to the region of the optic chiasm and the pre-optic area. It has been well described the vascular organization of hypothalamus constituted by a series of capillaries that end in a capillary bed in the hypophysis, from which these vessels flow out with a singular vein into the systemic circulation.

It is well known that hypothalamus receives different types of neuronal fibres, GABAergic, cholinergic and catecholaminergic; indeed, several transmitters, such as acetylcholine, gamma amynobutyric acid, dopamine, serotonin, substance P, adrenaline, histamine have been isolated from this area.

It has become clear that the secretory activities of the cells of the anterior lobe of hypophysis are widely controlled by substances secreted in the hypothalamus and transported in the adenohypophysis by the portal vessel system. As stated previously, the hypothalamus integrates many functions and is involved in a series of reactions essential to the maintenance of homeostasis and to the initiation and control of many behavioural responses, such as reproduction, thirst and control of water balance, control of body weight, temperature regulation, reactions to stress, control of emotional reactions, sleep and arousal, control of somatic reactions.

The hypothalamus exerts its control over body functions through endocrine, hypothalamic-pituitary axis, and somatic neuronal efferent systems. It has a close relationship with the hypophysis and this hypothalamic-pituitary axis regulates the activity of nearly all endocrine

organs. Discussion of the role of the hypothalamus in the control of the body economy through the endocrine system can be divided in at least three parts: *reproductive functions*, *water balance*, and *metabolic processes*. The hypothalamus exerts its function through several peptides and biogenic amines, so-called *releasing factors*, which in turn lead to stimulation or inhibition of hormones secretion of pituitary gland. Among these substances we can recognize *gonadotropins-releasing hormone* (GnRH), *thyrotropin-releasing hormone* (TRH), *corticotropin-releasing hormone* (CRH), *growth hormone release-inhibiting hormone or somatotropin* (GHRH or STH), *somatostatin-releasing hormone* (SRIF). Each of these releasing factors secreted by the hypothalamus, reaches the anterior lobe of the pituitary gland and promotes or inhibits pituiry hormones release. *Pituitary gland or hypophysis* is divided in two parts: anterior lobe or *adenohypophysis* and posterior lobe or *neurohypophysis*. Histologically, the adenohypophysis consists of a large cells that contain secretory granules. As it has been already said, adenohypophysis is strictly related with the hypothalamus by a portal system of vessel, through which the releasing factors secreted into the hypothalamus reach this area and can exert their functions. The secretory cells of adenohypophysis show on the cellular membrane specific receptors with which the releasing factor interact leading to activation or inhibition of pituitary hormone secretion. The adenohypophysis secretes at least seven recognized hormones, four of which directly control the functioning of their particular target glands, the adrenals, thyroid, and gonads. These hormones are defined tropic or *trophic*.

Unlike to the adenohypophysis the neurohypophysis contains no large epithelial cells full of secretory granules. Instead, it consists primarily of unmyelinated nerve fibres and modified glial cell, called *pituicytes*. Although it was thought for many years that the pituicytes were the source of the posterior pituitary hormones, it is now recognized that these hormones are neurosecretory products of the hypothalamus conducted to the neurohypophysis by carrier proteins (neurophysins) via neuronal axons and stored into secretory vesicles in the neuron terminal and released directly in the systemic circulation. While the adenohypophysis release, as aforementioned, several hormones under the hypothalamus stimulating factors, neurohypophysis is responsible of secretion of *antidiuretic hormone or vasopressin* (**ADH**) and *oxytocin*, which escape to the hypothalamus regulation. It seems to be likely that the role of neurohypophysis is just storage and release of these substances; indeed the posterior pituitary gland contains almost 15 unit of ADH, which is enough to maintain a man in maximum antidiuresis for more than 1 week. By contrast, the hypothalamus contains very little hormone less than 5% of the total amount stored in the posterior lobe of pituitary. While

the hypothalamus may be capable of meeting some demands for ADH, it may not be able to support intense and sustained antidiuresis.

Pituitary hormones show a *feedback* mechanism of regulation, and in most cases it is a *negative feedback*; this means that releasing of these hormones is subjected to an auto-limitation process and the hormone itself inhibits further secretion by the pituitary. Nevertheless, when the plasma concentration of the target gland hormone falls below under critical level, the negative feedback is relieved, and the trophic hormone is again secreted until the target gland produces enough hormone to shut off the pituitary. However, there are particular conditions during which this mechanism may be a *positive feedback*, leading to a continue releasing of the specific substance. Assumed that each of the pituitary hormones exert an important role in the regulation of several pathways involved in the maintenance of the homeostasis of our body, I want to focus only on the role of those hormones, whose activity is directly correlated with the blood volume, ions absorption or excretion and blood pressure regulation. Among all the hormones aforementioned, ADH, ACTH, TSH, have been demonstrated to work in synergism with nervous system to maintain blood pressure within a physiologic range. Currently it is well know the functional mechanism of TSH, released from the adenohypophysis under the stimulation of specific releasing factor, to stimulate the thyroid gland to produce Thyroxin (T4) and Triiodothyronine (T3) which stimulates the metabolism of almost every tissue in the body. Furthermore, thyroid gland is also involved in the secretion of a polypeptide hormone, *calcitonin*, also named *thyrocalcitonin*, whose role seems to be important for the Ca^{2+} regulation. Its activity is not completely clear but to date we can say that this hormone is involved in the reduction of Ca^{2+} circulating at a three different levels:

- inhibition of Ca^{2+} absorption by the intestine
- inhibition of osteoclast activity in bones
- inhibition of renal tubular reabsorption of Ca2+ allowing it to be excreted in the urine

Furthermore, calcitonin is also involved in the inhibition of phosphates reabsorption by the kidney. The role of this hormone is very important considering the key function of Ca^{2+} ions in the cellular responses.

Similarly, the adrenocorticotropic hormone, ACTH, is secreted by the anterior lobe of the hypophysis in response to the CRH released by hypothalamus and exerts a key role not only in the metabolism but also in the blood pressure regulation. ACTH secretion is increased by reduced level of circulating cortisol and under conditions of stress such as fever, acute

hypoglycaemia, major surgery or by noxious stimuli; furthermore, its release resulted to be affected by the diurnal rhythm, increasing during the first few hours of sleep and reaching the peak at the time of awakening while it decrease during the evening. ACTH hormone, once released, by the systemic circulation reach the adrenal gland, that is the major target, where activates the first enzyme involved in the first step of synthesis of glucocorticoids and mineralocorticoid hormones (see chapter 2). Aldosterone is one of these, whose hypertensive role was completely clarified and will be deeper discussed in the next chapter. Furthermore, as an indirect consequence of its actions, ACTH, also maintains the size and blood flow of the adrenal cortex. Indeed, when present in excessive amounts, it causes enlargement of the two zones of the adrenal cortex, *fasciculate and reticularis*, while in its absence adrenal cortical atrophy occurs. Noteworthy is the aspect that ACTH has a lot of extracortical activities, modulating numerous organs and tissues.

Antidiuretic hormone, ADH, or vasopressin is clearly involved in the urine production and especially in the regulation of the urine osmolarity and consequently is indirectly responsible of blood volemia and blood pressure regulation. As widely explained in the last paragraph, one of the essential functions of the kidney is control of the total body water content so that the total solute concentration of the body fluids in normal humans is maintained within a very narrow limits despite wide variations in the in fluid intake and extrarenal water losses. Thus, urine volume and osmolarity directly reflect changes in the same parameters of blood flow. The osmolarity of human urine can vary widely between 85 and 1400 mOsm/kg of water. These changes in osmolarity are realized mostly in the last part of distal tube and along the collecting duct where ADH exerts its activity. I have already explained the urine flow over the nephron; briefly here I want just to remember how the urine changes in volume and concentration. In the distal tube almost two third of the filtered liquid is reabsorbed in the proximal tube, and the liquid has the same concentration of the plasma of almost 285mOsm/kg. Along the descending trait of the Henle's loop, only water is reabsorbed into the interstitial space, with a consequent increase of filtered liquid osmolarity that riches 1200mOsm; at this point the fluid start to flow up along the ascending trait of Henle's loop, where Na+ and Cl- ions are reabsorbed and the fluid's osmolarity get off again to 285mOsm. In the last two traits, distal tube and collecting duct, urine osmolarity will be changed by ADH hormone, according to the body's needs, producing hypo or hyperosmotic urine. Plasma ADH levels, under normal condition, range approximately between 1 and 3µU/ml and plasma osmolarity is maintained around 285-290mOsm. When this value is changed as little as 2%, ADH output is significantly increased. ADH secretion is influenced by many neural and

humoral stimuli that excite or inhibit hypothalamic supraoptic nuclei, from which this hormone is released. Blood volume and pressure are constantly monitored by numerous receptors, *osmoreceptor and baroreceptors*, which afferent fibres reach the CNS, which in turn integrates the informations and send appropriate responses to the periphery. The location of the volume receptors and their afferents pathways are not known with certain. It seems to be like that atrial and ventricular stretch receptors, receptors located in the great veins of the chest and probably from the pulmonary circulation, may be involved in the blood pressure and osmolarity control. Furthermore, there are particular type of cells of the supraoptic nuclei of hypothalamus that have been recognized to be involved in this mechanism acting as osmoreceptor. ADH releasing undergoes the negative feedback regulation, similarly to others hormones. Thus, increased concentration of solute in the plasma evokes the release of ADH, which travels in the blood to the kidney, where it increases water reabsorption. The water thus reabsorbed dilutes the solutes in the plasma and thereby shuts off the signal for ADH secretion. When the solute concentration of plasma is too low, as might occur after drinking large volumes of water, ADH secretion is inhibited and free water is excreted until the solute concentration in plasma retains some optimal level that again initiates basal secretion of ADH. When the volume of blood decreases following haemorrhage, dehydration, or sodium depletion, signals originating from receptors that monitor either the central venous pressure or the arterial pressure are transmitted to the CNS and initiate the release of ADH. In addition, renin released from kidney may trigger increased ADH secretion through the intermediate AG-II. Increased water reabsorption reduces loss of volume in the urine, and in the presence of water intake increases plasma volume, thereby shutting off the signal for ADH secretion. The mechanism of action of ADH hormone is not completely understood, even if it seems to work activating its receptors V_2 located over the basolateral membrane of epithelial cells in the last part of distal tube and collecting duct. These receptors are Gs-coupled proteins, whose activation lead to an increased level of cAMP, that stimulates the up-regulation of the gene expressing for the *aquaporin channels* and in the same time triggers their insertion by exocytosis mechanism.[5] Thus ADH makes both tubules more permeable to water, that diffuses freely across the collecting duct epithelium into the medullary interstitium, and the urine equilibrates the interstitial concentration of 1200mOsm. The water reabsorbed from the cortical collecting ducts and distal convolution enters the peritubular capillaries of the cortex and is removed from the kidney. In the absence of ADH the epithelium of the distal convolution and collecting duct has a very low permeability to water, the tubular urine flows through the remainder of the tubule without achieving osmotic equilibrium with the

surrounding interstitial fluids (1200mOsm), and hypoosmotic urine is produced. It has been seen that ADH hormone exerts its hypertensive action activating also V_1 receptors localized over the membrane of smooth muscle cells and leading to an increased vascular resistance.

From these informations results quite clearly that alteration of mechanisms that lead to the ADH release are responsible of pathological conditions. Deficiency of ADH results in a continuous, copious flow of as much as 15L of dilute urine/day. This disease state is known as *diabetes insipidus*. It is accompanied by a profound thirst to compensate for the water loss in the urine. Otherwise, overproduction of ADH produces a disease state that is characterized by abnormal retention of water and dilution of plasma sodium. Renal compensation for the change in volume results in increased sodium loss, which aggravates the hyponatremia.

1.5 Pharmacology of Hypertension

As already said blood pressure regulation is realized by several integrated mechanism that involve heart functionality, vessels resistance modulation and water and ions reabsorption or excretion. Central and peripheral nervous systems, kidneys and endocrine systems work in a coordinated manner in order to keep blood pressure parameters within physiologic values ranging between 80/120 mmHg. Thus, the pharmacological therapies of hypertension can be realized at a different levels targeting one or more of these physiological mechanisms. Drugs currently available act either by decreasing CO or TPR. However, reduction of blood pressure by any of these mechanisms can trigger compensatory reflex responses that may prevent drug-induced decrease in blood pressure.

Over the years it has been well clarified the complexity of hypertension and it has been described as a multifactorial pathology where others diseases such as dyslipidaemia, diabetes, renal dysfunction and endocrine alterations were included. Sometimes, antihypertensive therapies need an integrated treatment in order to reduce pathological risks and prevent cardiovascular complications too. Thus, the choice of therapy for a patient with hypertension depends on a variety of factors such as age, gender, race, body build, and lifestyle of patient; etiology of disease; other coexisting diseases; presence or absence of risk factors (smoking, alcohol consumption, obesity, etc...).

In some cases patients show borderline blood pressure values for which a no pharmacological approach can be sufficient to restore them. Low sodium chloride diet, weight reduction, cessation of smoking, decrease in alcohol consumption, sports, etc... may be useful

to achieve this goal. Unfortunately, the most part of hypertensive patients need a pharmacological intervention because of their higher blood pressure values.

Antihypertensive drugs can be divided into seven classes, based on mechanism of action; ACE inhibitors, α_1 antagonists, β-blockers, angiotensin receptor-blockers, Ca2+-antagonists, central adrenergic inhibitor, diuretics, direct vasodilators and are summarized in the table n.2.

Drug	Advantages	Disadvantages	CO	HR	TPR
Angiotensin-Converting Enzyme (ACE) Inhibitors ***Captopril, Zofenopril, Benazepril, Enalapril, Lisinopril, Ramipril, Fosinopril***	Renoprotective in type 1 diabetics Profoundly antiproteinuric Decreased morbidity and mortality and symptomatic improvement in CHF patients Decreased ventricular remodeling and mortality post-MI Leftward shift of pressure-natriuresis curve Safely combined with other anti-hypertensive, especially diuretics or calcium antagonists	Cough (~9%) Rare angioedema (~3/1000) Rare hypokalemia Higher dose requirements in some African-Americans BP lowering effect very sensitive to level of dietary sodium intake	Reduced cardiac output by reducing blood volume	These drugs have not effects on the heart rate	Reduced vascular resistance
Alpha$_1$ Antagonists ***Non-selective:*** ***Phenoxybenzaminep hentolamine, tolazoline, ergot-derivates;*** ***Selective:*** ***prazosine, doxazosine, alfuzosine, terazosine, indoramine, uropidil***	Positive effect on all lipoprotein fractions Improved insulin sensitivity Unchanged to improved sexual function in men Blunts thiazide-induced rise in cholesterol Improves maximal urine flow in men with BPH	Orthostatic hypotension (particularly in the elderly, in combination with other vasodilators, in the setting of autonomic dysfunction, and in volume depleted patients) Slightly higher doses needed in African-Americans	Reduced cardiac output by reducing cardiac activity and lowering preload	Not effect	Marked reduction of vascular resistance

Angiotensin II (AT_1) Receptor Antagonists or Sartan: ***Losartan,*** ***Telmisartan,*** ***Valsartan,*** ***Irbesartan***	Lesser peak BP lowering compared to ACEs Nearly complete blockade of A-II effect No effect on bradykinin, enkephalins, or substance P Uricosuric (losartan) Profoundly antiproteinuric Metabolically neutral Rare angioedema No cough	Relatively flat BP dose-response curve No effect on bradykinin Limited data available in African-Americans Absence of hard clinical end-point (i.e., CHF, MI, renal) data Rare hyperkalemia	Reduced cardiac output, by inhibiting sympathetic outflow	Not effect	Reduced vascular resistance by inhibiting vascular AT_2 receptors
β_1-Blockers non-selective: ***Propranolol,*** ***Pindolol,*** ***Bucindolol,*** ***Sotalol*** ***Timolol*** **Selective:** ***Atenolol*** ***Bisoprolol,*** ***Acetobutolol,*** ***Nebivolol*** ***Esmolol*** ***Betaxolol***	Differential cardiac and hemodynamic effects Proven to lower morbidity and mortality post-MI Selected agents useful in patients with migraine or angina	Can worsen CHF; however, at low doses may improve CHF Low cardiac output symptoms Can worsen or precipitate depression Can aggravate PVD symptoms Lowers HDL and raises TGs Delays recovery from and masks symptoms of hypoglycemia Abrupt discontinuation can lead to rebound hypertension	Reduced cardiac output by, reducing volemia, inhibiting central sympathetic outflow	Inotropic, chronotropic and dromotropic negative effects	Increased vascular resistance, mediated by inhibition of β_2 receptors

β2-agonist short-cting: ***Salbutamol*** ***Terbutaline*** ***Metaproterenol*** ***Ritodrine*** ***Isoprenaline*** ***Long-acting:*** ***Salmeterol*** ***Clenbuterol***		Loss of K^+, alteration of cardiovascular electrical conductivity, sympathetic rebound	Not effects	Not effect	Reduced vascular resistance
Calcium Antagonists: ***Nifedipine,*** ***Nicardipine,*** ***Isradipine,*** ***Verapamil,*** ***Diltiazem***	Unqualified efficacy profile (African-Americans, diabetics, elderly) Heterogeneous electrophysiologic, inotropic, hemodynamic and SNS effects Useful in diastolic dysfunction Metabolically neutral Minimal erectile dysfunction in men BP lowering effect is robust in setting of high dietary sodium intake BP lowering effect not attenuated by NSAIDs	Immediately post-MI or during unstable angina (short-acting dihydropyridines) may increase CHD risk Rate-limiting CCBs can worsen CHF and increase mortality in patients with systolic heart failure Side effect profile varies (constipation-verapamil, pedal edema, and vasodilatory symptoms-dihydropyridines) Combination with beta blockers can result in profound bradycardia and depression of myocardial contractility	Small effect	Small effect	Markedreduction of vascular resistance
Central Adrenergic Inhibitors ***α_2-agonist:*** ***Methildopa,*** ***Clonidine,*** ***Guanabenz***	Relatively cheap Useful in hypertensive urgencies (clonidine) Methyldopa is drug of choice for pregnant women	Sedation Depression Orthostatic hypotension Decreased heart rate (clonidine) Rebound hypertension.	Reduction of CO by inhibiting the sympathetic outflow	Reduction of heart activity by inhibiting the sympathetic outflow	Decrease of vascular resistance by inhibiting the sympathetic outflow

Direct Vasodilators NO-releasing: Nitroglycerin and nitrates, hydralazine, sodium nitroprusside, minoxidil (also activator of K+-channel)	Relatively cheap Effective in severe hypertension (minoxidil > hydralazine)	Best suited for adjunctive therapy Salt and water retention necessitate use of loop diuretics Reflex tachycardia necessitates use of rate-limiting CCB or beta blocker Lupus-like syndrome (>200 mg/d of hydralazine) Edema (minoxidil)	Not effects	Not effects	Marked reduction of vascular resistance
Diuretics ***Osmotic diuretics: Mannitol, Glycerol, Isosorbide.*** ***Carbonic anhydrase inhibitors: Acetazolamide, Methazolamide, Diclorophenamide*** ***Thiazide diuretics: Clorothiazide, Indapamide, Quinethazone, Hydrochlorthiazide, Chlorthalidone*** ***Loop diuretics: Furosemide, Bumetanide, Ethacrynic acid*** ***Potassium-sparing diuretics: Spironolactone, Canrenone, Triamterene, Amiloride***	Cheap acquisition cost Highly effective and lowers BP similarly in all demographic groups Proven to lower BP-related morbidity and mortality Enhances the BP lowering effect of all other BP drugs Thiazides and metolazone have relatively long half lives	Thiazides ineffective when GFR <40 mL/min Dose-related hypokalemia Raises cholesterol and triglycerides Glucose intolerance especially in setting of K+depletion Increased uric acid Erectile dysfunction in men	Reduction of CO	Increase of heart rate	Reduction of vascular resistance

Table n.2 *Most important antihypertensive drugs used in therapy, CO (cardiac output), HR (heart rate), TPR (total peripheral resistance)*

2.0 Adrenal gland and hormones synthesis

2.1 Physiology of the adrenal gland

The adrenal glands are small, paired structures lying on either side of the midline of the abdominal cavity above the kidneys. Each gland consists of two major components: the outer cortical area and the inner medulla. The mature adrenal cortex consists of three morphologically distinct zones. These zones differ in enzymatic content, histologic and ultra-structural morphology,[6,7] mitotic rates, lipid distribution, and functional activity. The outer layer is the *zona glomerulosa*, the middle layer is the *fasciculate* and the inner layer is the *zona reticularis*. The adrenal gland may be considered as a double endocrine glands, because of different nature of released hormones. The medulla area secretes the water-soluble neurotransmitter, adrenaline, and receives innervation by preganglionic, cholinergic, sympathetic nerve fibres. The medullary cells, called chromaffin cells, are considered neuroendocrine cells because the two neurotransmitters adrenaline (80%) and noradrenaline (20%) are secreted directly into the systemic circulation, like other hormones, rather than act in the synaptic space and activate the post-synaptic adrenergic receptors. The adrenal cortex secretes substances moderately soluble in lipids of a chemical class known as steroids and is not controlled by secremotor nerves. Specifically, the *zona fasciculate* is responsible of glucorticoid hormones secretion, cortisol and corticostirone; *the zona glomerulosa* releases the most important mineralcorticoid hormone, **aldosterone**; and the *reticularis* leads to secretion of androgenic and estrogenic important precursor, androstenedione and dehydroepiandrosterone sulphate.[8] Although, these hormones show some differences in structure and activity, they originate from the same substance, cholesterol. Cholesterol is stored in the adrenal cortex in both free and esterified form. Furthermore, cholesterol may be synthesized by the adrenal cortex from acetate and it can also be acquired from blood, where it appears from the dietary intake, or from the liver, in which cholesterol may be synthesized *de novo*.

Synthesis and secretion of adrenal cortex hormones are stimulated by an important hormone, ACTH and the stimulation persist as long as it is present. As it was well explained in the previous chapter, ACTH is released by special neurons of the medial basal

hypothalamus that act as neuroendocrine transducers and signal integrators. These neurons receive and integrate neural inputs from other regions of the CNS and chemical inputs from blood and cerebrospinal fluid. The output of these neurons is the corticotropin-releasing hormone (CRH), which is carried from the hypothalamus, via the infundibular stalk, to the corticropic cells of adenohypophysis. After stimulation by CRH, corticotropic cells release ACTH, which then enters the general circulation and finally stimulates corticosteroidogenesis and increased glucocorticoid secretion at the adrenal cortex. It has been well established that ACTH exerts not only secretory effects but also trophic effects on the adrenocortical tissue; it is the major, but not the only, physiologic signal that determines the rates of glucocorticoid synthesis and release as well as trophic and secretory effects, including compensatory adrenal hypertrophy.[9,10] However ACTH remains the most important stimulus in the glucocorticoid hormones secretion. The synthesis of adrenal hormones is realized by members of the CYP450 superfamily (steroid hydroxylase) and by steroid dehydrogenases. In the adrenal cortex of human five distinct steroids hydroxylases take place in the steroidogenic pathways leading to the production of the cortisol, corticostirone, androstenedione and dehydroepiandrosterone and aldosterone. Even though it was determined that the increase in the steroid hormones biosynthesis in response to ACTH/cAMP involves the delayed increase in steroidogenic gene transcription, it was also realized that ACTH/cAMP in the adrenal cortex contains two distinct components. Upon ACTH release from the anterior pituitary gland, the peptide hormone binds to its specific cell surface receptors on the adrenocortical cells. This interaction activates the adenylyl cyclase leading to increased cAMP concentration in the cytoplasm resulting in the activation of cAMP-dipendent protein kinase (PKA). Once PKA is activated there are two responses, acute and chronic, that occur to increase steroid hormones biosynthesis. During the acute response, an essential site of phosphorylation by PKA is cholesterol ester hydroxylase (CEH), which upon activation catalyses the conversion of cholesterol ester to free cholesterol.[11] The acute response leads to mobilization of cholesterol, the initial substrate for all steroidogenic pathways, from cellular stores to the inner mitochondrial membrane where CYP45011A1 resides. This enzyme is in common between the three adrenal cortex areas, *glomerulosa*, *reticularis and fasciculate*. CYP45011A1 is the first enzyme of this pathway and converts cholesterol in pregnenolone, which in turn is converted in glucorticoid or mineralcorticoid hormones depending on the enzymes and cortical area. Glucocorticoid are lipid-soluble substances, thus, they reach the target organs and tissues flowing in the blood bound to plasmatic protein; albumin and globulin are the two main proteins that exert this role. Because of their lipophilic

characteristics glucorticoid hormones are able to cross the phospholipid double-layer of the cell membrane and enter into the cytoplasm where are localized their receptors. Upon interaction the resulting ligand-receptor complex leaves the cytoplasm and reaches the nucleus where modulates gene expression. These receptors have been seen in a large number of cells over the organs and tissues, leaving to imagine a widespread effects for these hormones:

- inhibition of the inflammatory response
- **maintenance of normal arterial systemic blood pressure and volume**
- effects on metabolism including maintenance of blood sugar levels during starvation
- support resistance to many noxious stimuli

Amongst these effects, maintenance of blood pressure is of our interest and will be discussed below. Glucocorticoids are not usually thought to have any important effects of their own, except possibly in the kidney, where they increase the glomerular filtration rate by an unknown mechanism, but they are required for the vasoconstrictor action of norepinephrine. In the absence of glucorticoids the vasopressor action of catecholamine hormones is diminished or lost and blood pressure falls. It has been sought to explain this particular role of glucorticoids, analysing its inhibitory effects on the catecholamine-O-methyl-transferase (COMT) enzyme that inactivates catecholamine hormones and transmitters. If this enzyme is not inhibited by corticosteroids, catecholamines are metabolized so rapidly that they cannot reach physiological level to exert vasoconstriction.[12]

It is well known the major role in the blood pressure regulation of the mineralocorticoid hormone, aldosterone, secreted by the outer section, zona glomerulosa, of the adrenal cortex.

Aldosterone is defined a mineralocorticoid hormone, because it is involved in some pathways that have ions such as Na^+, K^+, and water as the major protagonists. This hormone controls salts balance and blood volume by binding to the mineralocorticoid receptors and activating genes involved in the Na^+ reabsorption as well as K^+ release from the kidney. The mineralocorticoid receptors are widely expressed in several organs and tissues, such as kidney, heart, brain, vascular smooth, and other tissues.[13,14] Activation of the mineralocorticoid receptor by aldosterone in these tissues can trigger responses such as increased vascular resistance, increased volemia, increased cardiac output. Thus, all these effects lead to an hypertensive condition. Primary hypertension results from an overproduction of aldosterone that is independent of excess of renin-angiotensin stimulation and affects a significant portion

of patients.[15] Furthermore, prolonged exposure to high level of aldosterone can result in an inflammatory responses, followed by damage to vascular and myocardial tissues and hypertensive effects. Thus, overproduction of aldosterone lead to deterioration of target organs.[16] For all these reasons, aldosterone production is highly regulated by nervous, endocrine, physical integrated stimuli.

2.2 Aldosterone synthesis mechanism

Aldosterone synthesis takes place starting from the conversion of cholesterol to pregnenolone by CYP450 side-chain cleavage enzyme; pregnenolone by an isomerase enzyme is converted in progesterone, which in turn is converted in 11-deoxycorticostirone and corticostirone before to convert the latter in aldosterone. Aldosterone released into blood binds to plasma albumin and globulin. In this respect, its behaviour is similar to that of the glucorticoids, although the binding affinities are not as great for aldosterone as they are for glucorticoids. Under physiological conditions, approximately the half plasma aldosterone is bound to these proteins, whereas the other half is free. Just as in the case of the glucorticoids, there is no appreciable storage of aldosterone within the adrenal cortex, and the rate of synthesis is essentially equal to the rate of release, therefore its regulation involves signals that alter the rate of synthesis.

Many hormonal and paracrine factors are involved in the regulation of aldosterone production; low and high concentration of circulating Na^+ and K^+ ions respectively, ACTH-induced AG-II releasing, whereas it is inhibited acutely by circulating atrial natriuretic peptide and by locally produced dopamine and somatostatin.[17] Considerable evidence supports the role of two key regulatory steps in the control of adrenal cell aldosterone synthesis. The first has been termed the early rate-limiting step. AG-II and K^+ control this step through regulation of both protein expression and activities of the steroidogenic acute regulatory protein (StAR), which facilitates cholesterol transport from the outer to inner mitochondrial membrane where conversion to pregnenolone occurs.[18,19] The second limiting reaction has been termed the late rate-limiting step, which represents the conversion of deoxycorticosterone to aldosterone by the mitochondrial enzyme aldosterone synthase, CYP11B2.[20,21] CYP11B2 is expressed almost solely within the zona glomerulosa of the adrenal cortex. The main action of ACTH on glomerulosa cells is connected with the activation of transmembrane Gsα-coupled ACTH receptors and the generation of cAMP as a second messenger.[22,23] Until the recent discovery

of a new family of cAMP binding protein, exchange protein directly activated by cAMP, that can directly activate small GTPase, RAP1 and RAP2, cAMP signalling was mainly linked to the activation of cAMP-dependent protein kinase and subsequently phosphorylation of target protein.[24] Hydrolysis of cholesterol and activation of StAR, which are both critical steps in steroidogenesis, are regulated by PKA phosphorylation. Phosphorylation of CEH and StAR markedly decreases cholesterol ester levels and increase CEH activity. Although ACTH in zona glomerulosa cells acts primarily through cAMP, it has also been seen a stimulation of Ca^{2+} influx by activation of L-type Ca^{2+} channel.[25] Increased Ca^{2+} concentration lead to an activation of Ca^{2+}/calmoduline-dependent protein kinase.

Similarly to the ACTH, also both agonists AG-II and K^+ increase intracellular calcium levels and activate calmoduline and calmoduline kinase which leads to increased CYP11B2 transcription.[26,27] Adrenal glomerulosa cell stimulation occurs predominantly through AG-II; acute activity of AG-II is realized through activation of AG-II type 1 receptor (AT1R) via a q-subunit of G-protein coupled receptors (Gq), whose activation increases the cAMP levels, which in turn activates a phospholipase C enzyme.[28] This enzyme stimulates the hydrolysis of plasma membrane phosphoinositide inositol-1,4-bisphosphate with production of inositol-1,4,5-trisphosphate (IP_3) and DAG. IP_3 increases cytoplasmatic Ca^{2+} concentration, activating calcium/calmoduline-dependent kinase, (CaMK)[29] which, as already said, increases the gene expression of CYP11B2 and the aldosterone production.[30,31,32] Chronically, AG-II acts to alter the expression of various genes,[33] including CYP11A1, CYP11B2 and StAR protein. [34]

K^+ ions concentration with AG-II represents the major signal for the aldosterone secretion. It has been well clarified over the years that alteration of circulating K^+ ions concentration has a great influence on the zona glomerulosa cells, because of depolarizing shift in the K^+ equilibrium potential, that leads to a K^+-induced membrane depolarization. Depolarization of the resting potential membrane of glomerulosa cells is the major stimulus for the voltage-dependent Ca^{2+} channels opening, which together with the Ca^{2+} concentration gradient gives the driving force of Ca^{2+} transport inside the cell. Similarly for the other cases, the increase of Ca^{2+} concentration into the cytoplasm triggers the CAMK enzyme, whose activity results in the CYP11B2 expression.

Thus, the three most important stimuli for aldosterone secretion, ACTH, AG-II, K^+, and in particular the last two, although acting through different mechanism, trigger steroidogenesis by a process involving the calcium messenger system. It will be better clarified in the next paragraph the enormous importance of the resting membrane potential for aldosterone production.

2.3 Aldosterone activity

Aldosterone regulates blood pressure through its activity on the kidney and cardiovascular system and it is a crucial component of Na^+, K^+ and H^+ homeostasis. Aldosterone regulates the concentration of these important ions through activation of mineralcorticoid receptor (MR), which is widely distributed in epithelial cells of target tissues, such as kidney and colon,[35] smooth muscle, endothelium, cardiomyocytes and hippocampal neurons.[36,37] MR is activated not only by the steroid aldosterone but also by cortisol in cells that do not express the enzyme 11β-hydroxysteroid dehydrogenase type 2.

It has been well clarified that the main activity of aldosterone on the blood pressure regulation is realized by activating this receptor in the aldosterone-sensitive distal nephron, comprising the distal convoluted tubule (DCT) and both principal cells and intercalated cells of the connecting tubule (CNT) and collecting duct (CD).[38] In common with other steroid hormone receptors, MR operates as a ligand-dependent transcription factor, indeed, in the distal nephron the expression of aldosterone-epithelial Na^+ channel (ENaC) is tightly regulated by aldosterone, which promotes the MR-dependent transcription of ENaC. MRs are localized inside the cell into the cytoplasm where the aldosterone easily enters because of its lipophilic nature. The binding of aldosterone stabilizes the interaction of MR with specific DNA sequence elements in the promoter regions of aldosterone-responsive genes to promote or suppress their expression through the recruitment of specific coactivators or corepressor.[39] Aldosterone-bound MR links to the DNA regulatory elements that are also recognized by GR. The transcription effects stimulated by aldosterone through MR are augmented by the rapid activation of signal transduction cascades, which are most often initiated through the interaction of aldosterone with MR. Even though some of these rapid signalling responses have been known for some time, it is only recently that their relevance to the physiological effects of aldosterone has been characterized and their synergism with the transcriptional responses to aldosterone has been established.[40]

The Aldosterone is able to increase the gene expression of ENaC in the apical membrane of the distal nephron cells increasing the Na^+ flow from the tubular lumen to the cytoplasm of these cells raising the cytoplasmatic concentration of Na^+. Thus, ENaC is a key regulator of aldosterone-mediated Na^+ conservation by the kidney. These channels continually cycle between the cell membrane and a sub-apical, intracellular pool.[41] Thus, equilibrium exists between the various processes that contribute to ENaC subunit transcriptional regulation,

subcellular trafficking, and expressed protein stability to achieve a steady state in ENaC surface expression. It has also been seen that aldosterone exerts its activity also increasing the expression and the functionality of the Na^+/K^+ ATPase pump on the baso-lateral membrane supporting the Na^+ reabsorption and K^+ excretion. The activity of Na^+/K^+ ATPase provides a favourable electrochemical driving force for aldosterone-induced apical Na^+ reabsorption through ENaC.[42] This provides a favourable electrochemical gradient for K^+ transported into the cytoplasm to be secreted across the apical membrane into the ultrafiltrate through K^+ channels, or alternatively recycled back into the blood through basolateral K^+ channels. The increased level of Na^+ ions into the systemic blood circulation leads to an increase of osmolarity, which in turn stimulates ADH release and activity which is responsible of water reabsorption from the collecting duct in order to restore the osmolarity within a physiological range. A consequence of this process is a marked increase of volemia and blood pressure. Because aldosterone has an opposite effect on the Na^+ and K^+ excretion, the urinary Na^+/K^+ concentration ratio falls when aldosterone acts. Changes in this parameter are often used as an indication that the hormone's level has changed.

Alterations of aldosterone secretion, low production or high production, have very important pathological implications because of its role over the organs and tissues. On one hand removal of aldosterone by adrenalectomy leads to an overall reduction of Na^+ reabsorption and a powerful and uncontrolled loss of extracellular fluid volume. This marked loss of liquid leads the subject to an hypovolemic shock condition, which is a frequent cause of death in adrenally insufficient animals. On the other hand, hypersecretion of aldosterone leads to a very complicated pathological conditions that often do not respond to the classical antihypertensive therapy.

2.4 Resting potential membrane of zona glomerulosa cell and its role in the aldosterone secretion

The mechanisms that control aldosterone secretion are not fully understood, although the negative membrane potential of adrenal ZG cells seems to be pivotal. Membrane depolarization has long been hypothesized to be an important component of the cellular mechanism mediating AG-II and K^+ stimulation of aldosterone secretion from the glomerulosa cells. This mechanism has been extensively studied in an *in vitro* model constituted by isolated human glomerulosa cells (H295R), rat and bovine glomerulosa cells,

by patch-clamp technique. These studies demonstrated that both elevated extracellular K^+ ions and AG-II depolarize the resting potential membrane (V_m), supporting the hypothesis that Ca^{2+} influx through voltage-gated channel plays a primary role in stimulation of secretion. Several lines of evidence have demonstrated that stimulation of Ca^{2+} influx is necessary to sustain AG-II and K^+ induced secretion,[43,44] and several Ca^{2+} influx pathways have been identified in glomerulosa cells, T and L-types voltage-gated channels, capacitative Ca^{2+} channels,[45] Ca^{2+} permeant nonselective cation channels and Na^+/Ca^{2+} exchange.[46] In this regard, potential membrane (V_m) is a critical determinant of Ca^{2+} influx trough these pathways by virtue of its role in control of channel gating and its contribution to ionic electrochemical gradients. Pharmacological studies have led to the hypothesis that T-channels are the primary Ca^{2+} influx pathways leading to stimulation of aldosterone secretion.[47] However, L-type Ca^{2+} channels doesn't seem to be involved in the Ca^{2+} flow in the same cells.

As already said above, AG-II and K^+ ions trigger the calcium messenger system and raise cytosolic free Ca^{2+} concentration, although through very different mechanisms. It has well demonstrated by several in vitro experiments that AG-II is able to decrease the resting membrane potential of the zona glomerulosa cells and increase the Ca^{2+} concentration in the cytoplasm of these cells. AG-II induces phosphoinositide breakdown through activation of a specific phospholipase C. This leads to the formation of DAG and IP_3, which in turn mobilizes Ca^{2+} from intracellular storage organelles. Increased Ca^{2+} concentration is responsible of membrane depolarization which in turn can start a positive feedback, opening voltage-dependent Ca^{2+} channels on the cell membrane. In the same time, AG-II contributes to the membrane depolarization through inhibition of both leak and voltage-gated K^+ conductance.[48] Pharmacological inhibition of leak K^+ conductance was reported to be sufficient to depolarize glomerulosa cells and induce Ca^{2+}-dependent stimulation of aldosterone secretion.[49] Most probably other channels may also contribute to AG-II-induced depolarization. Indeed, Lotshaw et al. reported that AG-II activates Ca^{2+}-permeant nonselective cation channels, which may contribute to membrane depolarization.

K^+ involvement in the steroidogenesis has been demonstrated too. The ZG cell membrane is selectively permeable to K^+ giving it the characteristics of a K^+ electrode over a wide range of extracellular K^+ concentrations. The K^+ flow from the outer to the inner of glomerulosa cells causes an increase of the resting potential membrane, that leads to a secondary rise in intracellular Ca^{2+} through T-type Ca^{2+} channels, which is thought to be the primary stimulus for increased production and release of aldosterone.[50] Whereas after AG-II stimulation the source of Ca^{2+} is both the intracellular store and the extracellular space, the Ca^{2+} signal

evoked by K^+ flow depends exclusively on the Ca^{2+} influx. A distinctive feature of adrenal zona glomerulosa cells is the high resting K^+ conductance, responsible for the negative membrane potential (-80mV) that closely follow the K^+ equilibrium potential. At a this value of membrane potential two families of K^+ channels may give rise to K^+ conductance: inward-rectifying-potassium channel and two pores domain K^+ channel TASK-1 and TASK-3. TASK1 and TASK3 are encoded by KCNK3 and KCNK9 genes respectively and highly expressed in the adrenal cortex.[51,52] It has been seen in the human adrenal cortical cell line H295R that inhibition of these two-pores K^+ channels with antisense oligonucleotides, decreases the K^+ current and consequently the resting potential membrane, resulting in increased intracellular Ca^{2+} levels, CYP11B2 expression and aldosterone secretion, consistent with their role in regulating aldosterone production. Furthermore, it is noteworthy to emphasize that AG-II, under physiological conditions, binding with its receptors AT_1, is able to close TASK K^+ channels. This closure in turn causes the depolarization of the plasma membrane, the activation of voltage-gated Ca^{2+} channels, and an elevation of cytoplasmatic Ca^{2+} levels that promotes CYP11B2 transcription and aldosterone production. [53,54]

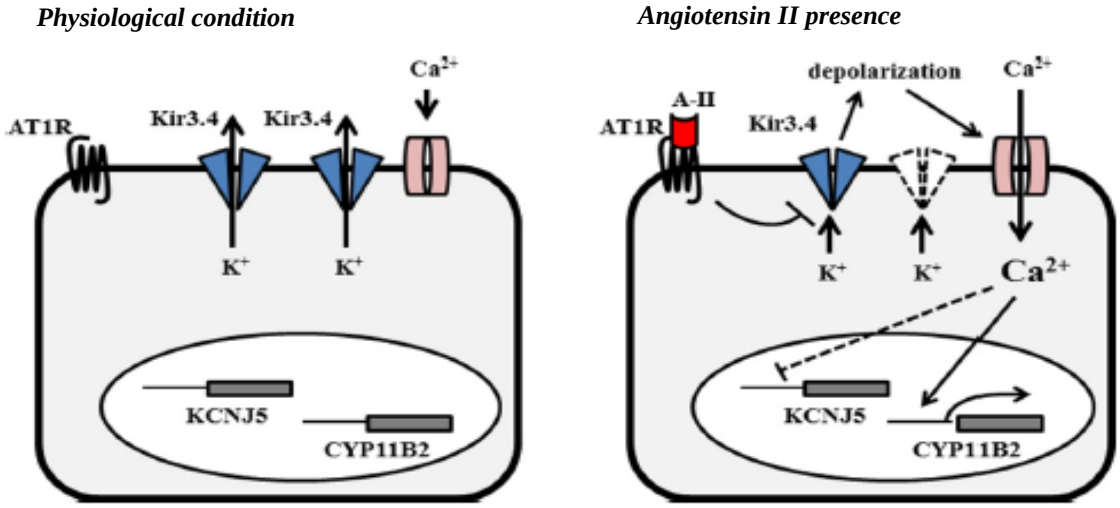

Fig. 3 *Schematic representation of KCNJ5 channel activity across the membrane of H295R cells in a resting condition (figure on the left of the panel) and in a presence of AG-II (figure on the right of the panel). It has been proposed this mechanism: in the absence of AG-II KCNJ5 channels seem to be opened with an outflow of K^+ from the inner to the outer of cells and a consequent membrane hyperpolarization; when AG-II is present activates its G-coupled ATIIR1 receptor and the two subunits α and γβ are released with the second one responsible of KCNJ5 interaction and inhibition. KCNJ5 inhibition leads to a loss of K+ outflow with a consequent resting membrane depolarization*

and a consequent opening of voltage-dependent Ca2+ channels, increased cytoplasmatic Ca^{2+} concentration and CYP11B2 up-regulation.

2.5 KCNJ3/KCNJ5 (Kir3.1, Kir3.4 or GIRK1 and GIRK4) implication on resting potential membrane and aldosterone production

The inward-rectifying potassium channel KCNJ5, also called Kir.3.4 or GIRK4, is a G protein-coupled inwardly rectifying potassium channel, belonging to the seven Kir channel subfamilies denoted as Kir1 to Kir7. Kir3.x is gated by ligand-stimulated G protein-coupled receptors and activated by a large number of neurotransmitters, including acetylcholine, adenosine, ATP, dopamine, serotonin and somatostatin. The Kir3 gene family has four members including Kir3.1 to Kir3.4, with a widespread distribution at the plasma membrane in various different tissues, including heart,[55] central and peripheral neurons,[56] various endocrine tissues, as well as no excitable cells, such as platelets.[57] These genes are highly conserved between species with Kir3.1 and Kir3.3 subunits having 98 and 99% of homology and Kir3.4 with 94% of sequence identity (Fig. 4).

Fig. 4 *KCNJ5 gene homology over several species, from ; both Kir3.1 and Kir3.4 show an amino acid sequence equality ranging from 94 to 99%, demonstrating their importance in the cell functionality and viability.*

The ATP-dependent Kir channel and the classical Kir channel were isolated for the first time in 1993 from the outer medulla of rat kidney and a mouse macrophage cell line. These channels were structurally characterized and it has been seen that their primary structure is constituted by two putative membrane-spanning domains, TM1 and TM2, linked by an extracellular pore-forming region (H5) and cytoplasmatic amino (-NH2) and carboxyl (-COOH) terminals domains.[58] This structure seems to be common to all types of Kir channels (Fig. 5)

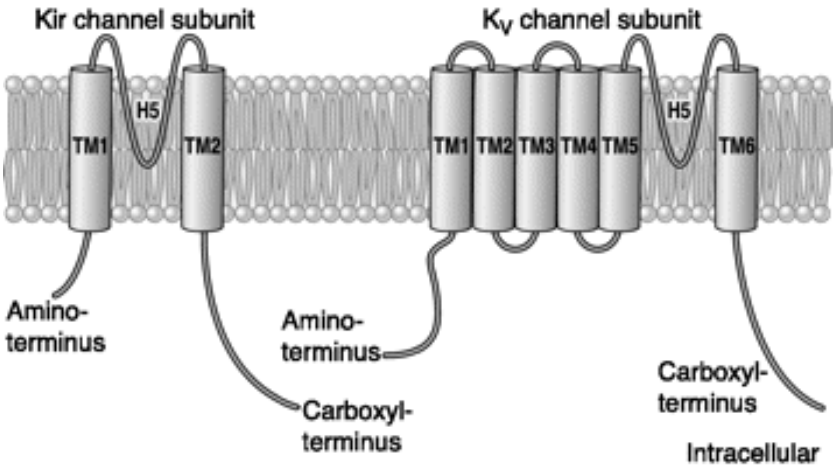

Fig. 5 *Primary structure of Kir channel; it shows two transmembrane domains TM1 and TM2 linked by a pore-forming H5 loop and cytosolic amino and carboxyl terminals (left image). On the right it is showed the structure of voltage-gated potassium channel which is constituted by six transmembrane domains, two of which, TM5 and TM6 are linked by a H5 loop, similarly to the previous one.*

The H5 region it is likely to be the most important structure of the Kir channel, because it seems to act as a "ion-selectivity filter" and shares with other K^+ -selective ions channels the signature sequence Gly-Tyr-Gly, highly conserved over the species. This filter of selectivity allows the only K^+ flow across the membrane in or out the cytoplasm.[59]. Another important characteristic of the Kir channel is the absence of the S4 region, conserved in voltage-gated Na^+, Ca^{2+} and K^+ channels. As a result, Kir channels are insensitive to membrane voltage and, when activating stimuli are absent, this channel would be opened to the membrane potential (E_m). The architecture of the transmembrane domain of Kir channels has been elucidated only after the crystal structure resolution. Protein structure of these four Kir channels show a 36% sequence identity, that reaches the 80-90% in the transmembrane and pore regions, demonstrating their importance in the channel activity. The Kir3.1 is the most abundant subunit and in the same time is also the most divergent of the four since it shares only 44% sequence identity with the other three channels. Kir3.2, Kir3.3 and Kir3.4 are more closely related and share 62% of amino acid structure.[60,61] It is well known that the primary structure of Kir channel made up by two transmembrane domains is insufficient to form a complete and functional channel, whereas it becomes active in a tetrameric structure. Heterodimerization generally occurs between members of the same family as for example Kir3.4 forms heteromeric complex with Kir3.1. It has been seen that the tetrameric structure is organized with the two TM2 domains opposite one another in order to form the channel pore, while the others two TM1 are localized to the side of each TM2. The ion conduction pore can be

divided in three distinct zones that consist of the selectivity filter, the water-filled central cavity and the internal face of the pore made up of the internal bases of the four inner (TM2) helices. Analysis conducted to explain the mechanism underlying the activation of several potassium channels has provided clear evidence for mobility of the TM2 helices contributing to Kir channel gating.[62]

Inwardly rectifying potassium channels (Kir) transport potassium ions into and out of cells, and play a key role in a cell's ability to generate and transmit electrical signals. In general, Kir channels tend to hyperpolarize the membrane potential in excitable cells such as neurons and cardiac myocytes, whereas they carry outward current in no excitable cells such as those in the anterior pituitary gland.[63] The most important biological fingerprint of Kir3.x channel is their characteristic inward rectification which depends critically on positively charged polyamines that reside into the cytoplasmic side of the cell.[64,65] This characteristic is totally different from the voltage-gated K^+ channel current; instead, their relationships with membrane voltage don't follow Hodgkin-Huxley kinetics but their behaviour seems to be depended more on the electrochemical gradient for K^+ (membrane potential (E_m) minus equilibrium potential of K^+ (E_K)). Therefore, under physiological conditions, Kir channels show a large K^+ conductance flow at potential negative to E_k but permit less current flow at a depolarized potential. This limited potassium flow at a depolarized potential is due to the blockade by intracellular magnesium (Mg^{2+}) and polyamines. Thus, the inward rectification turns out not to be an intrinsic function of the channel protein but a result of the block of outward K^+ flux by Mg^{2+} and polyamines. Cells that show a large Kir conductance are expected to show the resting membrane potential (E_{res}) close to E_k and no spontaneous electrical activity. This and their essential voltage independence, permits Kir channels to play a key roles in the maintenance of E_{res} and in regulation of the action potential duration in electrically excitable cells.[66]

The G protein-coupled receptor rectifying potassium channel KCNJ5 is, similarly to other members of the KCNJ family, a ligand activated inward rectifier, although the ligand for a KCNJ5 channel does not bind to the ion channel itself. Indeed, activation of $G_{i/o}$ protein-coupled receptor (GPCR) lead to releasing of two G-protein subunits, G_α and $G_{\gamma\beta}$, which in turn binds to and activates the KCNJ-type channel itself, whereas in the absence of the ligand the Kir channels are closed. Upon interaction of $G_{\gamma\beta}$ subunit with the internal face of the Kir channel there are great conformation changes in the channel structure allowing its closing and a consequently blockade of K^+ ions outflow.[67] It has well established that KCNJ5 potassium channel is associated with several GPCR over different tissues and organs of our body. While

its activity in mediating the modulation of acetylcholine (Ach) in the heart and dopamine in the pituitary gland is almost completely understood the same cannot be said for KCNJ5's role in adrenal gland. It has been seen that the inhibitory effect of Ach on the rate, frequency, and strength of contractility of the heart is negatively modulated by M_2 receptor and furthermore by muscarinic-gated potassium channel I_{KAch}. Ach interacts with its muscarinic receptor M_2, G protein-coupled receptor, whose activation release the two subunits G_α and $G_{\gamma\beta}$. The latter subunit activates in turn the I_{KAch} channel contributing to the negative inotropic and chronotropic effects of Ach.[68] Cardiac I_{KAch} is comprised of two related protein GIRK1 and GIRK4, whose presence is necessary to form a functional I_{KAch}.[69] It has been seen that activation of I_{KAch} in KCNJ5 knock out (KO) mice demonstrated the pivotal role that this channel has in heart rate regulation. These mice exhibited resting tachycardia, incapacity to respond to parasympathetic stimuli and a long ventricular effective refractory period compared with wild type (WT) animals. Moreover, in KCNJ5 KO mice, I_{KAch} was shown to play a central part in the generation of atrial fibrillation.

It has well established by several experimental activities, such as *in situ* hybridization, semi quantitative PCR, molecular cloning, immunocytochemistry and coimmunoprecipitation, an abundant presence of inward-rectifying-potassium channels GIRK1 and GIRK4 in the pituitary gland. Indeed, it has been seen that dopaminergic D_2 receptors are coupled with inward rectifying potassium channel (IRK), whose activation seems to mediate dopamine's activity in the prolactin (PRL) release. Dopamine is a physiological regulator of PRL secretion from the pituitary gland, exerting a tonic inhibitory control.[70] However, the dissociation of DA from its D_2 receptors seems to be another important physiological stimulus for the PRL secretion.[71] In addition, subnanomolar concentrations of DA have been reported to directly induce a PRL release.[72,73] Molecular characterization of the G protein-gated IRKs in pituitary tissue is a necessary step in elucidating the physiological role of the DA-activated K^+ channels in the regulation of PRL secretion. Firstly, DA is able to inhibit PRL secretion by directly activating pituitary dopaminergic D_2 receptor, that is coupled to a Gi protein, whose activation releases the two G protein subunits, G_α and $G_{\gamma\beta}$.

From in vivo studies it has been demonstrated that a DA-activated K^+ conductance plays a critical role in the PRL secretion and it has been seen that this K^+ conductance is voltage and calcium concentration independent, but can be activate at a normal resting potential membrane. K^+ conductance can also be positively modulated by $G_{\gamma\beta}$ complex, which interacts with the cytoplasmatic chain of IRK channel and induces a IRK opening and K^+ flow from the inner to the outer of the cells with a consequent hyperpolarization of the resting potential

membrane. Furthermore, it has been seen that in some systems KCN-family channels can also be activated by dopaminergic D4 receptors. As it has been anticipated below, the role of KCNJ5 in the glomerulosa cell is not completely understood; it has been hypothesized these potassium channels can act as a dopamine-activated GIRK because of the presence of high quantity of DA in the adrenal gland area. To date, not too much is known about the physiological role of inward-rectifying potassium channels in the adrenal gland, even if a role in maintaining a hyperpolarized potential membrane may be hypothesized.

Jin et al. demonstrated that there are several amino acid residues over the TM2 domains of KCNJ5 that exert a crucial role in the channel activation and in the same time they demonstrated that substitutions of these residues would change TM2 helix flexibility and form constitutively active channel. Similarly, mutations in the carboxyl and amino-terminals lead to a loss of capacity of $G_{\gamma\beta}$ to interact and activate this channel. Several studies conducted over the last decade on KCNJ5 potassium channel demonstrated a strong association of this mutated channel with several cases of hypertension. Indeed, was reported that mutations in or around the selectivity filter of KCNJ5 lead to a marked loss of K^+ ions selectivity in the glomerulosa adrenal cells with a consequent inflow of Na^+ ions from the outside to inside of the cell and an increase of resting membrane potential. Membrane depolarization opens voltage-gated Ca^{2+} channels leading to a marked increase of Ca^{2+} ions into the cells, which in turn activates the Ca^{2+} dependent pathways, one of which leads to a great production of aldosterone. In this circumstance the synthesis of aldosterone doesn't undergo the normal physiological control mechanisms but it is only regulated by membrane depolarization and increased cytoplasmatic calcium levels. A such condition is fully elucidated in the most common secondary form of hypertension, primary aldosteronism.

2.6 Hyperaldosteronism

Primary aldosteronism is the most common cause of secondary hypertension, with a prevalence of 5-15% depending on the severity of blood pressure levels. The most important feature of primary aldosteronism is the hypersecretion of the mineralocorticoid hormone aldosterone in a manner that is fairly autonomous of the renin-angiotensin system. The hypokalaemia that was once considered a prerequisite for primary aldosteronism suspicion is in fact only present in a minority of affected patients.[74]

Primary aldosteronism is a heterogeneous group of disorders that may be divided in two main groups:

- Familial primary aldosteronism
- Sporadic primary aldosteronism

The two most common subtypes of sporadic forms are **bilateral adrenal hyperplasia (BAH)** and **Aldosterone-Producing Adenoma (APA)**, which together accounts for over 90% of all cases of primary aldosteronism, which in turn show a 60% and 30% of frequency respectively. Although the majority of primary aldosteronism is sporadic, there are monogenic familial forms of the condition, familial form type-I (FH-I), type-II (FH-II) and type-III (FH-III) that account for almost 10% of all cases. The diagnosis of primary aldosteronism and subsequent subtype identification is imperative because it enables the use of a targeted therapy, either the surgical removal of APAs or the pharmacological treatment of BAH. Furthermore, the relevance of primary aldosteronism diagnosis is consolidated by observations that patients with primary aldosteronism are prone to cardiovascular and cerebrovascular complications and to metabolic syndrome in comparison with patients with essential hypertension that have similar cardiovascular risk profiles.[75,76] Several guidelines and algorithms for the diagnosis and treatment of primary aldosteronism have been developed and proposed over the last 4 years.[77] The diagnostic steps include:

- **case detection testing**
- **confirmatory testing**
- **subtype classification**
- **localization**

Patients with a positive detection test are recommended to undergo confirmatory testing to either confirm or exclude the diagnosis of primary aldosteronism. The Endocrine Society's clinical practice guidelines recommend the use of any of four confirmatory testing procedures, *oral sodium loading test*, *saline infusion test (SIT)*, *fludrocortisone suppression test*, and *captopril challenge test (CCT)*. Finally, subtype differentiation should be undertaken to establish the site of aldosterone hyperproduction in the adrenal glands. Subtype differentiation is performed by adrenal computed tomography scanning (CT) and adrenal venous sampling, which is the only reliable method to distinguish unilateral from bilateral primary

aldosteronism in the differentiation of primary aldosteronism. It is the method of choice because enables identification of the source of increased secretion of aldosterone. This is an important aspect because the detection of adrenocortical adenoma secreting the excess of aldosterone is an indication for surgical treatment, whereas bilateral adrenal hyperplasia is an indication for conservative treatment.[77] In the last ten years, significant progresses in understanding the genetic background of primary aldosteronism has been observed, adding new informations that helped to characterize the different forms of primary aldosteronism. Familial hyperaldosteronism type-I also called glucocorticoid-remediable aldosteronism, described for the first time in 1966 by Sutherland et al., is an autosomal dominant monogenic form of primary aldosteronism that accounts for <1% of all cases of primary aldosteronism[78] and characterized by early onset hypertension, elevated ACTH-dependent aldosterone concentrations, suppresses plasma renin concentrations and increased urinary excretion of the hybrid steroids, 18-hydroxicortisol and 18-oxocortisol. Despite the elevated aldosterone level, hypokalaemia is uncommon.[79,80] The molecular basis of the condition is attributed to an unequal crossover between the genes *CYP11B1 (*which encodes steroid 11β-hydroxylase) and *CYP11B2* (which encodes aldosterone synthase). The resulting hybrid gene encodes an enzyme *chimera* with aldosterone synthase activity that is expressed in the adrenal *zona fasciculata* under control of ACTH hormone instead ofAG-II.[79] Therefore, in patients with FH-I, aldosterone concentrations are persistently suppressed by glucocorticoid administration. Most affected individuals develop severe hypertension in early life and are prone to cerebrovascular events, although patients with mild hypertension or blood pressure within the normal range have been described in many families.[81] The phenotype of the disease can vary not only between affected families but even within the same family.[82] Diagnosis of FH-I is usually made through use of a long PCR technique. As recommended by the Endocrine Society Guidelines, genetic testing for FH-I should be considered in patients with hypertension and a family history of early onset hypertension or a cerebrovascular accident at a young age.[83] Familial hyperaldosteronism type-II is a non-glucorticoid-remediable form of primary aldosteronism that is clinically and biochemically indistinguishable from sporadic forms. The prevalence of FH-II was reported to be as high as 6%. The pattern of transmission is autosomal dominant in some families, but this cannot be determined in all cases because of the small number of affected family members.[78] The molecular basis of FH-II is still unknown, but genetic analyses of five cohorts from Australia, Italy and South Africa have demonstrated a linkage of FH-II with chromosome 7p22;[84] however, this linkage has not been demonstrated in other families, thus indicating that FH-II could be a heterogeneous disease.

The first family affected by FH-III was described by Geller et al. in 2008 as an early-onset and particularly severe form of primary aldosteronism with distinctive clinical and biochemical features. Affected patients displayed marked aldosteronism, hypokalaemia and severe hypertension that was resistant to medical therapy, necessitating bilateral adrenalectomy. Furthermore, FH-III shows marked bilateral adrenal hyperplasia, high levels of 18-oxocortisol. The molecular genetics features of FH-III and some cases of FH-II have been only recently resolved with the discovery of a germline mutations in the KCNJ5 gene, which is located on chromosome 11q24 and encodes the G-protein-activated inward rectifier K^+ channel 4.[85] The first mutation that was described in this family by Choi et al. was an inherited **Thr158Ala** mutation in the GIRK4 and, succeeding this original finding, an additional six families with FH-III caused by KCNJ5 mutations were rapidly discovered.[86] Two families of these showed a **Gly151Glu** mutation and a milder phenotype, treatable with medical therapy and with a normal appearance of the adrenals at CT scanning. Others two families showed a **Gly151Arg** mutation and one family a **Ile157Ser** mutation. These families, in contrast to the previous described, display marked bilateral hyperplasia and require bilateral adrenalectomy to treat hypertension.[87,88] The clinical features of families carrying the Gly151Glu mutation indicate that they show a milder phenotype compared with the other mutations. These mutations that lie in or near the highly conserved selectivity filter of KCNJ5 potassium channel lead to a loss of selectivity for K^+ ions and increase Na^+ conductance with a consequent membrane depolarization, voltage-gated Ca^{2+} channels opening, increase of cytoplasmatic levels of Ca^{2+}, that is the normal signal for aldosterone production and maybe cell proliferation in adrenal cortex. Gly151Glu alters the K^+ selectivity and the Na^+ permeability in a similar way to Thr158Ala. It seems to be likely that these characteristics of Gly151Glu mutation may be responsible of osmotic shock and cell death in adrenal cells. This finding potentially explain the lack of adrenal hyperplasia and the paradoxically milder phenotypes of families carrying the Gly151Glu mutation.[88] The FH-III Thr158Ala mutation carriers display massive production of hybrid steroids, atypical responses of aldosterone and cortisol to dexamethasone, and reduced androgen production in contrast to the milder FH-III Gly151Glu phenotype. This extreme phenotype of T158A could be the mutation itself or an unidentified alteration that was not seen in Gly151Glu family.

As it has been said previously, the most common form of PA is the sporadic forms APA and BAH which account for the 90% of all cases; amongst the all cases of APA a 40% was affected by KCNJ5 mutation. It has been seen that in patients with APA, the KCNJ5 mutations are remarkably more prevalent in female than in male individuals (>70%) and the

mutation carriers are significantly younger compared with non-carriers (age 42 years vs 48 years).[89]

As well as for the familial form also for the first description of APA Choi et al., reported somatic mutation of KCNJ5, Gly151Arg and Leu168Arg, in 8 of 22 APAs in a group of Swedish patients with apparently non-familial PA[85] Another mutation deletion **Ile157del** has been identified in Cambridge, from an Australian pedigree and it has been well characterized.[90,91] Subsequently Mulatero et al. reported a new germline mutation, G151E, in two affected members mother and daughter, in one of 21 investigated families. Both showed an early-onset hypertension and moderately sever biochemical primary aldosteronism, but, unlike the family studied by Geller et al., the adrenals appeared normal and hybrid steroid level were only slightly higher than those for apparently non-familial primary aldosteronism patients. This new mutation showed like others loss of Na^+/K^+ selectivity.[92] Scholl et al., showed other two families with the same germline G151E mutation with an early-onset primary aldosteronism.[93] Furthermore, another G151R mutation has been undertaken from two different pedigrees and they showed a much more severe phenotype, characterized by a very early-onset and severe primary aldosteronism that was poorly responsive to spironolactone.

To date, it is possible to say that mutation of selectivity filter of KCNJ5 potassium channel is a key player in sporadic as well as the much rarer syndromic forms of PA, since up to 40% of APAs. It seems to be one of the most important genetic cause of ions flow alteration, membrane depolarization and aldosterone production.

3.0 Lipid metabolism

3.1 Lipid metabolism

Lipids are a group of natural molecules that include fats, waxes, sterols, of which the most important is cholesterol, soluble vitamins (such as A, D, E and K), monoglycerides, diglycerides, triglycerides, phospholipids. Dietary lipids of physiological and especially pathological importance include triglycerides, which account for 90% of dietary lipid, cholesterol esters, phospholipids and the fat soluble vitamins. Lipids represent the most concentrated source of calories in the diet, yielding more than twice as many calories per gram as either carbohydrate or protein and requires less than half the amount of intracellular

water for storage (Ex. 9 calories/gr as compared to 4 calories/gr of carbohydrates). Both properties depend on its long chain fatty acid constituents.

It is well know that the human diets contain variable amount of triglyceride and cholesterol provided by diet. Typical Western diets provide as much as 40% of the total calories in the form of triglyceride, but, on a worldwide scale, this type of diet is geographically and historically unusual. Most dietary triglyceride is absorbed in the duodenum and proximal jejunum after undergoing partial hydrolysis in the gut lumen. Triglycerides are mechanically mixed with the aqueous secretions of gastrointestinal tract to form large fat droplets. Then a smaller emulsion of smaller fat droplets is formed as bile acids, and phospholipids from the diet and bile become associated with the droplet surface. These substances are amphophilic, i.e., partly hydrophilic and partly hydrophobic, and thus promote the formation of a stable oil-water interface.[94] These fat droplets are continuously exposed to the enzymatic activity of lipases, including the lingual lipase that begins to act in the stomach and a lipase in pancreatic liquid that acts within the intestinal lumen. This enzymatic activity releases monoglycerides and free fatty acid (FFAs). At the same time, action of a pancreatic phospholipase on the phospholipid associated with the droplet surface leads to the formation of lysophospholipids and FFAs. The hydrolytic products leave the droplet surface and, in association with the bile acids, form aggregates termed micelles. The micelles are small enough to be absorbed through spaces present between the microvilli of intestinal epithelial cells, and once released monoglycerides and FFAs will be able to cross the cell membranes. Within the cells the FFAs and the monoglycerides and the small amounts of free glycerol that are formed during digestion are reconverted into triglyceride, reacting with ATP and coenzyme A (CoA) to form fatty acyl-CoA, which in turn can react with lysophosphatidilcholine to form phosphatidilcoline or with cholesterol to form cholesteryl esters. All these lipids will be packaged with proteins to form large particles called chylomicrons and then secreted into lymph. The FFAs and monoglycerides that are converted back to triglycerides are those that have chains at least 14 carbon atoms long. Shorter chain fatty acid largely pass through the epithelial cells and are transported via the portal vein to the liver.[95,96] Once reached the bloodstream, chylomicrons are hydrolysed by lipoprotein lipase (LPL) releasing triglycerides, which in turn will be absorbed to the luminal surface of capillaries. Most of FFAs released in this way are taken up by adipose cells, and stored as adipose tissue triglyceride. Over the passages chylomicrons change in composition; they lose phospholipid and apo-lipoproteins AI, AII and AIV and take up cholesterol and apo-lipoproteins CI, CII, CIII and apo E. Apo CII it is known to be particularly important because it affects the clearance of chylomicron

triglyceride from the plasma, by activating LPL. Thus, patients with a familial apo CII deficiency develop hypertriglyceridemia and hyperlipidaemia when they ingest fat because chylomicrons accumulate in the plasma.[97,98]

The other lipid particularly important by a physiological and pathological point of view is a sterol, named cholesterol, which is a key component of cell membrane and lipoproteins and a precursor of bile acids and steroid hormones. Its role in the membrane composition and architecture seems to be due apparently to its amphophilic character and its unique, rigid structure, that allows it to intercalate between membrane phospholipids. This intercalation markedly decreases the permeability of membranes to water-soluble molecules and also decreases membrane fluidity.[99] Although a dietary requirement for cholesterol doesn't exist, cholesterol introduced with diet significantly participates to a pool of cholesterol and its bile acid products that circulate several times each day between the intestine and the liver. Cholesterol introduced daily by diet can range up to 0.5/1.0 g/day and most part of this cholesterol is in the form of cholesteryl ester and probably is not directly absorbed; but pancreatic fluid contains a cholesteryl ester hydrolase that in the presence of certain bile acids catalyses the hydrolysis of cholesteryl esters in the intestinal lumen to release FFAs and unesterified cholesterol, which is ready to be absorbed. The process of absorption is not completely clear, even if it seems to be likely a passive rather than an active mechanism. Likely to triglyceride absorption also cholesterol is taken up by micelles of bile acid and FFAs, monoglycerides and phospholipid; as a component of these micelles, cholesterol enters the spaces between the microvilli of the mucosal cells and becomes available for net transfer into the cell. If net transfer of cholesterol into mucosal cells does not occur but FFAs, monoglycerides, or lysophospholipids are taken up, the micelles are disrupted and cholesterol precipitates, no longer capable of being absorbed. Overall, cholesterol absorption is incomplete and only 30-60% seems to enter body pools.[100] It seems to be likely that cholesterol absorption is realized by soluble, lipid-carrier protein that bring it from the microvillus membrane to the intracellular site of lipoprotein synthesis. The cholesterol absorbed, once within the epithelial cells, mixes with the intracellular pool of cholesterol, is esterified again with fatty acid and enters the chylomicrons, which also contain small amount of free cholesterol. After the chylomicrons enter the plasma and are attacked by LPL, much of this cholesterol, particularly the cholesterol ester, becomes associated with chylomicron remnants. The remainder of the cholesterol and also the remaining phospholipid apparently become associated with high density lipoprotein (HDL). Chylomicron remnants contain apo-E, which interacts with its receptors localized on the surface of hepatocytes, and thanks to this

interaction chylomicrons are rapidly removed from plasma into hepatocytes.[101] The ingested particles are hydrolysed within secondary lysosomes to yield amino acids, FFAs, and unesterified cholesterol. One effect of the influx of chylomicron remnant cholesterol into the liver is the decreased synthesis of endogenous cholesterol because of reduced hydroxyl-methylglutaryl-CoA reductase (HMGCoA reductase) activity. This is the rate-limiting enzyme of cholesterol biosynthesis and catalyses the conversion of HMG CoA to mevalonic acid, the first committed metabolite in the biosynthesis of cholesterol. It is subject to multivalent feedback suppression by sterols and by non-sterol products of mevalonic acid,[102] as well as to phosphorylation and dephosphorylation.[103] Down-regulation and inactivation of HMG-CoA reductase by these mechanisms leads to diminished formation of hepatic cholesterol, which limits the tendency of dietary cholesterol to increase hepatic cholesterol levels. The most part of cholesterol synthesised ex novo by the liver or introduced with the diet is either converted into bile acids or secreted directly into bile.[104] It is well known that bile acids come into play during the fat digestion and absorption along the intestinal tract and in the same time it is well known as our body is not able to increase bile acid production in response to a dietary cholesterol. Furthermore, each component of these substances are subjected to an enterohepatic circulation, i.e., they are efficiently absorbed by an active mechanism in the distal ileum and return to the liver complexed to the albumin of the portal blood. Nevertheless, a small portion of the bile acids and a larger portion of the bile cholesterol escape reabsorption during each recirculation of the bile, that in adding to the loss of cholesterol by desquamation of skin and intestinal mucosa counterpoises the amount of cholesterol that is daily synthesized and absorbed. Furthermore, the liver exerts an important function in maintaining the pool size by synthesizing new bile salts to replace those that are lost. This synthetic capacity of liver is limited so that the bile salt pool in the jejunum falls below the critical concentration, with resultant impairment of lipid absorption.

It is known that most peripheral cells can synthesize cholesterol but few cells can even partially degrade it, thus, special mechanism are required to keep cholesterol balance in peripheral tissues. The liver contributes to these mechanism secreting at least two lipoproteins and one plasma enzyme that together contribute to the cholesterol transport to and from peripheral cells. One type of lipoprotein secreted is very low density lipoprotein (VLDL), also involved in the triglyceride transport; indeed, VLDL of hepatic origin consists of a core of triglyceride stabilized by a thin film of phospholipid, unesterified cholesterol, apo-lipoproteins B100, C, and E. Into the peripheral tissue VLDLs are metabolized by LPL, that catalyses the partial hydrolysis of the triglyceride, producing remnant lipoproteins. These

cholesterol-rich remnants may be taken up directly by the liver or may continue to circulate in the plasma and be gradually converted into small lipoproteins known as low-density lipoproteins (LDLs) that delivery cholesterol to peripheral cells. LDL show a diameter that is one fourth to one third of the diameter of the parent VLDL, and show a core that is made chiefly by cholesteryl ester surrounded by phospholipid, unesterified cholesterol, and apo-B. The net effect is the formation of a cholesterol-rich lipoprotein that is small enough to be transported across the endothelial cells of peripheral capillaries. The conversion of VLDL to LDL usually requires about 12 h, after which LDLs are gradually cleared from the bloodstream, principally by two mechanisms, one receptor-dependent, and another nonspecific mechanism such as pinocytosis. The best understood mechanism is that mediated by LDL receptor, which bind lipoproteins that contain apo-B and/or apo-E with a high affinity. Once interaction between receptor and lipoprotein is realized, complex is internalized by adsorptive endocytosis. Vesicles formed by this process subsequently fuse with lysosomes, whereupon the lipoproteins are degraded by lysosomal hydrolases and unesterified cholesterol is released into the cytosol. As cytosolic cholesterol accumulates, it is esterified by an intracellular acyl CoA-cholesterol acyltransferase (ACAT) or used to form membranes. At the same time, increased cytoplasmatic cholesterol levels, activates feedback mechanisms that reduce intracellular HMG-CoA reductase activity and down-regulate the LDL receptor. These feedback mechanisms clearly limit both the intracellular synthesis of cholesterol from acetyl CoA and the uptake of excessive amounts of LDL cholesterol. In the LDL clearance process an important role is played by LDL-receptor and apo-B and E, whose importance is emphasized by the strikingly high concentrations of LDL that are typically found in the plasma of patients affected by familial hypercholesterolemia.[105] Familial hypercholesterolemia may be caused by incapacity of our body to form functional apo-B and E receptors and/or inability to internalize lipoproteins. Cells that normally show LDL receptors include fibroblast, smooth muscle cells, adrenocortical cells, luteal cells from the ovary. Thus, these receptors are widely distributed over tissues and organs, accounting for about two thirds of the removal of LDL particles from plasma.[105] The LDL receptors are usually localized into cytoplasmatic vesicles and are subject to a recycling process, according to the intracellular requirements for cholesterol. For example, since LDL it has been seen to play a key role in delivering cholesterol to endocrine cells that synthesise steroid hormones, under corticotropin stimulation the LDL receptor number increases highly.[106]

In addition to secreting VLDL, the liver secretes lipoproteins referred to as nascent high density lipoprotein (HDL), a heterogeneous class of lipoproteins, which have in common a

high density (>1.063 g/mL) and a small size. These lipoproteins contain phosphatidilcoline, unesterified cholesterol, and apo-AI or E. Mature HDL3 and HDL2 are synthesised from lipid-free apo-AI or lipid-poor pre-β1-HDL as the precursor. These precursors are released from lipolysed VLDL and chylomicrons or are produced as a nascent HDL by the liver or intestine. After being secreted into plasma, nascent HDLs interact with a plasma enzyme that also is synthesised and secreted by the liver. This enzyme, lecithin-cholesterol acyltransferase (LCAT), forms cholesteryl esters in plasma by transferring fatty acids from HDL phosphatidilcoline to HDL unesterified cholesterol. The LCAT enzyme is activated by apo-lipoproteins, in particular by apo-AI, that is the principal apo-lipoprotein component of mature, circulating HDL. Differences in the quantitative and qualitative content of lipids, apo-lipoproteins, enzymes, and lipid transfer proteins result in the presence of various HDL subclasses, which are characterized by differences in shape, density, size, charge, and antigenicity.[107] Lipoproteins not only deliver cholesterol to cells but apparently also contribute to reverse cholesterol transport (RCT). Cholesterol in peripheral cells in the form of cholesteryl ester is hydrolysed by an intracellular cholesteryl ester hydrolase.[108] Then, on interaction of cells with HDL that is transiently bound to HDL receptors on the cell surface,[109] the liberated unesterified cholesterol transfers to the plasma membrane and becomes associated with the HDL. RCT describes the metabolism and an important anti-atherogenic activity of HDL, namely, the HDL-mediated efflux of free cholesterol from non-hepatic cells and its subsequent delivery to the liver and steroidogenic organs, in which cholesterol is used as a precursor for the synthesis of lipoproteins, bile acids, vitamin D, and steroid hormones.[110,111] Approximately 9 mg cholesterol per kg body weight is synthesized by peripheral tissues every day and must be moved to liver for effective catabolism.[112] Alteration of RCT can lead to a deposition of free cholesterol (FC) within the arterial wall and thereby contributes to the development of arteriosclerosis. Evidence that HDLs contribute importantly to reverse cholesterol transport is provided by the abnormalities that accompany Tangier disease. This inborn error of metabolism is characterized by abnormally low level of HDL in the plasma and by the presence of cholesteryl ester-rich foam cells (macrophages) in peripheral lymph nodes.

Several lines of evidence suggest that cholesterol efflux may be realized by three different mechanisms: aqueous diffusion, SR-BI-mediated FC efflux, and ABCA1-mediated efflux. Cholesterol molecules are sufficiently water-soluble to transfer from cell membrane to an acceptor by the aqueous diffusion mechanism.[113] This process involves desorption of cholesterol molecules from the donor lipid-water interface and diffusion of these molecules

through the intervening aqueous phase until they collide with and are absorbed by an acceptor. The aqueous diffusion mechanism involves a simple diffusion process, and, as such, cholesterol transfer is passive and driven by the cholesterol concentration gradient. In this mechanism process, the unstirred water layer surrounding cells creates a significant diffusion barrier.[113] The other mechanism of cholesterol efflux is based on the interaction between acceptor and SR-BI receptors of donor. These receptors are members of the CD36 family of proteins and share about 30% sequence homology with the other members of this family.[114,115] Besides stimulating the efflux of free cholesterol, expression of SR-BI also facilitates the influx of free cholesterol, thus, the movement of free cholesterol is bidirectional and the net movement depend on the cholesterol concentration gradient, like to the aqueous diffusion. Furthermore, besides mediating the bidirectional flux of free cholesterol, SR-BI is able to induce a selective uptake of other lipoprotein lipids, including cholesteryl ester, phospholipid, and triglyceride.[116] This movement is unidirectional, and by promoting the net flux of HDL cholesteryl ester and triglyceride, SR-BI induces depletion of HDL core lipids. SR-BI receptors interact with a wide range of acceptors, including HDL, LDL, oxidized LDL, acetylated LDL, and small unilamellar vesicles.[117] The last mechanism of free cholesterol efflux is mediated by ABCA1. This receptor is a member of a large family of ATP-binding cassette transporters that have common structural motif and use ATP as an energy source to transport a variety of substrate, including ions, lipid, and cytotoxins. In contrast to SR-BI, the preferred cholesterol-acceptors for ABCA1 is a lipid-poor apo-lipoproteins. All of exchangeable apo-lipoproteins, such as apo AI, apo AII, apo AIV, apo E, and apo C can act as phospholipid and cholesterol acceptors for ABCA1.[118,119] ABCA1 promotes the unidirectional efflux of cholesterol and phospholipids to lipid-free or lipid-poor apo-lipoprotein, and is also involved in the lipidation of apo-lipoprotein AI in the formation of nascent HDL. It has been demonstrated by several studies that a direct interaction between cholesterol transporter and apo-lipoprotein play an important role in ABCA1-mediated efflux. ABCA1 has been seen to cross-link with the apo-AI, indicating a very specific association between them. These results are confirmed by recent studies conducted by Fitzgerald et al., that show as four different mutant ABCA1 transporters with missense mutation exhibited little or no apo-AI-induced efflux or cross-linking to apo-AI. This result suggest that a direct interaction between apo-AI and ABCA1 is necessary for efflux.

Plasma lipoprotein can also be removed by less specific mechanisms. Macrophages and endothelial cells possess a scavenger receptor that recognizes modified LDL (i.e. oxidized

LDL). The uptake of modified lipoproteins by this scavenger pathway has been hypothesized to contribute to the deposition of cholesterol in atherosclerotic plaques.[120]

3.2 Disorders of lipid metabolism

Hyperlipidaemia is characterized by an accumulation of one or more of the major lipids transported in plasma and is a manifestation of one or more abnormalities of lipid metabolism or transport. Speaking of hyperlipidaemia or dyslipidaemia, we are referring to hypercholesterolemia, hypertriglyceridemia or both. These hyperlipidaemias may be primary or secondary. Secondary hyperlipoproteinaemias are the complication of underlying, metabolic disturbances, drug-induced effects, or the result of dietary excesses. Otherwise, primary hyperlipoproteinaemias are genetically determined and may be classified by lipoprotein phenotype or genotype. Furthermore, the genetic classification may be subdivided into monogenic (caused by single-gene inheritance) and polygenic-multifactorial (caused by multiple subtle genetic factors that act together or in combination with environmental factors).[121] The biochemical parameters of primary and secondary hyperlipoproteinaemias are very similar, thus, their differentiation is sometimes difficult and represent the cornerstone of successful therapy, because the secondary form may be corrected simply through changes in lifestyle. Primary hyperlipidaemia has been classified into six types, based on the specific patterns of the various lipoproteins in plasma.(Table n.3)

Phenotype	Lipoprotein(s) elevated	Serum cholesterol concentration	Serum triglyceride concentration	Relative frequency, %
I	Chylomicrons	Normal to ↑	↑↑↑↑	<1
IIa	LDL	↑↑	Normal	10
IIb	LDL and VLDL	↑↑	↑↑	40
III	IDL	↑↑	↑↑↑	<1
IV	VLDL	Normal to ↑	↑↑	45
V	VLDL and chylomicrons	↑ to ↑↑	↑↑↑↑	5

Table n.3 *Primary Hyperlipidaemia classification based on lipoprotein phenotype*

However, these types of pattern are not specific, and the plasma lipoprotein pattern may change with the time in any individual.

Abundant evidence supports the importance of abnormal lipoprotein metabolism in atherosclerotic disease and more in general in cardiovascular disease. Several epidemiological studies have documented that diets reach of saturated fats and cholesterol accelerate atherogenesis. The prominent alterations that are consistently related to atherogenesis include hypercholesterolemia (reflecting increased concentration of LDL), hypertriglyceridemia (reflecting increased concentration of VLDL and/or remnants and triglyceride enrichment of LDL and HDL), increased apo-B levels, and reduced levels of HDL and its apo-AI.[122] Hypercholesterolemia is the major risk factor associated with the increased incidence of atherosclerosis in the United States and in Europe. Although many atherosclerotic lesions are fibrous and contain relatively little lipid, the effects of lipid on the endothelium, monocytes, and smooth muscle, and the accumulation of lipid in the lesion of hypercholesterolemic individuals, are critical components of the process of atherogenesis. Since different animals are able to form lesion of atherosclerosis similar to human when they develop hypercholesterolemia, it has been possible to understand the cellular changes that come into play in the atherosclerotic plaque formation. Faggiotto et al. focused on this particular issue adding new important informations.[123] These data show that the first and most striking event occurs after 7 to 14 days of diet-induced hypercholesterolemia. This consists of the attachment of large number of leukocytes, principally monocytes, to the surface of the arterial endothelium. The monocytes attach to the endothelial cells in clusters that appear to be located throughout the arterial tree in all large and medium-sized arteries. These monocytes then migrate over the surface of the endothelium and use the junctional site to slip between the cells and localize in the sub-endothelial space. In the sub-endothelial intima space, monocytes become converted into macrophages, so that within 1 month large number of foam cells, or lipid-filled macrophages, are found beneath an intact endothelium. These cells continue to enlarge as they fill with lipid. Such accumulation of foam cells represent the establishment of the first and ubiquitous lesion of atherosclerosis, the fatty streak. The fatty streaks continue to grow, and because of this process the surface of the artery becomes highly irregular and convoluted. Over the time, small numbers of smooth muscle cells begin to appear beneath the accumulated macrophages within the intima and also begin to accumulate deposits of lipid and take on the appearance of foam cells. After approximately 6 to 12 months at the very high cholesterol and LDL levels, changes occurred at branches and bifurcation of the main arteries. These changes consist of retraction of the endothelial cells covering some of the fatty streaks caused by endothelial cell-cell detachment. Endothelial retraction exposes the numerous lipid-filled macrophages to the blood flow and permits them

to remove the lipid they have ingested from the lesion by taking it with them into the circulation to the spleen and to lymph nodes. In this way, the macrophages play a role in the lesions of atherosclerosis similar to their role at site of injury, where they are the principal scavenger cells. Thus, in very real sense, the progressing lesions of atherosclerosis represent a special kind of inflammatory response in which macrophages (and perhaps T-lymphocytes) attempt initially to protect the tissues. With the continuing insult (hypercholesterolemia, diabetes, and so on), the response becomes excessive, and the resultant fibro-proliferative events themselves become the disease process. When macrophages become exposed to the circulation they can act as a deposit site for platelets, which start to form a fibrotic cap that overlay areas of extensive proliferation of smooth muscle cells intermixed with lipid-filled macrophages, beneath which were found cell debris, lipid accumulation, and sometimes calcification.

Thus, in keeping with these informations and The Lipid Research Clinical Trials results it seems quite clear as lowering of plasma LDL levels would be extremely beneficial. Furthermore, it has been seen either in animal models either in human patients aggressively treated with lipid-lowering regimens a regression of atherosclerotic plaque, fatty streaks, significant reduction in the size of the smooth muscle proliferative lesions, with a consequent benefits in atherosclerosis, myocardial infarction, and coronary artery disease.

Brown and colleagues have demonstrated a statistically significant regression of semi-occlusive lesions of human coronary atherosclerosis in association with decreases in plasma cholesterol and LDL levels.[124] These results provide clear evidence of pathological role of cholesterol and more in general of lipids.

3.3 Kinesin like protein 6 role in the lipid pathway

Three major superfamilies' of microtubule motor proteins have been identified, kinesin, myosin, and dynein. Kinesin is a wide superfamily constituted by 45 members expressed in mammalian cells, which share analogies in the motor domain but differ considerably in their cargo-binding tail domain.[125] Although KIFs are conserved between species, many different types exist within a single organism, suggesting that KIFs might have diverged through molecular evolution to mediate different cellular functions. Conventional kinesin, also known kinesin-1, and most of the other vesicles motors in the kinesin superfamily move unidirectionally toward the plus end of the microtubule.[126] Thus, kinesins are likely to be

involved in the in trafficking events directed toward the cell periphery, such as motility from the Golgi to the plasma membrane. However, other minus end-directed kinesins also contribute to intracellular trafficking events, such as the minus end-directed transport of early endosomes.[127] The intracellular transport is essential for appropriate cellular morphology and function. Kinesin proteins have been seen to transport organelles, protein complexes, and mRNA to specific site along the microtubule while hydrolysing ATP for energy. Kif6 is a member of the kinesin 9 family but the precise molecular function of kif6 is still not known, and in particular its association with the circulating cholesterol level is not understood. It is likely to play a role in the cellular transport of proteins along microtubules and could be involved in cellular transport in the cardiovascular system.[128] To date, all studies have focused on investigating polymorphism in the KIF6 gene within the context of coronary artery disease even if its molecular pathway needs to be clarified. Indeed, there has been considerable debate regarding the role of KIF6 in coronary artery disease. Several lines of evidence that used a candidate gene based approach in several atherosclerosis population cohorts have observed an increased risk of adverse coronary events in carriers of the rs20455 C variant in the KIF6 gene which lead to a Trp719Arg substitution in the Kif6 protein.[129,130] However, these results were refuted by a large part of Genome-Wide-Association-studies metanylisis using 19 case-control studies where cases were defined either by a history of prior myocardial infarction and/or the presence of coronary artery disease at angiography.[128] Furthermore, no association between the KIF6 variant and this pathological condition has been found in the most recent large meta-analysis.[131] Nevertheless, evidence from international randomized controlled trials with statins associated carriage of Tpr719Arg genotype with a greater clinical response to statin therapy.[132,133] Another study, JUPITER trial, has been realized on a wide population of patients, men and women, without prior cardiovascular disease or diabetes, were randomly allocated to rosuvastatin 20 mg/day or to placebo and followed for first major vascular events and for all causes of mortality. The effects of Tpr719Arg polymorphism have been evaluated. Outcomes obtained from this study didn’t show any correlation between genotype and major cardiovascular risk factors, as well as no clinically meaningful differences in pharmacological response have been seen between carriers and non-carriers.

3.4 Pharmacology of lipid metabolism

As it has been mentioned above, several lines of evidence from epidemiological and biochemical studies have demonstrated the importance of reducing to physiological level lipids concentration and in particular total cholesterol and LDL concentration. Indeed, it has been well demonstrated over the years that a linear relationship between LDL-C levels and relative risk for CHD exists, as showed in Fig. 6 below:

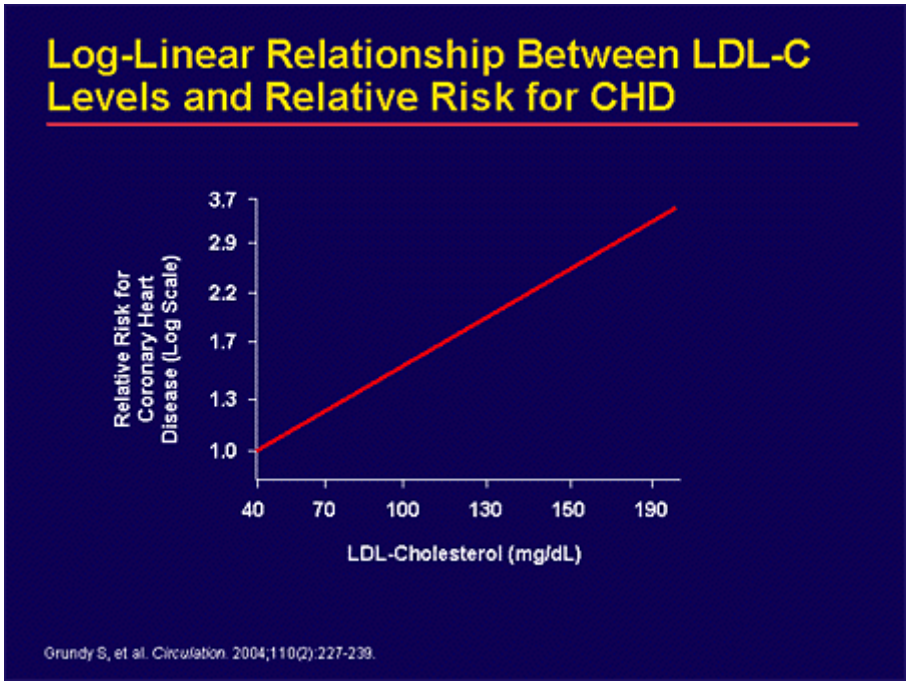

Fig.6 *Linear correlation existing between LDL-C and Relative Risk for CHD demonstrates the high importance to keep total cholesterol level and LDL within a physiological range suggested from the international guidelines for cholesterol level (Grundy et al. Circulation 2004; 110(2):227-239).*

According to the World Health Organization (WHO), **LDL** cholesterol level ranging from 100-130 is considered optimal, >160 high, >190 very high; **Total Cholesterol** is considered desired <200, borderline between 200-239, and high ≥240; **HDL** cholesterol <40 is believed low, ≥60 high (Table n.3).

LDL Cholesterol level (mg/dl)	*Acceptance Degree*
<100	**Optimal**
100-129	**Near optimal/above optimal**
130-159	**Borderline high**
160-189	**High**
≥190	**Very high**
Total Cholesterol	
<200	**Desirable**
200-239	**Borderline high**
≥240	**High**
HDL Cholesterol	
<40	**Low**
≥60	**High**

Table n.4 *Classification of lipids level in the blood and acceptance risk according to the international guidelines.*

Risk factors such as diet, smoking, physical inactivity, obesity, high blood pressure, lipids (high LDL and low HDL cholesterol and raised triglycerides), diabetes, family history of coronary heart disease are responsible of a huge number of cases of coronary heart disease. The World Health Organization (WHO) believes that 60% of coronary heart disease and 40% of strokes are due to elevated LDL cholesterol levels. Reducing LDL-C has long been the primary target of cholesterol policy and this remains the case today. The two major approaches of LDL-lowering therapy are therapeutic lifestyle changes (TLC) and drug therapy. Dietary therapy should be the first approach in all form of lipid disorders. Although there is a great variation in individual response to a low-fat, low-cholesterol diet, changes of alimentary habits can be expected to play a significant role in improving most lipid profile. The minimal goals of dietary therapy should be to reduce LDL cholesterol to under 160 mg/dl and lower the total cholesterol to under 240 mg/dl in patients without CAD or two additional risk factors and to less than 130 mg/dl if any of these conditions apply. In the same time, if the patient result to be also overweight, the dietary approach should provide also a total caloric intake, as well as the intake of saturated fat and cholesterol.

Drug treatment of lipid disorders should not be used until a precise diagnosis is firmly established and the presence of treatable of secondary causes has been ruled out. Before to

start the drug lipid-lowering therapy, maximum dietary effort should be done. To date, there are several classes of lipid-lowering drugs which act at a different levels of cholesterol biosynthesis, such as bile acid sequestrants (Cholestyramine, Cholestipol), Nicotinic acid, Fibric acid derivatives (Gemfibrozil, Clofibrate), HMGCoA-reductase inhibitors (Statins).

Cholestyramine and Cholestipol are quaternary ammonium salts that act as anion-exchange resins, binding bile salts in the intestine tract. This sequestration leads to a reduction of reabsorption of bile salts and consequently to a decrease of their enterohepatic recycling; in the same time there is an increase of bile salts excretion in the stool. The positive effects on lipid-lowering of this class of drugs is represented by the fact that cholesterol will be used for an ex-novo synthesis of bile salts rather than be used for LDL or VLDL synthesis. Cholestyramine and Cholestipol are considered first line agents and are highly effective in decreasing LDL cholesterol.[134] The bile acid sequestrants are difficult to be used because of their side effects, related to nausea, abdominal pain, constipation, and indigestion. Because of their site of action, intestine, these drugs have been seen to interfere with the pharmacokinetics of other drugs such as digitalis, phenobarbital, thiazides, etc..., reducing their intestinal absorption.

The B vitamin nicotinic acid or niacin is able to reduce the LDL levels by about 25% and VLDL levels by about 75%. The mechanism with which this drug acts is complex and most probably related to a decreased cholesterol and VLDL hepatic synthesis. Nicotinic acid has been also seen to decrease the release of free fatty acid from adipose tissues and thus decreases the level of substrate involved in the triglycerides synthesis. Furthermore, this drug is able to increase the HDL levels by 20 to 40%. Similarly to bile acid resins, nicotinic acid's activity, is accompanied by frequent side effects, that include cutaneous flushing and gastrointestinal (GI) symptoms.

Gemfibrozil and clofibrate are the fibric-acid derivatives widely used in USA; in Europe, fenofibrate, benzafibrate, and ciprofibrate are also used. The fibric-acid derivatives are used in VLDL and triglycerides lowering. Their mechanism of action is not completely clarified, even if they seem to act by increasing lipoprotein lipase activity via peroxisome proliferator activated receptors-α; this action leads to an increase of VLDL clearance.[135] In the same time, fibric acid derivatives also increase the cholesterol secretion into the bile. Fibric-acid derivatives usually are well tolerated, with nausea and GI discomfort the major side effects.

The last class, not for importance, of lipid-lowering drug is represented by 3-hydroxyl-3-methyl-glutaryl-CoA-reductase inhibitors, the so-called statins: atorvastatin, pravastatin, lovastatin, rosuvastatin, and simvastatin. This class of drugs is the most extensively used in

the treatment of hypercholesterolemia. The efficacy, safety, and benefits of statins make them the drugs of choice. Statin are able to lower the serum low-density lipoprotein and triglycerides levels, and in the same time they increase the HDL levels. Furthermore, it has been seen that statins offer an important contribution to the reduction of the risk for the major cardiovascular events, such as MI and death for CVD in selected population. Side effect of statins are principally muscle-related (such as muscle weakness) and in general mild in nature. More serious side effects, such as myopathy and fatal rhabdomyolysis are rare and often can be seen when statins are used in combination with other medicines that affect statin pharmacokinetics.[136] Usually statins are used singularly, even if there are several cases in which statins are used in combination with other lipid-lowering drugs. However, doubts about the benefits of statins, alone or in combination with other lipid-lowering drugs, remain. In patients with a very high LDL levels or mixed dyslipidaemia that fail to reach the desired lipids levels on statin monotherapy, other classes of drugs are often added in order to improve clinical outcomes. Besides the co-administration, another aspect of statin therapy that remains difficult for prescribers is the ability to predict individual patient's response to therapy, in terms of efficacy and genetic predisposition to possible adverse effects.[137] Plasma concentration of statins as well as the pharmacological efficacy can vary widely amongst patients that receive the same dose of the same statin. Recent results obtained from pharmacogenomics and pharmacogenetics studies have demonstrated a wide number of candidate genes (40 gene) that are involved in the modulation of statin pharmacokinetics and pharmacodynamics. Doubtless, the most important is the gene expressing for the HMGCoA reductase enzyme. Statins act binding to the catalytic domain of HMGCoA-reductase enzyme that, as above said, catalyses the rate-limiting step of cholesterol biosynthesis. All statins share rigid, hydrophobic groups that are covalently linked to the HMG-like moiety. All statins are competitive inhibitor of the natural substrate HMGR, and it has been seen that they act occupying the HMG-binding pocket and part of the binding surface for CoA, thus sterically preventing substrate from binding. Comparing the complex obtained between the natural substrate and the enzyme or between a statin and the enzyme, a clear rearrangement of the substrate-binding pocket has been seen after the statin interaction. The strong efficacy of statin's interaction with the catalytic site of HMGCoA-reductase is probably due to the large number of van der Waals interaction, which in turn reflects the importance of each amino acid that takes part in the catalytic site's constitution. Several studies have shown as structural changes of catalytic site of HMGCoA-reductase, principally caused by genetic alteration, lead to a loss of statin treatment efficacy.

4.0 Genetic background

4.1 Pharmacogenetics, pharmacogenomics, and individualized therapy

Individual variability in drug efficacy and drug safety is one of the most important challenge in current clinical practice of this century and the personalized therapy seems not to be a dream anymore.

In a large patient population, a medication that is demonstrated efficacious and safe in many patients, it is proven to be inefficacious and sometimes lethal in some other peoples. Although large individual variability in drug efficacy and safety has been well documented amongst peoples since the beginning of the medicine, its understanding still represents an unmet need. The demonstration of individual variation in drug response has proven difficult, even if the demand to overcome such variation has received more attention and become the goal of modern medicine. It is well known that large variability of drug efficacy and adverse drug responses in patients is a major determinant of the clinical use, regulation, and withdrawal from the market of clinical drugs. Drug response is influenced by several factors, some of which involve important aspects that are intrinsic to the biology itself.

Genetic variation in humans was recognized as the most important determinant of individual variability of drug response from clinical observations in late 1950s.[138,139] From these studies it has been observed amongst a certain population differences in plasma concentration of specific drugs and in the similar way also differences of metabolites concentration in urine, demonstrating that variances in pharmacodynamics and pharmacokinetics are present and exert a very important role. It has been seen that in some patients a very low or a very high concentration could be present and the biochemical traits leading to the variation of drug concentration were found inherited. These clinical findings fostered the formation of pharmacogenetics. Differences of drug response and behaviour reflect sequence variations of specific genes coding for drug targets, such as proteins, drug-metabolizing enzymes, receptors, drug-transporters.[140,141] Starting from these outcomes, the availability of the complete human genome sequence has made it possible to analyse the impact of variations of human genome sequence on the pathogenesis of important diseases and the response to drug therapy. The amount of knowledges and informations acquired over the years on genome-disease and genome-drug interaction has brought to a new area so-called pharmacogenomics starting from the previous pharmacogenetics. This goal provided a

rationale for the hope that the personalized medicine can be achieved in the near future. Both pharmacogenomics and individualized drug therapy are increasingly influencing medicine, biomedical research, in many areas, including clinical medicine, drug development, drug regulation, pharmacology, toxicology.

As already said drug efficacy and adverse drug reactions dose-dependently determine the clinical outcome of clinical therapy. To date, it is well known as these parameters change widely over the cohort and the personalized therapy, based firstly on a genetic screening and then on using of the right drug and concentration, may represent the only possible way to avoid to use an inefficacious drug and increase adverse drug reactions. We know that a higher dose boosts drug therapeutic effect but in the same time it increases the possibility to have undesirable side effects. The difference between the drug therapeutic effect and its side effects defines the so-called therapeutic window (Fig. 7)

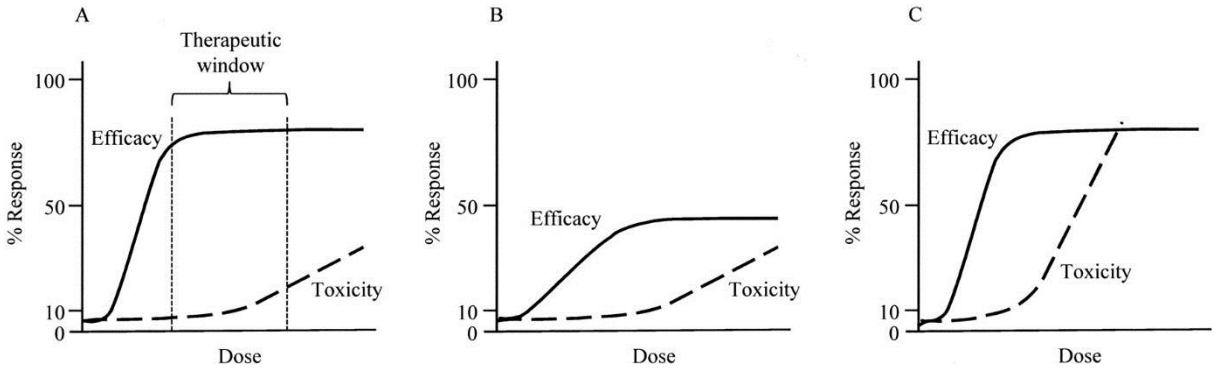

Fig.7 *Representation of dose-response curve. Panel A shows the therapeutic window, that is the difference between the efficacy and the toxicity. This window may vary from patient to patient, B and C, requiring dose-adjustment (*Qiang Ma and Anthony Y. H. Lu, Pharmacogenetics, Pharmacogenomics, and Individualized Medicine, *Pharmacol Rev 63:437–459, 2011).*

For many drugs, the optimum dose required for effective and safe therapy varies significantly from patient to patient, because the minimum therapeutic dose can be too low or too high. Such a situation can be characterized by an atypical therapeutic window, dose-response curve, and toxicity resulting in unexpected and undesirable outcomes.[142] Genetic and nongenetic factors affect individual variability of a drug response by modulating the dose-response curves of drug efficacy and drug toxicity of patients. Clinical outcome is altered if drug dose is not adjusted accordingly.

4.2 Single nucleotide polymorphisms (SNPs), important genetic variation

Inherited differences in DNA sequence contribute to phenotypic variation, influencing an individual's anthropometric characteristics, risk of disease, and response to the environment. A central goal of genetics is to localize the DNA variants that contribute most significantly to population variation in each trait.[143]

The idea that genes control some drug responses was suggested for the first time in the 1950 thanks to a correlation between inheritance or ethnicity and aberrant drug responses. This idea was strengthened by family and twin studies in 1960s 1970s, extended by biochemical studies in 1980s, and solidified by molecular genetics in the 1990s.[144,145,146] Cloning and characterization of the first human gene containing DNA sequence variations, singular nucleotide polymorphisms, that influence drug pharmacodynamics and pharmacokinetics did not take place until the end of 1980s.[147] Although there are a number of different types of polymorphic markers, most attention recently has focused on single nucleotide polymorphism (SNPs, pronounced snips), and the potential for using these to determine the individual drug response profile. Polymorphisms are genetic variations of human genome that occur at a frequency of 1% or greater in the population. These variations can appear as insertion or deletion of short fragments of nucleotide, even if the most common variations are SNPs. A SNP is a DNA sequence variation characterized by a singular nucleotide substitution among members of the same species or between chromosomes in an individual. SNPs are the most abundant genetic variants in mammalian genomes and specifically in the human genome they account for 3.2 million and are responsible for the 90% of genetic human differences.[148] SNPs are classified in three different groups depending on where they are located in the genome:

1. c-SNPs, variations located in coding region, exons, whose presence could modify or not the amino acid sequence in the protein structure, defined as synonymous and non-synonymous respectively
2. p-SNPs are located in perigenic region, i.e., in regulatory regions such as promoter, enhancer, etc… Such polymorphism can modulate both the expression level and the stability of the protein, by influencing the mRNA stability.
3. r-SNPs, random SNPs that are located in the intergenic region, i.e., in those regions that do not show genes but that constitute the 98% of our genome. These variations are not able to

influence the genetic expression. However they may alter the DNA structure by influencing the tertiary structure of DNA, and its capacity of interaction with chromatin or with enzymes such as topoisomerase. For these reasons, to date, we cannot exclude their implication in the pharmacogenetics.

Currently there is a growing list of polymorphisms found in genes encoding of drug transporters and targets, drug-metabolizing enzymes, as well as disease-modifying genes, that have been linked to drug effects in humans. Pharmacogenetics has indeed proven to be a potential source of biomarkers able to predict drug response and adverse drug reaction. For this reason, during the last decade a rapid development of techniques in the area of genome analysis has been seen and recognized as needful for the identification of new pharmacogenomics biomarkers. Such biomarkers mainly originate from genes encoding drug-metabolizing enzymes, drug transporters, and drug targets. Some of these are now integrated by the USA Food and Drug Administration (FDA) and the European Medicines Agency (EMA) into drug label inserts.[149]

Although the numerous potential benefits of pharmagenomics and pharmacogenetics have been announced and sometimes also demonstrated, to date, this innovative area of research is so rarely used in clinical practice. This seems to be a particularly relevant question that in the next year needs to be answered. It seems that the failure to use of this type of approach in the clinical practice is related not only to scientific issues but also to cultural limits. For example, individualizing dosages, even based on easily assessed patients characteristics, such as sex, age, renal functionality, has not embraced by medical or pharmaceutical communities. Furthermore, there is a strong resistance to relying on tests for every medical decision. Not only does pharmacogenomics require a laboratory test, it also requires an interpretation of genotypes, which will probably require clinicians to receive further training in molecular biology or genetics. Lastly, it also difficult to demonstrate that individualization of drug therpy on the basis of genetics improves clinical outcomes, given the multi-genic nature of most drug effects. These represent just some of limits that can hinder the clinical use of this discipline.

4.3 An overview on the genetic variations

The replication of genome before cell division is a remarkably precise process. Nevertheless, some errors during DNA replication can happen and lead to a new mutations. It is well known that genetic errors can occur at different levels of cellular growing. If they occur during mitosis, after conception, for instance, and during life, they are defined *somatic mutations*. Somatic mutations can happen at any time, and they usually lie in non-coding regions. For all these reasons they are said to be phenotypically silent. Because they do not occur in cells that give rise to gametes, the mutation is not passed along to offspring. Somatic mutations are thought to be the cause of most sporadic cancers, that are not inherited. In cancer there are usually somatic mutations of important genes like Ras, involved in the cell proliferation and survival, as well as other genes p53, etc… These mutations are restricted to an individual cell and any daughter cells that may have been produced (when the cell divides). Thus, the mutation can be seen only in the tumour tissue but not in the healthy tissue of the same patient.

If the mutation occurs during meiosis, in producing the egg or sperm, they are referred to as *germ line* mutation. If the mutant sex cell participates in fertilization, then the mutation will be passed to the next generation. An individual of perfectly normal phenotype and of normal ancestry can harbour undetected mutant sex cell. The mutation will be detectable only if it participates to the zygote formation.

The point mutation is the simplest type of mutation, which is characterized by the substitution of a singular nucleotide, addition or deletion of a small number of nucleotides Amongst the point mutations we can recognize, besides singular nucleotide polymorphism, other mutations that are here listed: *missense mutation, non-sense mutation, deletion, insertion or addition mutation*.. Depending on where the mutation occurs and which base is changed we can divide these mutations in *missense or non-synonymous* and *sense or synonymous* mutation.

With 61 codons for 20 amino acids, many of the codons are synonyms, coding for the same amino acid. This means that each amino acid can be expressed by several codons, up to six in the case of leucine, while methionine and tryptophan have two codons. In most cases it has been seen that synonyms differ by only one base, so it's quite plausible that a point mutation can lead to a new codon that encodes for the same amino acid. This type of mutation is called *synonymous*, silent mutation or sense-mutation. *Missense mutation* happens when a single

base substitution leads to a new codon that encodes for a different amino acid; for this reason is defined also non-synonymous because of a different product. This substitution may have a different degree of influence on protein structure and its functionality, according to the chemical nature of the new amino acid and the position where the substitution occurs in the protein structure. In case the amino acid shows the same chemical and physical properties, its influence on the protein structure and above all on the activity can be considered negligible, but whether the new protein has different characteristics we can obtain a non-functional protein or a protein with a reduced activity with a consequent health problems.

A *non-sense mutation* (UAG, UGA, UAA) is obtained when a point mutation substitution leads to the formation of one of the three stop-codons, which are known to be important because of their role in the peptide chain elongation, signalling the end of the process. If a point mutation changes a normal coding triplet in a stop-codon, the resulting protein will be cut off early and consequently will result to be incomplete and plausible not working. This has health implication, as it happens in the cystic fibrosis, a non-sense mutation caused pathology.

5.0 Aim of the study

This study was realized focusing our attention on three different diseases such as hypertension, hypercholesterolemia and hyperaldosteronism, trying to add new informations on their etiology. To date, hypertension is the most studied pathology worldwide because of its epidemiology. Recent reports indicate that 1 billion of peoples in 2000 had hypertension, and this number is predicted to increase to 1.56 billion by 2025. New reports also suggest that hypertension prevalence is increasing in the developing countries and is becoming to be one of the leading causes of death and disability. It is well know that hypertension is a multifactorial disease where several risk factors such as diet, smoking, alcohol consumption, cholesterol levels, aging, and the presence of other pathological conditions come into play. Nevertheless, this huge amount of information is not enough to justify the numerous cases of ictus, heart attack, and cardiovascular diseases in an non-negligible percentage of patients. Thus, to date there is an urgent need to identify new metabolic and above all genetic markers that can be used in the predictive medicine. Starting from genetic characterization of each patient, predictive medicine may be able to quantify the risk of particular subject to develop hypertension or another cardiovascular disease, and in this way it may personalize the therapy and low the annual worldwide cost of hypertension treatment quantified in $370 billion. Despite of the widespread availability of antihypertensive drugs, the blood pressure of many patients remains high and uncontrolled. This represents a very important issue especially in reference to the billions of dollar spent annually. Amongst all the causes that may be responsible of uncontrolled blood pressure, such as non-adherence to the therapy, diet, lifestyle, interfering medications, or underlying diseases, genetic variations have a determinant role.

The first part of the study takes in consideration a pathological condition, known as hyperaldosteronism, characterized by a dysregulation of aldosterone secretion by adrenal glomerulosa cells. It is well demonstrated that up to 10% of peoples with hypertension have abnormal aldosterone levels (primary aldosteronism, PA) and in a significant minority of these it arises from aldosterone-producing-adenomas (APA) in the adrenal cortex. Several potassium channels are expressed in the glomerulosa cells, even if we paid our attention on a specific inward-rectifying-potassium channel, KCNJ5, located across the membrane. This channel shows a specific characteristic, i.e. is extremely selective toward K^+ ions, giving them the possibility to flow across the membrane in both directions. It is well known that the ZG

cell membrane is selectively permeable to K^+ giving it the characteristics of a K^+ electrode over a wide range of extracellular K^+ concentrations. Depolarization causes a secondary (and transient) rise in intracellular Ca^{2+} through a T-type calcium channel, which is the primary stimulus for increased production and release of aldosterone. It has been recently reported that mutations of the selectivity filter of KCNJ5, showed either in familial form of PA either in much rarer syndromic form of PA, APA, allow other ions to flow across the membrane changing the electrolytic composition of cytoplasm. Because of selectivity loss, Na^+ ions, driven by their electrochemical gradient, start to flow from outside to inside depolarizing the potential membrane. The aim of this study is to understand the molecular mechanisms by which wild type (WT) and mutated forms of KCNJ5 can modulate the resting membrane potential, leading to an overproduction of aldosterone.

Starting from the awareness that genetics plays an important role in the development of several diseases, including hypertension, we moved our attention on investigating the role that singular nucleotide polymorphisms of important genes have in the blood pressure modulation and cholesterol biosynthesis in a rural unselected population. We focused our attention on several genes of the hypertensive pathway, such as angiotensing-converting-enzyme (ACE), angiotensinogen, beta-1 and beta-2 adrenergic receptors, angiotensin-II receptor-1 (ATII-R_1), and aldosterone synthase (CYP11B2); further we analysed other two genes, hydroxyl-methyl-glutaryl-CoA-reductase (HMGCoA-reductase) which catalyses the first step of the endogenous biosynthesis of cholesterol, and Kinesin-like-protein 6, which seems to be involved in the intracellular transport of organelles. Selected polymorphisms of these genes were analysed firstly by using Real-Time PCR and Real Time RFLP techniques for the genetic discrimination and then by using an informatics approach with the aim to find a statistically significant correlation between genotype and hypertensive phenotype for each subject.

6.0 Material and methods

6.1 Genomic DNA extraction and sequencing

Peripheral venous blood samples were collected from each patient and genomic DNA was extracted using standard method from fresh whole blood. In brief, we collected blood in either a heparin or EDTA-containing *Vacutainer* by venepuncture. The blood extracted was stored at room temperature and DNA was extracted within the same working day. We firstly from whole blood prepared a leucocytes pellet placing 3mL of blood in a 15 mL falcon tube and added 7mL of reagent A and mixed by inversion for 5 minutes. The lysis was considered completed when the solution had a clearish red colour. Subsequently, we centrifuged at 3000g for 5 minutes at room temperature (RT) and then we sucked the supernatant from the white pellet present on the bottom of the tube and added 1mL of reagent A and mixed to make the pellet completely dispersed. In the last step, we added 6mL of reagent A, mixed by inversion and centrifuged again at 3000g for 5 minutes at RT; we discarded the supernatant into waste container to get a clear/white/purer pellet. 500µL of 5 M sodium perchlorate were added and a 1000uL pipette was used to mix the pellet by drawing it up and down until it was fully dispersed. We added 2mL of reagent B and briefly inverted to mix; tubes were placed in water bath at 65°C for 2 hrs. We allowed the tubes to cool to RT and added 2mL of ice-cold chloroform and then mixed on rotating mixer for 30 to 60 minutes. The solution was centrifuged at 3000g for 10' and then 1.5/2.0 mL of aqueous layer were carefully removed into a new, clean microfuge tube. 3mL of ice-cold 100% ethanol were added; the solution was mixed by inversion to precipitate the DNA. Supernatant was discarded and DNA was washed with 2 mL of ice-cold 70% ethanol. Ethanol was then removed and DNA was left to air-dry. 200 µL of TE buffer were added to resuspend our DNA, which was stored at –20 °C to –80 °C for long term. Concentration and purity of DNA extracted were checked spectroscopically using a NanoDrop instrument.

The entire coding sequence (exon 2-3) and flanking regions of KCNJ5 were resequenced by using polymerase chain reaction technique in order to look for rare variants that might be important for the pathophysiology of sporadic primary aldosteronism. PCR was performed on 25ng of gDNA in a final volume of 30µl containing 1.5mM $MgCl_2$, 0.2mM of each primer forward (F) and reverse (R) (Table n.5), 0.2 deoxynucleotide triphosphate and 1.0 unit of Taq DNA polymerase (Life technologies corporation, Australia).

KCNJ5_1F	***GTGGCCTTCCATCTTGTGTT***
KCNJ5_1R	***GAGGGTCTCCGCTCTCTTCT***
KCNJ5_2F	***CCATTGAGACCGAAACAACC***
KCNJ5_2R	***GGCACAGACCTGCATCTTCT***
KCNJ5_3F	***ATTGATGGATGAATGGATGAATG***
KCNJ5_3R	***AGCCCCCAAACAGCACACTC***

Table. n.5 *PCR primers used to amplify the coding and flanking regions of human KCNJ5*

6.2 Mutagenesis site specific and X-Gold cells transformation

To reproduce and study the same mutations that have been previously identified by resequencing of coding and flanking regions of human KCNJ5 gene, we used an *in vitro* approach based on the site-specific mutagenesis technique. We used pCMV6-AC-GFP plasmid as a vector (Origen) (Fig. 8)

WT KCNJ5 cDNA and pCMV6-AC-GFP vector were undergone to the activity of the same restriction enzymes to produce in both sticky ends that are complementary each other. A subsequent reaction was catalysed by a ligase enzyme which is able to promote the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA. The plasmid used expresses for green fluorescent protein (GFP), which gives the advantage to monitor the expression of our cDNA, and also it expresses for an ampicillin resistance gene, giving us the possibility to select only bacteria and/or cells that have been actually transfected.

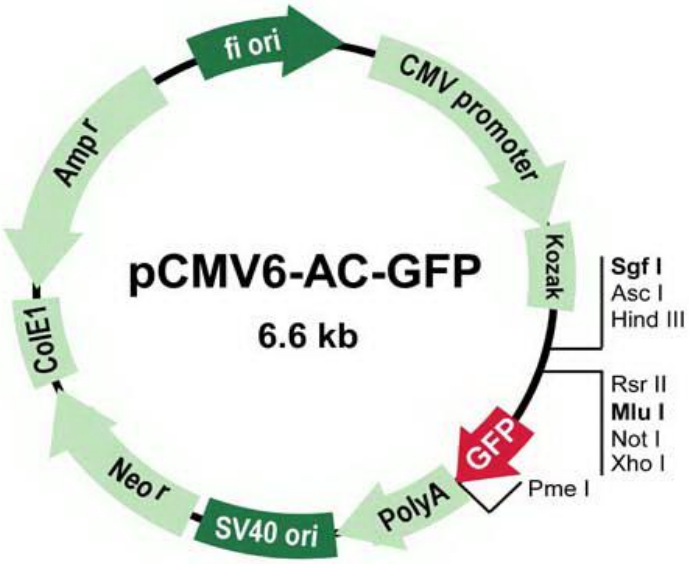

Fig.8 *Organization of pCMV6-AC-GFP plasmid used as a vector for our KCNJ5 cDNA*

Once WT KCNJ5 cDNA was added to the plasmid vector, the next step was to produce a site-mutated cDNA. This was done with a commercial kit *QuikChange II XL Site-Directed Mutagenesis Kit* (Agilent Technologies). Several approaches to this technique have been published, but these methods generally require single-stranded DNA (ssDNA) as the template and are labor intensive or technically difficult. *QuikChange II XL Site-Directed Mutagenesis Kit* shows many advantages comparing with others kit; indeed, it is specifically optimized for large, difficult constructs and allows site-specific mutation in virtually any double-stranded plasmid DNA (dsDNA) and it eliminates the need for sub-cloning and for ssDNA rescue. Furthermore the *QuikChange II XL site-directed mutagenesis kit* does not require specialized vectors, unique restriction sites, multiple transformations or in vitro methylation treatment steps. The rapid three-step procedures generate mutants with greater than 80% efficiency.

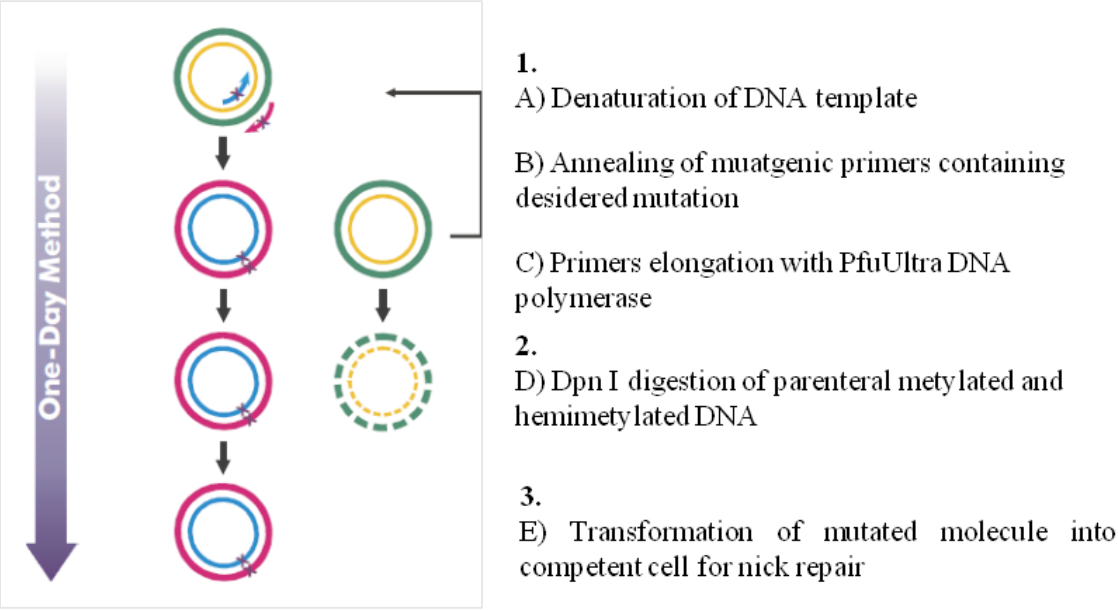

Fig.9 *Overview of the site-directed mutagenesis protocol: there are 3 different steps through which it is possible to produce a specific mutation, such as insertion/deletion, and point mutation.*

Site-directed mutagenesis approach is based on the use of supercoiled double strands DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers, both containing the desired mutation. The oligonucleotide primers, each complementary to opposite strand of the vector, are extended during temperature cycling by *PfuUltra* HF DNA polymerase. Extension of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. In last cycles, products are treated with Dpn I endonuclease, which is specific for methylated and hemi-methylated DNA, that will be digested.

For our mutagenesis reactions, we prepared a sample reaction solution constituted by a 5uL (10x) of reaction buffer, 1µL of dsDNA template (5-50ng set up tests), 1.25µL of primers mix (125ng), 1µL of dNTP, 1µL of polymerase, and water up to 50µL. Each sample reaction solution was undergone to PCR technique. The first step of this reaction is the denaturation of the double strands that is realized at 95 °C for 1 minute; during the annealing phase during which our primers interact with the DNA template temperature was maintained to 95 °C for another minute and then was lowered to 60 °C for 50'', and increased again up to 68 °C for 8 minutes (1min/kb). The reaction was continued for 18 cycles at the end of which the temperature was maintained at 68°C for 7' and then lowered to 4 °C to cool the reaction solution. Once the mutagenesis and the amplification were stopped, in each reaction solution

1µL of Dpn I endonuclease was added and left at 37 °C for 1h to digest the DNA template in order to leave the only site-mutated DNA.

The nicked vector DNA, incorporating the desired mutation is then transformed *into X-Gold ultracompetent cells*. These cells were thawed on ice and 30-50µL per reaction were aliquoted into pre-chilled 15mL tubes; 1µL of site-mutated DNA was added to cells and incubated on ice for 30'. Cells were then immediately incubated in a pre-warmed bath at 42°C for 45'' and on ice again for 2'. At the end of this incubation 250µL of *lysogeny broth (LB),* better known as LB medium, were added to the reaction cell solution and incubated at 37°C for 1h:30' with shaking 250rpm. 40-50µL of the medium containing cell were spread *according the dilution method* onto LB ampicillin agar dishes and cultured at 37°C overnight until colonies appeared (Fig. 10).

Fig.10 *Colonies of X-Gold ultracompetent cells transformed with site-mutated KCNJ5 cDNA, growth and selected onto LB ampicillin agar*

A single colony from each dish, as shown in *Fig.10,* was picked up and grown overnight in 4mL of LB medium containing ampicillin (stock solution 1mg/mL) diluted 1:1000µL at 37 °C and 250rpm (so called miniPrep). The plasmid DNA was then collected using the *Roche mini prep kit* and this DNA was sequenced to determine if the mutation was actually present, and to ensure that the rest of the KCNJ5 cDNA was not mutated by accident.

DNA sequencing was done starting from a reaction solution constituted by 300-400ng DNA, 1.5uL of primer, 2uL master mix, 2uL of diluent, H2O to 20uL. This solution was undergone to a PCR reaction (95 °C 30s, 50 °C 30s, 60 °C 4min for a 35 cycles) and the DNA amplified was then precipitated adding 1uL Glycogen, 2uL 3M Sodium Acetate, and 1uL

0.1M EDTA, followed by 60uL of cold 95% ethanol. The solution was mixed and transferred to a PCR 96 well plate, that was centrifuged (1100 rcf) at 4°C for 30'. The supernatant was discarded by inverting the plate onto the tissue paper and 200uL of cold 70% ethanol were added; these steps were repeated two times changing only the duration of centrifuge that was lowered to 10'. Plate was left to air dry at a room temperature for 10-15'and then filled with 40uL of loading buffer (formamide) and a drop of mineral oil to the top of each sample. Plate was so loaded in a sequencing machine.

Once the sequencing reaction was finished and mutation was ascertained, a large bacteria culture, so called maxiprep, was prepared. We added 100µL of miniprep solution in 100mL of LB medium (+ampicillin) and left overnight at 37 °C. In the next day bacteria were collected by centrifugation at 4000g at 4°C for 30'. The supernatant was threw away and pellet used for DNA extraction with a Qiagen Plasmid Maxi kit. Purity and concentration of DNA were checked spectroscopically using a NanoDrop instrument. The DNA extracted was in part used to make the bacterial stab by adding pure glycerol and stored at -80°C; and the remaining part was used for our experiment.

6.3 RNA synthesis

cRNAs of KCNJ5 and KCNJ3 constructs were created using the Ambion mMESSAGE mMACHINE T7 kit according to the manufacturers (*life technologies).* This commercial kit is indicated for the routine synthesis of capped RNA for oocytes microinjection, and *in vivo/in vitro transfection.* Constructs of human KCNJ5 and KCNJ3 under the T7 promoter in the pGEM vector (Fig. 11) were a generous gift from Dr. Nicole Schmitt (Danish Arrhythmia Research Centre).

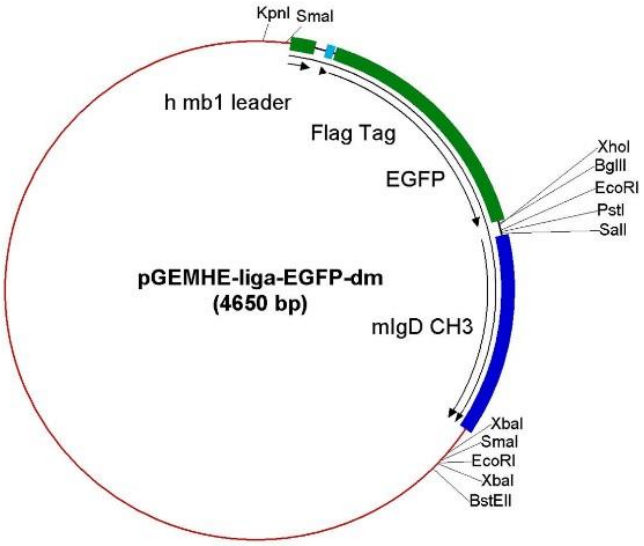

Fig.11 *pGEM vector structure used to run the cRNA transcription*

The pGEM constructs were linearized using the restriction enzyme *NotI*. 1 µg of purified, linearized DNA was used as starting material and all further steps were carried out following the manufacturer's instructions. The incubation period at 37 °C was increased from one hour to two hours to ensure maximum yield. The RNA was then purified using the RNeasy Mini kit (Qiagen) before it was analysed and quantified by UV spectrophotometry and gel electrophoresis in reducing conditions.

6.4 Oocytes preparation

Female *Xenopus laevis* frogs were anaesthetized with 3-aminobenzoic acid ethyl ester and the ovaries were removed through a midline abdominal incision. The oocytes were then placed into a Petri-dish with ND96 solution (96 mM NaCl, 2.0 mM KCl, 1.0 mM, $MgCl_2$ and 5.0 mM HEPES buffer, pH 7.4 without $CaCl_2$) containing 1 mg/ml collagenase (from *Clostridium histolyticum*, Sigma) and placed on a rocker for 45–60 minutes at RT. After the removal of collagenase by washing the oocytes 3 times in ND96, stage V and VI oocytes (sharp border between animal and vegetal poles, diameter 1–1.2 mm) were selected and manually defolliculated using ultra-fine forceps. The defolliculated oocytes were stored in ND96 overnight at 17 °C. Microinjection needles were pulled from 0.53 mm diameter glass capillaries (Drummond Scientific) using the Narishige PC-10 micropipette puller heated to 58.1° C. The needle tips were broken off manually by tapping them gently on a metal rod.

The size of the tip was assessed by the Narishige MF-900 microscope (tip diameter 15–25 µm). cRNA injections were performed using the Drummond Nanoinject automatic injector. Prior to loading the microinjection needle with the cRNA, it was backfilled with non-fluorescent mineral oil (Sigma) to seal the pipette from air. 2 µl cRNA was then loaded into the needle by suction and oocytes were placed onto a Plexiglas well under a microscope (Narishige). Each oocyte was injected with 10–20 ng of KCNJ3 + KCNJ5 wild type or mutant cRNA (injection volume 50nl). Coinjection is necessary because it has been seen that KCNJ5 heteroetramerizes with KCNJ3 to form a functional channel. Water-injected oocytes were used as a control. After injection, oocytes were incubated at 17 °C in HEPES-buffered ND96 containing 2 mmol/L sodium pyruvate and 0.1 mg/mL gentamicin at 18°C for 2 to 3 days before use. The ND96 solution was changed every two days and damaged oocytes removed. After 3–5 days, protein expression was assessed using confocal microscopy and the oocytes were used in two-electrode voltage clamp experiments.

6.5 Two electrode voltage clamp

Two-electrode voltage clamp technique was used for the electrophysiological studies. The instrument presents microelectrodes that were filled with 3 M KCl (1 to 2 MΩ) for voltage sensing and current passing. External voltage and current electrodes consisted of fine, chloride coated silver wires. Oocytes were held in a small chamber and perfused continuously with ND96 (2 ml/min) at room temperature (18 to 20°C). Current–voltage (I-V) plots were obtained from voltage step protocols ranging from −140 to +100 mV in 20 mV increments. Oocytes were held at each voltage step for 500 ms, with 100-ms intervals between the voltage steps. For the I-V plots, the steady state current at each voltage step was used. Oocytes were perfused continuously with the high-Na^+ ND96 (2 mL/min) at room temperature (18°C–20°C). To study selectivity, the voltage step protocol was performed first in the high-Na^+ ND96 (92 mmol/L Na and 2 mmol/L K) solution before the perfusing solution was switched to high-K^+ (45 mmol/L K) and the I-V plots recaptured. The high-K^+ bath was finally changed to high-K^+ containing 2 mmol/L $BaCl_2$, and a second set of I-V plots was obtained. The KCNJ5 current was the difference between these 2 curves. In experiments where the specific channel blocker tertiapin-Q was used (Ascent Scientific), currents were measured in high-K^+ in the presence and absence of the blocker (10 nmol/L). The clamp potential of the two-electrode voltage clamp amplifier (OC-725C amplifier; Warner Instruments, Hamden,

CT) was controlled using the program Pulse and Pulsefit (HEKA Electronic, Lambrecht, Germany) in conjunction with an ITC-16 interface (Computer Interface Instrutech Corp., Long Island, NY). Data were filtered at 1 kHz

6.6 H295R cell culture

Human adrenocortical carcinoma cell line, NCI-H295R, obtained from the American Type Culture Collection (ATCC), was used for *in vitro* studies. The cells were grown in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 Medium (Sigma). For routine maintenance the medium was supplemented with 5% of fetal bovine serum (FBS), 1% of insulin/transferrin/selenite mixture (1.0 mg/ml recombinant human insulin, 0.55 mg/ml human transferrin (substantially iron-free), and 0.5µg/ml sodium selenite, stock solution 100x), 1% of penicillin/streptomycin solution (100 units/ml penicillin, and 100 µg/ml streptomycin), 1x of L-glutamine (stock solution 100x). Cells were grown at a 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2.

Cells were grown in a 75cm^2 flask up to a 65-70% of confluence, after that cells were splitted in a new flask. For splitting, the medium was threw away and cells were washed two times with 5mL of Phosphate Saline Buffer (PBS). After washing, 2 mL of 1x Trypsin-EDTA solution (0.025%) were added and incubated for 5' into the incubator at 37 °C. Because of trypsin toxicity, after incubation a double volume of medium was added to the flask and cells were collected in a 15mL falcon tube and then centrifuged at 1000g for 5'. The supernatant was discarded and cells were resuspended into a complete medium and splitted according to the need in new flasks.

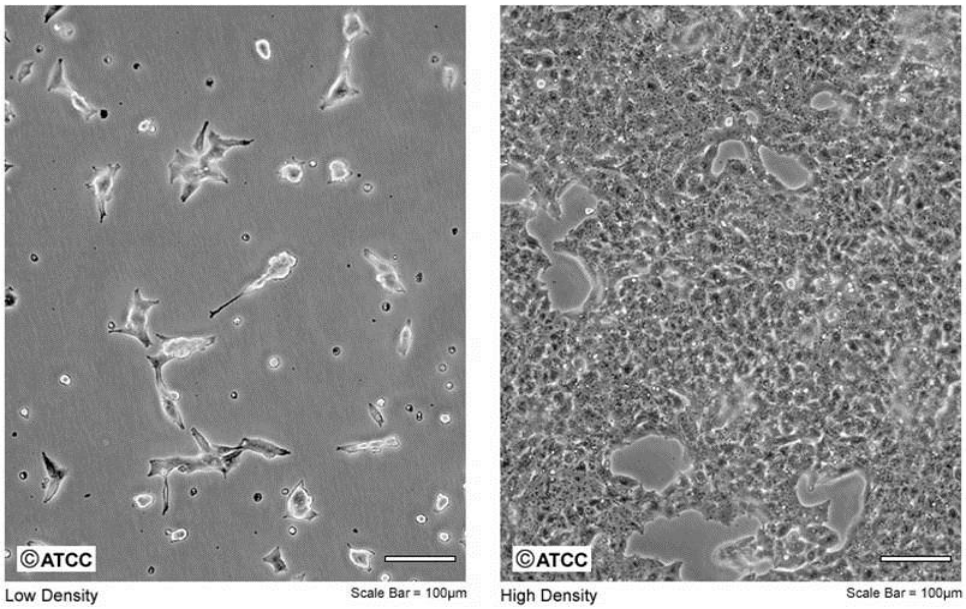

Fig.12 *H295R human adenocarcinoma cell line used for in vitro studies in two different state of confluence; low confluence (left panel) and high confluence (right panel). ATCC company.*

6.6.1 Cell counting

Cell counting was done whenever we needed to know the exact number of cells to seed in a 24-well plate. Cells were detached according to the protocol above mentioned. In brief, cells were washed two times with a PBS solution and then incubated for 5' at 37°C with 2mL of trypsin solution. After incubation ,cells were collected with normal medium, and centrifuged at 1000g for 5'; finally, cellular pellet was resuspended into 1mL of complete medium. Of the cellular solution 10µL were diluted in 90µl, and of this solution 10µl were taken and mixed with an equal volume of Trypan Blue solution, pipetting up and down. 10µL of this solution was loaded into a Neubauer chamber and cell number was counted under an inverted microscope (focus 10x).

6.6.2 H295R transfection

The plasmid constructs were transiently expressed in H295R cells by electroporation using the Nucleofector device and reagents from the Nucleofector kit R according to manufacturer instructions (Lonza).

The day of transfection NCI-H295R cells (60-70% of confluence) were counted as aforementioned and $2x10^6$ cells/well were aliquoted in a 1.5mL tube. Cells were centrifuged at 90g for 15 minutes. The supernatant was gently discarded and cellular pellet was carefully resuspended up and down with 100µL of pre-warmed nucleofection solution, to which 3µg of the plasmid KCNJ5 DNA (WT or mutated) were added. Transfection solution was then transferred into an Amaxa cuvette for the electroporation. The electroporeted solution was then resuspended with a volume of pre-warmed RPMI up to reach 500µL and incubated at 37 °C for 15'; this solution was seeded gently in the right well. Cells were incubated for 12hrs at 37 °C, after that the transfection solution was replaced with 500µL of fresh medium. Transfected cells were monitored to the fluorescence microscope and the transfection efficacy, typically 80% to 90% (Fig. 13) was assessed using the green EGFP fluorescence signal measured by fluorescence-activated cell sorting (FACS Calibur machine, bdbiosciences). The percentage of transfected cells (GFP-positive cells) was then used to normalize aldosterone release values from each well.

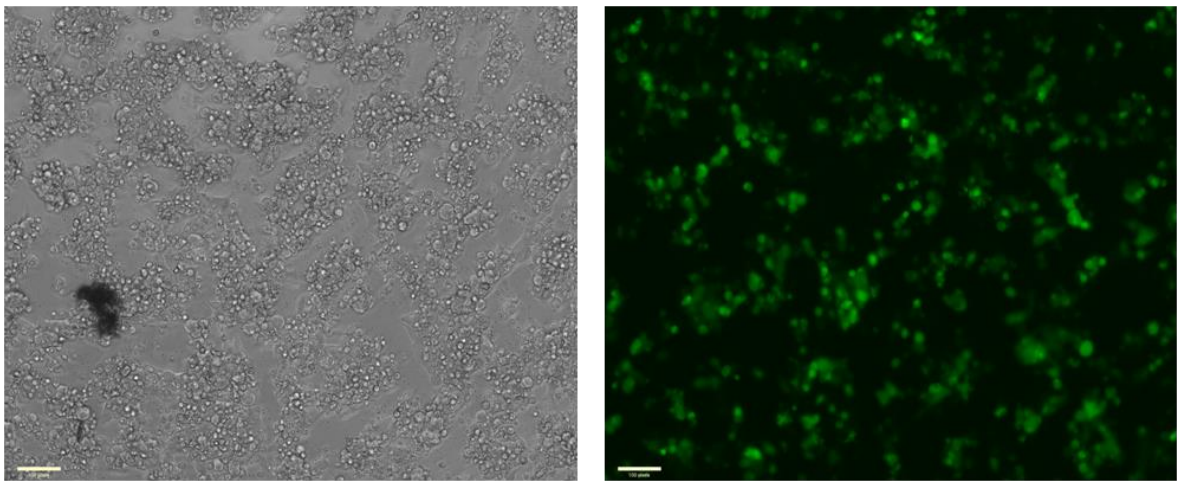

Fig.13 *Fluorescence microscope image of H295R transfected cells with a WT KCNJ5. The same picture was acquired with a fluorescence filter turned off (left panel), and turn on (right panel). Transfected cells are well characterized by the green fluorescent colour.*

6.6.3 Aldosterone release and radioimmunoassay technique

As aforementioned cells were transfected by electroporation and incubated for 24h at 37 °C before to replace the transfection solution with the normal medium. For the aldosterone

release experiment the electroporation solution was substituted with a complete medium without antibiotics and FBS, and they were grown on either in the presence or absence of ATII (10 nmol/L) for a further 24 hours. The supernatants were then collected for aldosterone assay using 200μL aliquots of the culture supernatant and the commercial Coat-A-Count [^{125}I]-aldosterone radioimmunoassay.

Once the aldosterone powder was dissolved with the right volume of deionized or distilled water, a serial dilutions (12.5, 25, 50, 100, 200, 600, 1200 pg) were done in normal plastic tubes and 200μL of stock solution were then transfered in the standard curve polypropylene tubes coated with antibodies (in duplicate). Furthermore, we prepared for each sample two tubes, where we transferred 200μL of the supernatant medium previously collected from the 24-well plate. Lastly, we added 1 mL of iodinated radioactive aldosterone solution (^{125}I Aldosterone) in each standard and sample tubes plus 2 blank and 2 total tubes. Our samples were incubated for 18h at RT, and at the end of the incubation period the radioactive solution was removed from each tube of standard curve, samples, and blank, except from total. The radioactivity was quantified through the beta-counter machine.

6.6.4 Cell viability and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The MTT (3,(4,5-dimethylthiazol-2)2,5-difeniltetrazolium bromide) assay is a standard colorimetric assay used to measure the vitality of the cells or a cytotoxicity of a drug/substance on your cell line. This assay is based on the capability of a particular enzyme, succinate dehydrogenase, located into the mitochondrial compartment and active only in the alive cells. This enzyme split the thiazolyl tetrazolium in a formazan salt, insoluble in hydrophilic solvent but soluble in DMSO or in HCl-isopropanol solution, obtaining a violet solution. The intensity of the solution is directly proportional to the concentration of the formazan salt, which in turn is an expression of cell vitality. Cell viability was measured using an MTT kit (http://www.millipore.com) according to manufacturer instructions. In brief, H295R cells (300.000 cells/well electroporeted with different KCNJ5 mutant forms as above) were seeded into 96 well-plates and incubated at 37°C. The medium was changed after 24 hours and grown on either in the presence or absence of ATII (10 nmol/L) for a further 24 hours before adding the MTT reagent solution. Medium was replaced with 100μL of MTT solution (1x) and cells incubated for 2hrs at 37 °C. At the end of incubation, 96-well plate was

centrifuged at 4 °C for 30minuts at 2500g to precipitate the formazan salts. Once the supernatant was carefully removed paying attention to no remove formazan salts, 100µL of DMSO were added to solubilize them. Absorbance was immediately read with the ELISA plate reader at 540nm. Results have been obtained as optical density OD.

6.6.5 Statistical analysis

All data are presented as mean±SEM. Comparisons between groups was by ANOVA with post hoc testing using GraphPad/Prism5 for Windows software. Statistical significance was set at $P < 0.05$.

6.7 Brisighella population recruitment

Brisighella Heart study is a prospective population-based study, started in 1972, involving 2939 randomly selected individuals, free of cardiovascular disease at enrolment, all resident in the northern Italian rural town of Brisighella. Brisighella was chosen as a typical example of a rural community in the process of industrialization, with a very low rate of immigration/emigration and above all with homogeneity of lifestyle habits. Participants were cyclically evaluated, every 4 years following enrolment, during which lifestyle informations, and physical and biochemical data were obtained and recorded into a database. Inclusion and exclusion criteria were used to select the population:

- being of historical population of Brisighella study,
- aging from 30-70 years old

were used as inclusion criteria, while

- peoples of different race from Caucasian,
- peoples with secondary hypertension or affected by severe diseases

were excluded from the study.

Brisighella heart study protocol was approved by the Ethical Committee of the Bologna's University, and from each patient we obtained the informal consent.

6.7.1 Brisighella blood samples collection

Blood samples were collected in the morning after a fasting period of 12h (overnight) in heparin-enriched tubes. DNA was extracted in the same date of collecting samples or after a short storage at -80 °C. Blood samples were used for biochemical and physical analysis, such as LDL, HDL, total cholesterol level (TC), triglyceride, APO-A1, APO-B quantification. Furthermore, anthropometrics parameters such as systolic and diastolic blood pressure, heart rate, breath rate, metabolic syndrome diagnosis, non-alcoholic fatty liver disease, were checked. Cholesterol level was defined high in case of TC > 200mg/dL, or lipid lowering therapy. Three blood pressure (BP) readings were recorded, and hypertension was defined as systolic BP $\geq$ 130 mmHg and diastolic BP $\geq$ 85 mmHg, or use of antihypertensive medication.

Genomic DNA was isolated from 200µl EDTA-anticoagulated whole blood using the QIAamp DNA Blood kit (Qiagen, Hilden, Germany) according to manufacturer. In brief, after

treatment with lysis buffer, the kit provides proteinase K, commonly used to digest proteins, to remove contamination and inhibits nucleases that might degrade DNA. The lysate buffering conditions are adjusted to allow optimal binding of the DNA to the silica membrane of the QIAamp spin column. To improve the purity of DNA eluted, column was washed two times with two different wash buffers (Buffer AW1 and Buffer AW2). Purified DNA was lastly eluted from the membrane with 100µl of elution buffer (AE). Concentration and purity of DNA extracted, were checked spectroscopically using a NanoDrop instrument at a wavelength. of 260nm and DNA was stored at -20 °C.

6.7.2 Genotyping analysis, PCR restriction fragment length polymorphism (RFLP) and Real time PCR methods.

DNA samples were diluted with a DNAase free water in 96-well plate in a concentration of 10ng/µl and stored at -20 °C. Genotypes were determined by RFLP and/or the Real-Time PCR approaches according to published methods or as recommended by manufacturer. The genotype results were confirmed by repetition of 70-80% of the samples. Negative controls were included in each reaction as quality control.

The principle components to perform a PCR are primers, containing sequences complementary to the target region, DNA polymerase, which is necessary to enzymatically assemble a new strand of DNA, deoxynucleoside triphosphates (dNTPs), the building blocks from which the DNA polymerase synthesize a new DNA strand, buffer solution and usually divalent cation that helps the reaction (i.e. $MgCl_2$).

Components	Final concentration
Water	**0.52X**
10x Buffer	**1X**
MgCl2 solution	**2.00 mM**
dNTPs	**0.15mM**
Forward primer	**0.13mM**

Reverse primer	**0.13mM**
Taq Polymerase	**0.03 U/µl**

Table n.6 *Reagent used to make a Real time RFLP master mix*

DNA was added at this mixture and the thermal cycling program was performed. The DNA is replicated in every cycles and the amount increased exponentially. After the amplification of the sequence of interest, the appropriate restriction enzyme was used to recognize the SNP, if present, and cut the DNA strand at this level, creating DNA fragments. The DNA restriction fragments, obtained after an overnight incubation at RT, were then separated electrophoretically at 200 Volt (V) for 30', on home-made or pre-cast polyacrylamide gel 10% TBE (Bio Rad, Hercules, CA, USA). The DNA band was then visualized by using ethidium bromide, an intercalating agent commonly used as a fluorescent tag. When exposed to the UV ray, its fluorescence is 20-fold higher after binding DNA. The image of the gel was acquired using a digital photo camera connected to VERSADOC-4000 (BIO RAD) and visualized with Quantity ONE software.

There are three different situations that can emerge from the analysis: homozygosis for the WT, homozygosis for the SNP, and heterozygosis in which only one of the two alleles shows the SNP while the other allele is WT.

Real time PCR or quantitative PCR (qRT-PCR) is a variation of the standard PCR technique used to quantify DNA or mRNA in a sample. Using sequence specific primers, the relative number of copies of a particular DNA or RNA sequence can be determined. Quantification of amplified product is obtained using fluorescent probes and specialized machines that measure the fluorescence while performing temperature changes needed for the PCR cycles. More specifically for our use, an allelic discrimination assay was used to detect variants of a single nucleic acid sequence. The presence of two primer/probe pairs in each reaction allows genotyping of the two possible variants of a polymorphism site in a target template sequence. One fluorescent dye detector is a perfect match to the WT (allele 1) and the other fluorescent dye detector is a perfect match with the SNP (allele 2). The allelic discrimination assay classifies unknown samples as: 1) homozygotes (sample having only allele 1 or 2) and 2) heterozygotes (sample having both allele 1 and 2). The allelic discrimination assay measures the change in the fluorescence of the dyes associated with the Taqman probes VIC$^{(R)}$ and FAM^{TM} (Applied Biosystem, Foster City, CA), that selectively bind wt or SNP allele. The reaction was prepared using 10ng DNA, 20X or 40X Taqman

genotyping assay mix, 2X Taqman(R) Universal Master Mix, No AmpErase(R)UNG and water RNAase free, for a total volume of 25µl. Each reaction was run in presence of a negative control, no template control (NTC). The analysis was performed using Real Time PCR System 7300 (Applied Biosystem).

The allelic discrimination is characterized by three different steps: **a)** a pre-read run, using an allelic discrimination plate that measures the fluorescence at the baseline; **b)** an amplification run, using a standard curve plate document to generate Real-Time PCR data that consist in 95 °C for 10 min, 40 cycles at 92 °C for 15 sec, and 60 °C for 1 min; **c)** finally, an allelic discrimination run (post-read run), using the allelic discrimination plate to analyse the data and discriminate the genotype, after removing the baseline fluorescence. Allelic discrimination assays use the fluorogenic 5' nuclease chemistry: fluorogenic probes, reporter (R), are used to detect specific PCR product as it accumulates during the PCR cycles. Additionally, the two probes at the 3' end bind the quencher (Q) and a minor groove binder (MGB) to improve the probe affinity for the target DNA.

The process could be described in 4 passages: *a)* polymerization: R and Q are attached to the 5' and 3' end of a probe; *b)* strand displacement, when both dyes are attached to the probe, R dye emission is quenched; *c)* cleavage, during each extension cycle: the DNA polymerase system cleaves the R dye from the probe; *d)* completion of polymerization: the R dye emits its characteristic fluorescence (Fig. 14). The signal is extremely specific because the probes do not interfere and the fluorescence signal is present only when the binding between probes and target DNA is correct.

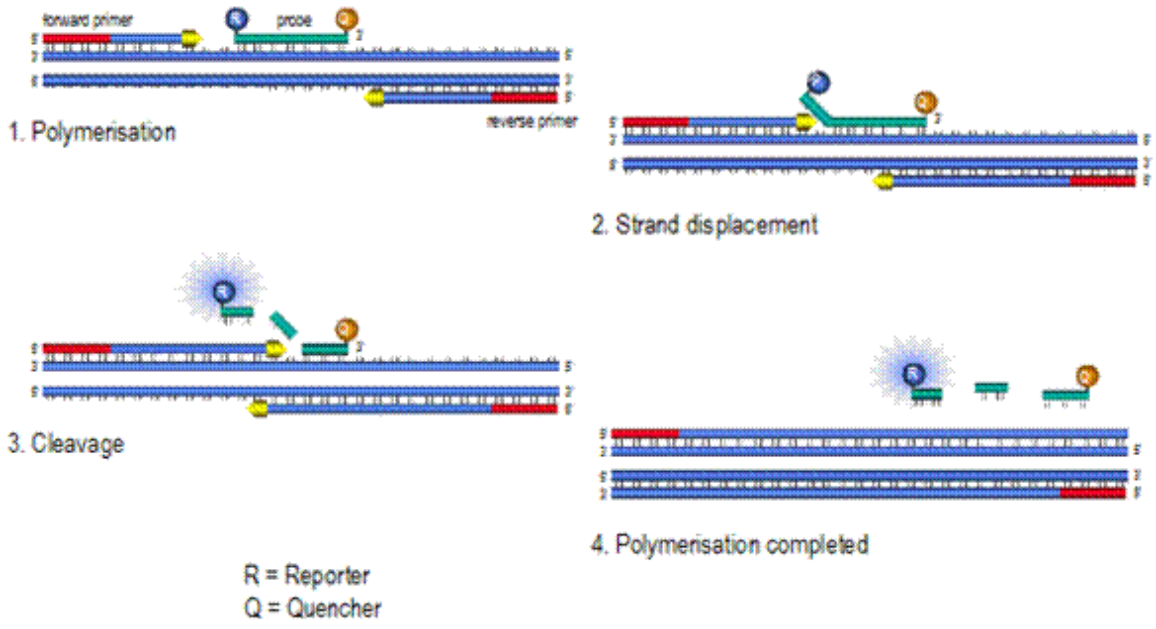

Fig.14 *Molecular basis for Taq-man based allelic discrimination processes: a) polymerization, b) strand displacement, c) cleavage, d) completion of polymerization.*

In the table below are reported the most important informations about the genes involved in the blood pressure regulation and lipid metabolism regulation, that we have analyzed in our population study.

Table n.7 *In this table are summarized the most important informations of our genes of interest: name of gene analyzed, singular nucleotide polymorphism variation, type of mutation (synonymous, and non-synonymous, etc…), primers ID, and the technique used to make the analysis.*

Name of Gene	***Primer assay***	***SNP***	***Mutation***	***Method of analysis***
HMGCoA	*rs3846662*	***C****/T*		*Real-Time PCR*
	rs3761740	*A/**C***		*Real-Time PCR*
KIF6	*rs20455*	***C****/T*	*Missense Arg>Trp*	*Real-Time PCR*
	rs9471077	*A/**G***		*Real-Time PCR*
	rs9462535	***A****/C*		*Real-Time PCR*
AGT	*rs699*	***C****/T*	*Missense Thr>Met*	*Real-Time PCR*
AGTR1	*rs5186*	***A****/C*		*Real-Time PCR*
ACE	*1r/1f 2r/2f*		*Inser/Del*	*PCR RFLP*
CYP11B2	*rs1799998*	*C/**T***		*Real-Time PCR*

6.7.3 Statistical analysis

To assess the association between genetics characteristics and blood pressure, odds ratios (HR) with a 95% confidence interval (95% CI) were estimated by using a multivariate logistic regression analysis. Gender, age, cholesterolemia, body mass index, and waist circumference were used as covariates in addition to the genotype.

The distribution of genotypes was tested for *Hardy-Weinberg equilibrium (HWE)*, and qualitative data were analyzed using a X^2 test. Similarly, allele and genotype frequencies in different groups were compared by X^2 analysis. *P-value* was calculated between two subgroups of patients, hypertensive and not hypertensive, and alleles: no hypertension (allele 2) vs hypertension (allele 0). The level of significance was set at $P < 0.05$.

7.0 Results and discussion

7.1 KCNJ5 resequencing

To realize this study we started from an accurate selection of population patients affected by PA. Our cohort is constituted by 251 white patients, recruited between 1999 and 2006 at either the Greenslopes or the Princess Alexandra Hospitals in Brisbane, Australia. Peoples were accurately investigated for PA phenotype, indeed, several biochemical and physical parameters were measured. Patients were identified on baseline hypokalaemia, a basal upright renin of <4 mU/L and an aldosterone:renin ratio (ARR) of >150. The genetic study was approved by the Ethics Committee of University of Queensland, and informed consent for DNA collection and the genetic study was obtained from all participants.

The entire coding and the flanking regions of KCNJ5 gene were resequenced in all patients and we identified 3 heterozygous missense mutations (Fig. 15), one of which ***(E246K)*** was novel. The other 2 are present on the dbSNP database (***R52H***, rs144062083; ***G247R***, rs200170681) but were not validated.

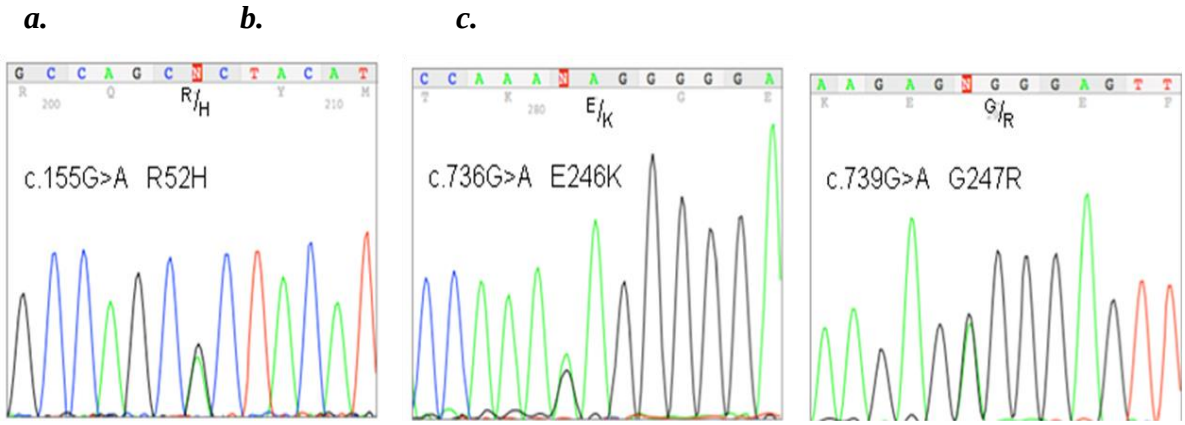

Fig.15 *Sequence chromatograms for the 3 heterozygous missense mutations identified in the cohort. A) It is showed the missense mutation 155G>A that leads to an Arginine (R) – Histidine (H) substitution at the position 52 of peptide chain; B) it is showed the 736 G>A that leads to a Glutamate (E) – Lysine (K) substitution at the position 247; C) in the last panel is represented the substitution 739 G>A, responsible of Glycine (G) – (Arginine) substitution at the position 247 in the protein structure.*

Mutations that we have identified are not present in the selectivity filter but in the immediate neighbouring parts as it is possible to see from the fig. 16 below.

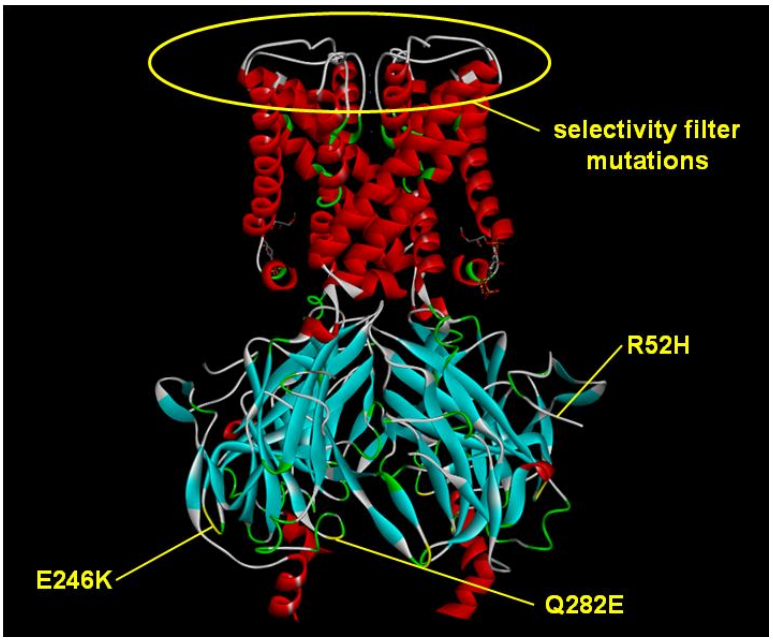

Fig.16 *KCNJ5 potassium channel structure: all the mutations characterized in our 251 population patient, R52H, E246K, Q282E, are not localized in the selectivity filter but in neighbouring region.*

Both mutations R52H and G247R resulted to be very rare, appearing just once (1/4296) in the National Heart and Lung Institute ESP6500, and G247R once (1/661) in the Clin-Seq-SNP cohorts, respectively. Another 12 subjects in the cohort were identified as carriers for the rare non-synonymous SNP (population frequency of 2% [22/1075] in the 1000 genomes cohort), rs7102584 (C/G), which causes a charge-changing amino acidic substitution ***E282Q (Glutamate/Glutamine)*** in KCNJ5. The phenotype of the rs7102584 heterozygous carriers was similar to non-carriers (Table 8), although the values of their aldosterone:renin ratios clustered at the lower end of the distribution for the cohort. The subjects with the missense mutations were noticeably younger (Table 8).

Missense mutation	Sex (F/M)	Age at diagnosis	Aldo (pmol/l)	Renin (mU/L)	ARR	Diagnosis* (APA/BAH)
R52H	M	46	835	5.3	157	BAH
E246K	F	37	365	1.3	290	BAH
G247R	F	38	863	1.5	575	BAH
rs7102584 genotype						
***GC* N=12**	**7/5**	**55.5±11**	**476±244**	**4.3±5.3**	**134±67**	**3/9**
GG N=239	132/107	56.0±9.4	688±446	4.9±4.7	273±321	54/185

Table n.8 *Clinical phenotype of the PA cohort by mutation and rs7102584 genotype*

7.2 Expression of WT and mutant form of KCNJ5 in Xenopus laevis, and its effect on the resting membrane potential

The wild type and mutant forms of KCNJ5 potassium channel were expressed in *Xenopus laevis oocytes* to investigate their influence on the resting membrane potential. Before to analyse the mutant channel behaviour on aldosterone release the electrophysiological activity of these mutations has been previously studied. Two electrode voltage clamp was realized as above in two most important solution, high Na^+ and low K^+ and vice versa.

From the electrophysiological studies resulted that two of three missense mutations, R52H and E246K , and the coding SNP variant, E282Q, widely depolarized the resting membrane potential of oocytes in a high Na^+ bathing solution. Furthermore, it has been seen that the other missense mutation, G247R, did not modify the resting membrane potential, indeed its effect is completely comparable with that of the WT channel, as you can see in fig. 17 below.

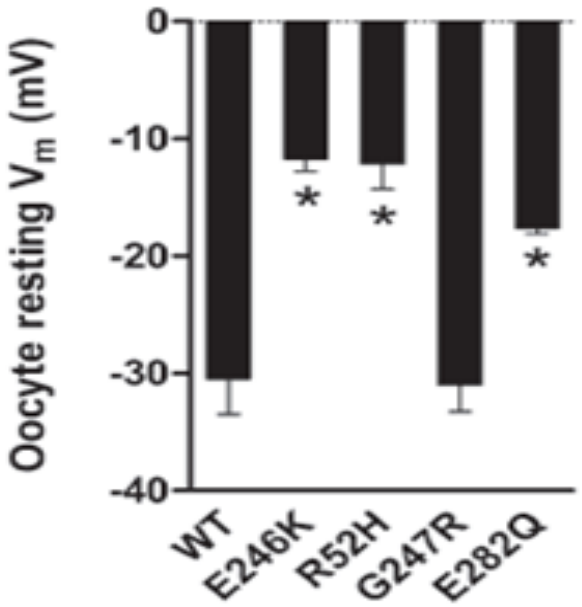

Fig.17 *Different activity of KCNJ5 WT and mutant forms on the resting membrane potential variation in Xenopus laevis oocytes. Great difference has been observed between EK, RH, EQ and WT form (mean±SEM; n=6). It has also been seen that the missense mutation G247R did not exert any effects on this parameter. *Significantly different from WT, P<0.01.*

The steady-state current–voltage curves were generated with high-Na^+ and low K^+ (92 mmol/L Na^+; 2 mmol/L K^+) with then high- K^+ (45 mmol/L K^+; 0 mmol/L Na^+) in the bath solutions to look for evidence of altered Na^+ permeability.

As you can see from the Fig. 17 WT KCNJ5 shows typical inward-rectification in the high extracellular K^+ concentration, while changing the bathing solution with a high Na^+ concentration it was not possible to measure any passage of current. Also as expected, the WT Ba-sensitive K^+ current was almost completely blocked by the specific Kir channel inhibitor tertiapin-Q (Fig. 18). A different result has been obtained with the missense mutations E246K, R52H, and the SNP E282Q. Indeed, the R52H channel showed no clear rectification, and the I-V curve was broadly unchanged by switching to a high extracellular Na^+ solution confirming a substantial loss of K^+ selectivity in this channel. Furthermore, R52H and E246K KCNJ5 mutant forms were not blocked by the specific inhibitor tertiapin-Q, unlike the WT channel, demonstrating that amino acidic substitution may induce a conformational change of the structure reducing the interaction between antagonist and its site.

The E246K mutation behaved similarly to R52H. However, the G247R mutation behaved like the WT channel with clear rectification, and almost no detectable current in the high-Na^+

bathing solution (Figure 17). The E282Q channel behaved like the WT channel in the high extracellular K^+; however, switching to the high-Na^+ solution left the I-V essentially unchanged again, suggesting a substantial loss of K+ selectivity.

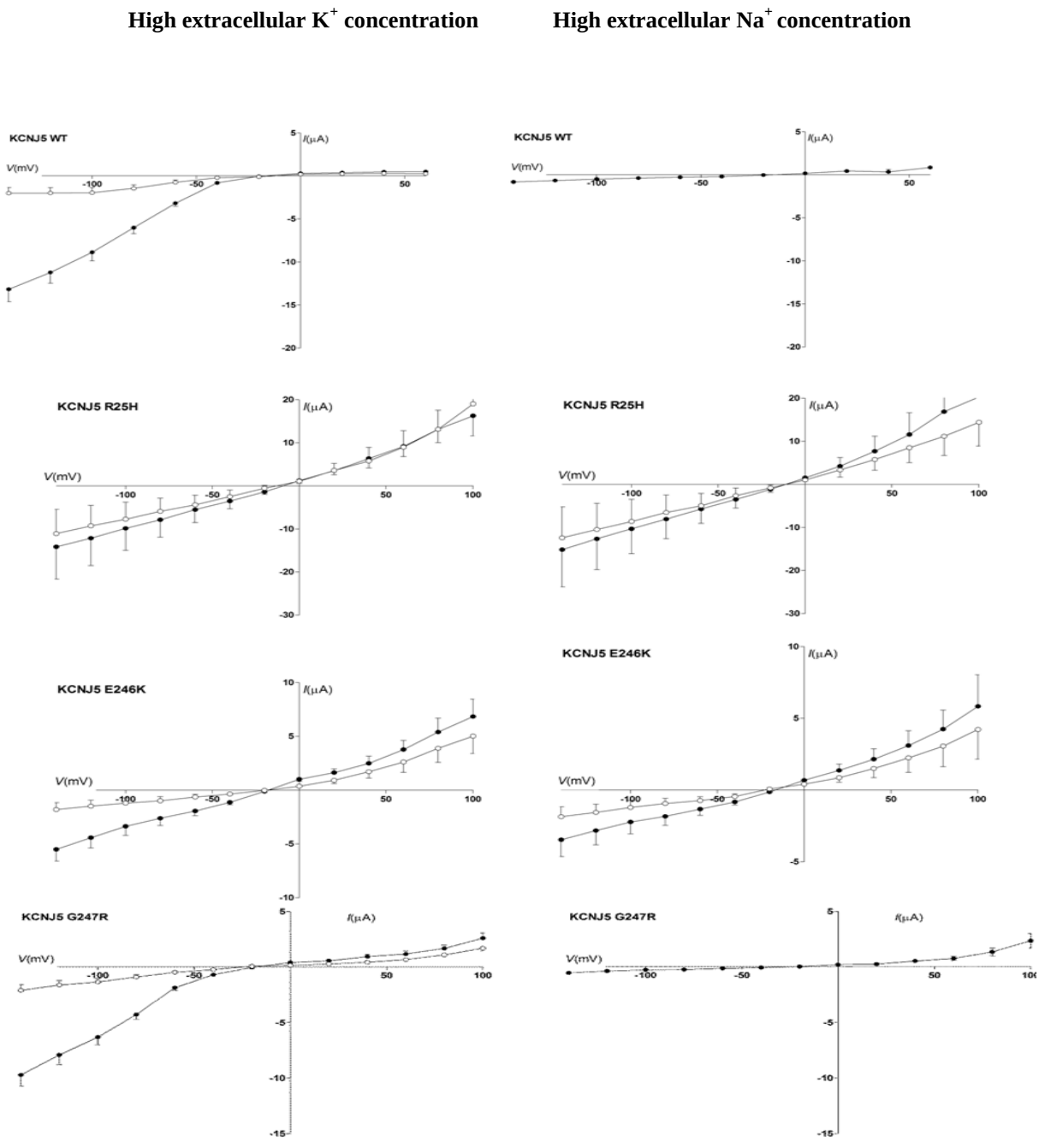

Fig.18 *Current-voltage curves of WT and mutant forms of KCNJ5 potassium channel obtained from clamped Xenopus laevis oocytes in two different bathing solution: high K+ and low Na^+(right) and low K^+ and high Na^+ concentration. The absence or presence of KCNJ5 antagonist, tertiapin-Q (50nM), are indicated respectively by ● and ○ (mean±SEM; n=6-8).*

7.3 Aldosterone release from transiently transfected H295R cells

Once missense mutations and SNP were identified, they were electrophysiologically characterized in the Xenopus oocytes. Furthermore, human adrenocortical cells, H295R, were transiently transfected with KCNJ5 plasmid cDNA opportunely site-mutated with the aim of studying how these genetic variations could affect basal and AG-II-stimulated aldosterone release. Human H295R cell line releases aldosterone either in basal either under AG-II stimulation condition and currently provides the most robust *in vitro* model for the human zona glomerulosa study.

H295R cells were transfected with pCMV6-AC-GFP empty vector as a negative control, and with the previously identified mutation, deletion ***del157,*** as a positive control. Unstimulated H295R cells transiently expressing the variant channels showed an upward trend in basal aldosterone release; however, this was only significant for E246K and the delI157 (Figure 19). In contrast, all the variants significantly increased ATII-induced aldosterone release. The E282Q variant produced a modest ≈1.5-fold increase, whereas the missense mutations produced a larger increase (2–3-fold) similar to the delI157 KCNJ5 control.

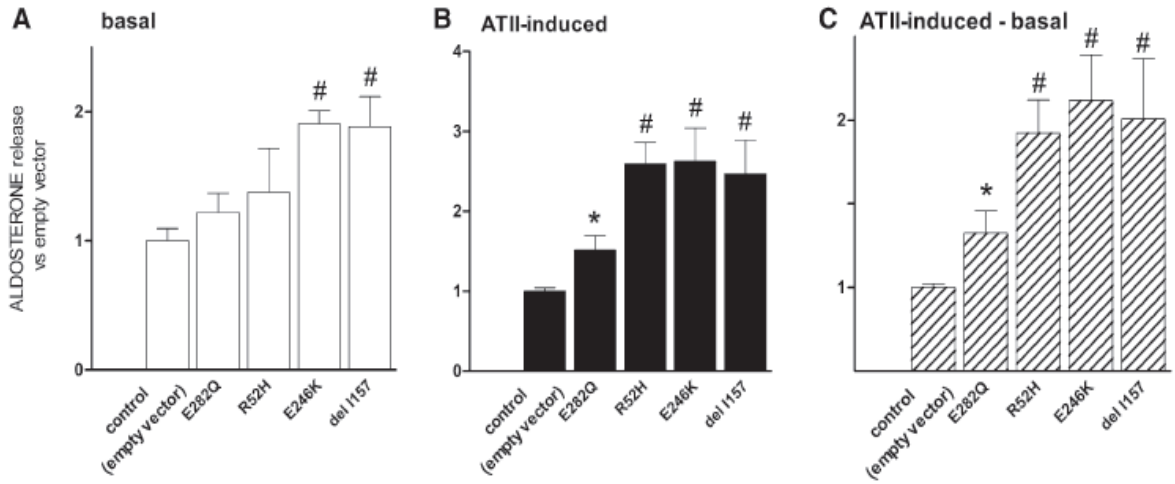

Fig.19 *Aldosterone release from H295R transiently transfected cells with empty vector or one of the KCNJ5 mutant forms. In the panel A are reported results of aldosterone release under basal condition; panel B shows the AT-II (10nM)-stimulated aldosterone secretion; panel C shows the difference between AT-II stimulated and basal aldosterone release. These results*

*are reported as mean±SEM; n=6. *P<0.05 and #P<0.01 vs empty vector control.*

In contrast to the other mutations, the G247R missense mutation that behaved indistinguishably from the WT channel in the oocytes did not affect basal or ATII-induced aldosterone release from H295R cells (Fig. 20).

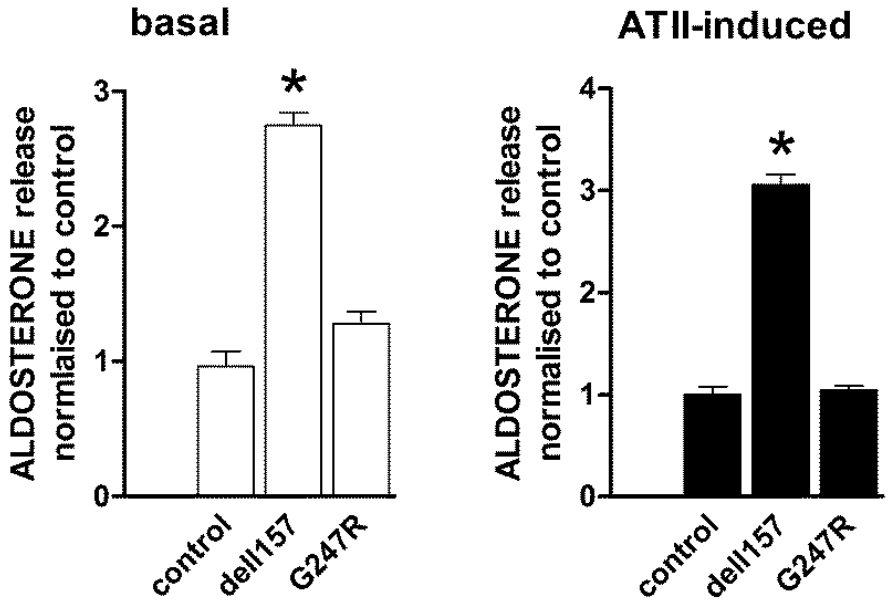

Fig.20 *Aldosterone release from H295R cells transfected with empty vector (negative control), del157 (positive control) and the missense mutation G247R. This missense mutation behaves similar to the negative control, empty vector, and do not produce any alteration in terms of membrane depolarization and aldosterone secretion. * P<0.01 versus control.*

7.4 KCNJ5 mutations and their effects on cellular viability

To add more information about the pathophysiological role of KCNJ5 potassium channel wt and mutant forms, we transfected the H295R cells as above and checked their viability 48h after transfection. To date, it is quite clear as alterations in ions composition in both intracellular and extracellular environment may be cause of cellular death. It was observed that all of the mutant forms were electrophysiologically active, and reduced cellular viability.

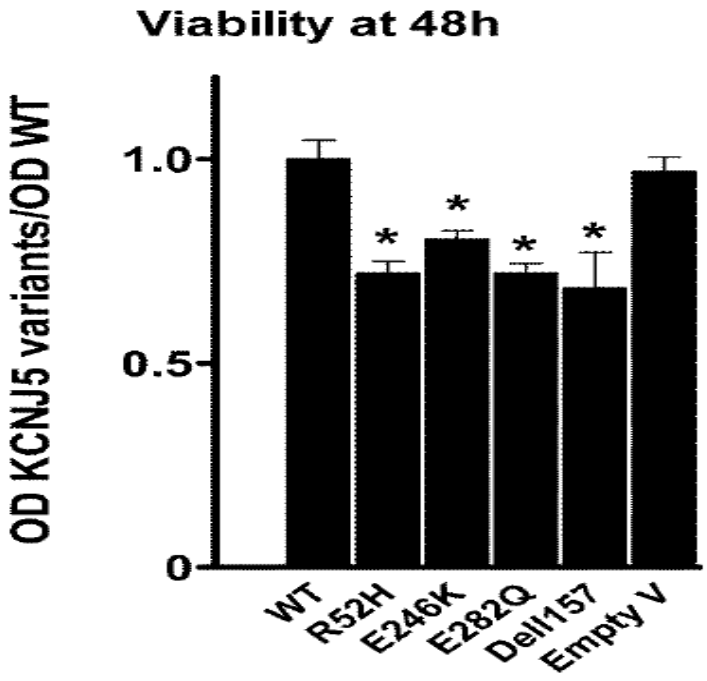

Fig.21 *Viability of H295R cells transfected with WT and electrophysiologically active mutant forms of KCNJ5 potassium channel. Cell viability has been evaluated 48h after transfection; results are indicated as fold changes of optical density (OD) versus control (WT or Empty vector). Results are an average of 4 different experiments. *P<0.05 versus WT or empty vector control.*

7.5 Allelic frequency and genotype distribution in the Brisighella population

Genetic analysis started with the evaluation of the allele frequency of each singular SNPs investigated in our cohort (Table 9). The frequency was considered in both hypertensive and not hypertensive groups for a total number of 1566 patients.

Table n.9 *Allele frequency of polymorphisms in hypertensive and not hypertensive patients within the Brisighella population; P<0.05 no Hypertension (allele 2) vs Hypertension (allele 0);OR was calculated with a confidence interval of 95%.*

Genotypes	**number %**	**number (%)**	***P*-value**	**OR (95%CI) ***
	Hypertension	*No Hypertension*		
AGT *rs699*				
0	511 (47.1)	907 (44.3)	***0.004***	1.294 (1.087-1.541)
2	575 (52.9)	*1139* (55.7)		1
$AGTR_1$ *rs5186*				
0	737 (68)	1442 (70.5)	0.052	0.831 (0.689-1.002)
2	347 (32)	604 (29.5)		1
CYP11B2 *rs1799998*				
0	644 (59.3)	*1108* (54.1)	***0.001***	1.331 (1.117-1.586)
2	442 (40.7)	940 (45.9)		
KIF6 *rs20455*				
0	687 (63.3	1302 (63.6)	0.333	0.915 (0.765-1.095)
2	399 (36.7)	746 (36.4)		1
KIF6 rs9471077				
0	425 (39.1)	785 (38.3)	0.524	1.059 (0.887-1.265)
2	661 (60.9)	1265 (61.7)		1
KIF *rs9462535*				
0	418 (38.5)	784 (38.2)	0.636	1.044 (0.874-1.247)
2	668 (61.5)	1266 (61.8)		1
HMGCoA *rs3761740*				
0	983 (90.5)	1849 (90.2)	0.567	1.089 (0.812-1.461)
2	103 (9.5)	201 (9.8)		1
HMGCoA *rs384662*				
0	592 (54.4)	1107 (54)	0.340	1.088 (0.915-1.294)
2	496 (45.6)	941 (46)		1

* Odds ratio (OR) age and gender adjusted

Allele frequency is one of the most important genetic parameters to be considered. Each

singular gene has a different distribution frequency among the same population and several human populations. Thus, individual can show different combinations of the variants with a different frequency. These levels of variation in DNA sequence has important implications for the individual medicine practice, for public health aspects of medicine, and for research design and data interpretation. The allele frequency can be considered as a measure of susceptibility to develop a pathology. In this regard, scientific communities need to know not only about the existence of DNA sequence variants but also the frequencies of those variants within several populations.

7.5.1 Distribution of our polymorphism in hypertensive and non-hypertensive individuals

For the genotype study we firstly started with the Hardy-Weinberg equilibrium evaluation, and its relative *P-value* was reported in the table below (Table 11). As it is possible to see, deviation from the HWE was observed for AGT, KIF6 (rs9471077), and for KIF6 (9462535). Because the genotypes of the 90% of duplicate samples included in the sample set agreed with each other, genotyping errors could be excluded. Deviation from the HWE leads to suppose that the statistically significant values found in the polymorphism distribution analysis are purely due to the case. Indeed, the allele frequency in a small population like our can be radically altered by random events; thus, the final results, even if significant, might be considered with particular attention. However, in this case the X^2 probability test seems to confirm the accuracy of our results, since its values are not small enough to reject the hypothesis.

Table n.10 *Comparison of Hardy-Weinberg P value between hypertensive and not hypertensive patients. P-value of HWE is reported and considered significant if <0.05. Accuracy of results was checked with the X^2 test. Deviation from the HWE can be seen for the only genes AGT and KIF6 rs9471077, and KIF6 rs9462535, whose P-values are 0.03, 0.005 and 0.006 respectively.*

Genotypes	**% n distribution**	**HWE P-value**	**% n distribution**	**HWE P-value**
	Hypertension		*No Hypertension*	
AGT *rs699* 0	122 (22.4)	0.759	184 (18)	**0.03**

1 2	267 (49.2) 154 (28.4)		539 (52.7) 300 (29.3)	
$AGTR_1$ *rs5186* 0 1 2	256 (47.3) 225 (41.5) 61 (11.2)	0.281	497 (48.6) 448 (43.8) 78 (7.6)	0.094
CYP11B2 ***rs1799998*** 0 1 2	194 (35.8) 256 (47.1) 93 (17.1)	0.587	297(29) 514 (50.2) 213 (20.8)	*0.732*
KIF6 *rs20455* 0 1 2	220 (40.5) 247 (45.5) 76 (14)	0.618	402 (39.3) 498 (48.6) 124 (12.1)	0.109
KIF6 **rs9471077** 0 1 2	90 (16.6) 245 (45.1) 208 (38.3)	0.218	129 (12.6) 527 (51.4) 369 (36)	**0.005**
KIF *rs9462535* 0 1 2	87 (16) 244 (45) 212 (39)	0.235	129 (12.6) 526 (51.3) 370 (36.1)	***0.006***
HMGCoA ***rs3761740*** 0 1 2	444 (81.8) 95 (17.5) 4 (0.7)	0.6584	833 (81.3) 183 (17.8) 9 (0.9)	0.763
HMGCoA ***rs384662*** 0 1 2	157 (28.9) 278 (51.1) 109 (20)	0.483	312 (30.4) 483 (47.2) 229 (22.4)	0.107

Besides the allele frequency, polymorphic distribution of each singular SNPs has been also evaluated in our population (Table 10). The polymorphic distribution gives an idea of how much diffused is a gene, within a population, as homozygous WT, heterozygous, or homozygous SNP, that we conventionally named 0, 1, and 2 respectively. Amongst the three different combinations, homozygous WT, heterozygous, and homozygous SNP, WT is considered the negative control.

Table n.11 *Distribution of SNPs within our cohort, divided in two groups, hypertensive and non-hypertensive. Through a statistical approach, a correlation between SNP and the hypertensive phenotype was evaluated, and results were considered significant for a P<0.05.*

Genotypes	**% n distribution**	**% n distribution**	***P*-value**	**OR (95%CI) ***
	Hypertension	*No Hypertension*		
AGT *rs699*				
0	122 (22.4)	184 (18)		1
1	267 (49.2)	539 (52.7)	0.006	0.633 (0.458-0.877)
2	154 (28.4)	300 (29.3)	0.002	0.562 (0.392-0.806)
0	122 (22.4)	184 (18)		1
1+2	421 (77.6)	839 (82)	**0.002**	0.607 (0.446-0.827)
AGTR$_1$ *rs5186*				
0	256 (47.3)	497 (48.6)		1
1	225 (41.5)	448 (43.8)	0.305	1.145 (0.884-1.485)
2	61 (11.2)	78 (7.6)	0.053	1.526 (0.995-2.342)
0	256 (47.3)	497 (48.6)		1
1+2	286 (52.7)	526 (51.4)	0.129	1.210 (0.946-1.547)
CYP11B2 *rs1799998*				
0	194 (35.8)	297(29)		1
1	256 (47.1)	514 (50.2)	0.028	0.732 (0.554-0.967)
2	93 (17.1)	213 (20.8)	0.002	0.574 (0.403-0.819)
0	194 (35.8)	297(29)		1
1+2	349 (64.2)	727 (71)	***0.004***	0.683 (0.526-0.888)
KIF6 *rs20455*				
0	220 (40.5)	402 (39.3)		1
1	247 (45.5)	498 (48.6)	0.769	0.961 (0.738-1.251)
2	76 (14)	124 (12.1)	0.163	1.316 (0.895-1.937)
0	220 (40.5)	402 (39.3)		1
1+2	323 (59.5)	622 (60.7)	0.812	1.031 (0.803-1.322)
KIF6 rs9471077				
0	90 (16.6)	129 (12.6)		1
1	245 (45.1)	527 (51.4)	0.022	0.655 (0.456-0.940)
2	208 (38.3)	369 (36)	0.186	0.778 (0.536-1.129)
0	90 (16.6)	129 (12.6)		1
1+2	453 (83.4)	896 (87.4)	**0.047**	0.707 (0.502-0.995)
KIF *rs9462535*				
0	87 (16)	129 (12.6)		1
1	244 (45)	526 (51.3)	0.036	0.677 (0.470-0.975)
2	212 (39)	370 (36.1)	0.254	0.805 (0.554-1.169)
0	87 (16)	129 (12.6)		1
1+2	456 (84)	896 (87.4)	0.075	0.732 (0.519-1.032)
HMGCoA *rs3761740*				

0	444 (34.8)	833 (81.3)		1
1	95 (34.2)	183 (17.8)	0.604	0.918 (0.665-1.268)
2	4 (0.7)	9 (0.9)	0.785	0.831 (0.221-3.130)
0	444 (34.8)	833 (81.3)		1
1+2	99 (65.2)	192 (18.7)	0.577	0.914 (0.666-1.254)
HMGCoA *rs384662*				
0	157 (28.9)	312 (30.4)		1
1	278 (51.1)	483 (47.2)	0.781	0.960 (0.722-1.277)
2	109 (20)	229 (22.4)	0.326	0.840 (0.593-1.190)
0	157 (28.9)	312 (30.4)		1
1+2	387 (71.1)	712 (69.6)	0.552	0.922 (0.705-1.205)

* Odds ratio (OR) age and gender adjusted

With regard to the analysis of genetic polymorphism distribution, comparing the presence of at least one variant allele (heterozygous or homozygous for the SNP) in both non-hypertensive and hypertensive groups, we observed a statistically significant difference in AGT, CYP11B2 (rs1799998) and KIF6 (rs9471077) (Table 10).

Afterwards, we moved our attention on the hypertensive group of individuals, and we recognized a limited group of subjects (470 individuals) undergoing antihypertensive therapy. We further divided this group in two subgroups, i.e. responder and non-responder, and then, we tried to find a correlation between the pharmacological response and the genetic characteristics. Pharmacological response was corrected for the age, sex, body mass index (BMI) and cholesterolemia.

Table n.12 *In this table is reported the SNP distribution of candidate genes within the hypertensive population undergone to a drug therapy. Significant P value<0.05 was calculated for no responder vs responder (allele 0 vs 1 and 0 vs 2).*

Genotypes	**number %**	**number (%)**	***P*-value**	**OR (95%CI) ***
	Drug therapy Responder	*Drug therapy No responder*		
AGT *rs699*				
0	9 (20.5)	94 (22)		1
1	26 (59)	213 (49.9)	0.550	1.277 (0.573-2.845)
2	9 (20.5)	120 (28.1)	0.655	0.802 (0.303-2.118)
$AGTR_1$ *rs5186*				
0	19 (43.2)	207 (48.6)		1
1	21 (47.7)	166 (39)	0.325	1.391 (0.721-2.681)
2	4 (9.1)	53 (12.4)	0.699	0.800 (0.259-2.472)

CYP11B2 *rs1799998*				
0	10 (22.7)	163 (38.2)		1
1	26 (59.1)	194 (45.4)	***0.050***	2.143 (1.000-4.591)
2	8 (18.2)	70 (16.4)	0.216	1.847 (0.698-4.886)
KIF6 *rs20455*				
0	16 (36.4)	174 (40.7)		1
1	21 (47.7)	198 (46.4)	0.723	1.312 (0.570-2.245)
2	7 (15.9)	55 (12.9)	0.535	1.349 (0.524-3.471)
KIF6 rs9471077				
0	7 (15.9)	69 (16.2)		1
1	22 (50)	194 (45.4)	0.785	1.134 (0.459-2.800)
2	15 (34.1)	164 (38.4)	0.858	0.917 (0.357-2.357)
KIF *rs9462535*				
0	8 (18.2)	65 (15.2)		1
1	21(47.7)	193 (45.2)	0.811	0.899 (0.375-2.153)
2	15 (34.1)	169 (39.6)	0.508	0.736 (0.297-1.826)
HMGCoA *rs3761740*				
0	36 (81.8)	354 (82.9)		1
1	8 (18.2)	70 (16.4)	0.735	1.152 (0.507-2.620)
2	0 (0.0)	3 (0.7)	na	na
HMGCoA *rs384662*				
0	8 (18.2)	127 (29.7)		1
1	25 (56.8)	218 (50.9)	0.157	1.819 (0.794-4.163)
2	11 (25)	83 (19.4)	0.124	2.12 (0.813-5.534)

* OR was adjusted for age, sex, cholesterolemia, and BMI

No correlation has been found between the considered *SNPs* and a positive response to the antihypertensive drug therapy as it is possible to see from the *P values* reported above (table 12).

We have repeated the same analysis as above, correcting the pharmacological response for the same variables age, sex, cholesterolemia, but changing the BMI with the waist circumference. Obesity is a well-documented risk factor for morbidity and mortality; however, the association between body fat and pathology has not been fully elucidated. Even though, body mass index is the most common measure of obesity, it does not reflect body shape. Moreover, it can be e misleading, thus, waist circumference, a more accurate measure of the distribution of body fat, has been shown to be more strongly associated with morbidity and mortality. For the same reasons, we decided to correct our results for BMI and waist circumference.

However, in both cases we didn't see any correlation between the genetic characteristic and the effect of antihypertensive therapy (Table 13).

Table n.13 *Similarly to the analysis as above, in this table is reported the SNP distribution of candidate genes within the hypertensive population undergone to a drug therapy. Significant P value<0.05 was calculated for no responder vs responder (allele 0 vs 1 and 0 vs 2).*

Genotypes	**number %**	**number (%)**	***P*-value**	**OR (95%CI) ***
	Drug therapy Responder	*Drug therapy No responder*		
AGT *rs699*				
0	9 (20.5)	94 (22)		1
1	26 (59)	213 (49.9)	0.622	1.224 (0.547-2.738)
2	9 (20.5)	120 (28.1)	0.630	0.787 (0.298-2.081)
$AGTR_1$ *rs5186*				
0	19 (43.2)	207 (48.6)		1
1	21 (47.7)	166 (39)	0.384	1.342 (0.691-2.607)
2	4 (9.1)	53 (12.4)	0.705	0.804 (0.260-2.483)
CYP11B2 *rs1799998*				
0	10 (22.7)	163 (38.2)		1
1	26 (59.1)	194 (45.4)	0.058	2.092 (0.975-4.491)
2	8 (18.2)	70 (16.4)	0.206	1.874 (0.708-4.956)
KIF6 *rs20455*				
0	16 (36.4)	174 ()		1
1	21 (47.7)	198 ()	0.846	1.071 (0.536-2.141)
2	7 (15.9)	55 ()	0.507	1.376 (0.536-3.536)
KIF6 rs9471077				
0	7 (15.9)	69 (16.2)		1
1	22 (50)	194 (45.4)	0.872	1.077 (0.434-2.674)
2	15 (34.1)	164 (38.4)	0.867	0.923 (0.359-2.372)
KIF *rs9462535*				
0	8 (18.2)	65 (15.2)		1
1	21(47.7)	193 (45.2)	0.705	0.844 (0.350-2.033)
2	15 (34.1)	169 (39.6)	0.503	0.733 (0.295-1.819)
HMGCoA *rs3761740*				
0	36 (81.8)	354 (82.9)		1
1	8 (18.2)	70 (16.4)	0.668	1.198 (0.525-2.736)
2	0 (0.0)	3 (0.7)	na	na
HMGCoA *rs384662*				
0	8 (18.2)	127 (29.7)		1
1	25 (56.8)	218 (50.9)	0.102	2.062 (0.865-4.913)
2	11 (25)	83 (19.4)	0.080	2.426 (0.899-6.547)

* OR was adjusted for age, sex, cholesterolemia, and waist circumference

Finally, we moved our attention on the pathological familial history, taking in consideration several diseases such as hypercholesterolemia, hypertension, acute myocardial infarction, and ictus. We investigated if in the both hypertensive subgroups, treated or not,

there was a familiarity between hypertension and the pathological conditions above mentioned. Also these last analyses are characterized by the lack of any statistically significant value, at least for ictus, hypercholesterolemia, and acute myocardial infarction, whereas the hypertension familiarity was confirmed into the drug-untreated subgroup. A borderline *P-value* was obtained for the hypercholesterolemia into the drug-treated subgroup, but not into the untreated subgroup.

Table n.14 *Familiarity was considered in the drug-treated subgroup referring to several pathological condition such as hypercholesterolemia, hypertension, acute myocardial infarction, and ictus. A borderline value was seen for the only hypercholesterolemia and not for the other conditions. P-value<0.05 was considered significant.*

	IPERTENSIONE			
	SI	**NO**	**TOT**	**P-value**
Hypercholesterolemia NO	35 (11.2) (79.5)	278 (88.8) (65.1)	313 (100) (66.5)	
SI	9 (5.7) (20.5)	149 (94.3) (34.9)	158 (100) (33.5)	***0.053***
Hypertension NO	21 (12.1) (47.7)	152 (87.9) (35.6)	173 (100) (36.7)	
SI	23 (7.7) (52.3)	275 (92.3) (64.4)	298 (100) (63.3)	0.112
Acute myocardial infarction NO	33 (9.4) (75.0)	318 (90.6) (74.5)	351 (100) (74.5)	
SI	11 (9.2) (25.0)	109 (90.8) (25.5)	120 (100) (25.5)	0.939
Ictus NO	32 (8.7) (72.7)	336 (91.3) (78.7)	368 (100) (78.1)	

SI	12 (11.7) (27.3)	91 (88.3) (21.3)	103 (100) (21.9)	0.362

Table n.15 *Familiarity was considered into the untreated subgroup referring to several pathological condition such as hypercholesterolemia, hypertension, acute myocardial infarction, and ictus. Hypertension familiarity was confirmed. P-value<0.05 was considered significant.*

	IPERTENSIONE			
	SI	**NO**	**TOT**	**P-value**
Hypercholesterolemia NO	614 (93.3) (57.8)	44 (6.7) (61.1)	658 (100) (59.9)	
SI	413 (93.7) (40.2)	28 (6.4) (38.9)	441 (100) (40.1)	0.824
Hypertension NO	585 (95.0) (57.0)	31 (5.0) (43.0)	616 (100) (56.1)	
SI	442 (91.5) (43.0)	41 (8.5) (57.0)	483 (100) (43.9)	***0.022***
Acute myocardial infarction NO	860 (94.0) (83.7)	55 (6.0) (76.4)	915 (100) (83.3)	
SI	167 (90.8) (16.3)	17 (9.2) (23.6)	184 (100) (16.7)	0.106
Ictus NO	895 (93.8) (87.2)	59 (6.2) (81.9)	954 (100) (86.8)	
SI	132 (91.0) (12.8)	13 (9.0) (18.1)	145 (100) (13.2)	0.207

7.6 Discussion of results

7.61 KCNJ5 results

We have resequenced the entire coding and flanking regions of KCNJ5 gene, expressing for the homonymous potassium channel in our 251 PA population patients. In this study we have characterized using an electrophysiological and biochemical methods 3 heterozygous missense mutations: *R52H, E246K, G247R*; one of these, ***E246K***, was described for the first time. Furthermore, an infrequent SNP, *E282Q*, rs7102584 in some 5% of the cohort (12/251), has been identified and characterized in the similar way to others. According to our knowledge, this is the first study, that try to characterize the infrequent non-synonymous SNP *E282Q*, therefore, our demonstration that the *Q282* channel variant encoded by this SNP does affect KCNJ5 behaviour in *in vitro* model is exciting and completely novel. We have shown that two of the missense mutations, *R52H* and *E246K*, influence the K^+ ions flow across the membrane and affect in this way the resting membrane potential in the Xenopus oocytes system and aldosterone release from the human H295R cell line. It has been seen that all these mutations occur in region of channel, whose amino acid sequence is highly conserved in evolution over the species, from the fly to the human, suggesting a key role in the cytoplasmic ionic composition. However, it has well been demonstrated that these mutations do not occur in the selectivity filter region, whose importance has been widely described, but in the other domains. Taken together, these results indicate for the first time that not only mutations that lie in the selectivity filter but also outside this important region may alter KCNJ5 behaviour and may be associated with sporadic PA.

To realize this study we employed two *in vitro* approaches based on using of *Xenopus* oocytes eggs and human H295R adenocarcinoma cell line. The human H295R cell line is a well-established model for the human zona glomerulosa cell, and it expresses the KCNJ5 channel. Expression of a typical selectivity mutant KCNJ5 channel in the H295R cell has been reported to depolarize the cell and enhance ATII-induced aldosterone synthesis and release (Fig.19). We saw the same increase in ATII-induced aldosterone release with another selectivity mutant, delI157, and in both the E282Q SNP and the 2 missense mutations that had altered channel behaviour in the oocytes (Fig. 19). Expressing the KCNJ5 channels in the oocytes gave a similar picture. The missense mutations and the E282Q channel behaved like typical selectivity mutants and depolarized the oocytes in high-Na^+ as we reported previously

with the typical delI157 selectivity filter mutant (Fig. 18). All of the germline mutations associated with FH-III and the somatic mutations within aldosteronomas, reported to date, have been localized within the selectivity filter of KCNJ5. This is a small but important domain in the protein that is expressed extracellularly and is highly conserved across the inward-rectifying K^+ channels. The effect of amino acid substitution within it is well understood, and the PA-associated mutations in KCNJ5 typically cause a marked reduction in K^+ selectivity. Hence the channels show a remarkable inward Na^+ current, which is thought to depolarize the zona glomerulosa cells triggering increased aldosterone synthesis and release. The discovery that a mutation in or near the selectivity filter of KCNJ5 K^+ channel is associated with a familial form of PA provides a novel molecular mechanism for the autonomous secretion of aldosterone. It is striking then that 2 of the KCNJ5 missense mutations we identified in our cohort and the SNP variant Q282 show substantial currents in a high-Na^+ solution. The R52H and E246K channels also lack the typical inward-rectification, which is striking because this is a defining feature of Kir channels with K^+ tending to move into the cells more easily inwards through the channel rather than in the reverse direction outwards. We reported similar behaviour before in a novel selectivity filter mutation in KCNJ5 delI157. Both in this earlier report and here (Fig. 17), the discontinuous I-V plot typical of a Kir channel is converted to a linear ohmic resistor in these mutants. Although the selectivity filter is the narrowest part of the channel and bears the important K^+ signature (TXGYG) at least in glomerulosa cells, it is not the only determinant of selectivity in Kir channels. For example, channels mediating the pacemaker current (if) in the heart have this canonical signature but show almost no selectivity for K^+ over Na^+, and mutations in both the transmembrane helix (M2) and the cytoplasmic domains of Kir channels have been reported to affect selectivity. Although the mechanisms for these substitutions are still poorly understood, they are thought to involve electrostatic effects, which are relevant to the E282Q and E246K mutations as they both introduce positive charges in the pore region on the cytoplasmic side of the channel. It is also relevant that R52H and E246K channels both lose inward-rectification and selectivity because of amino acid substitution. However, better structural data on the cytoplasmic side of the GIRK4 pore are needed to resolve how these substitutions might alter the structure of the pore because currently there is only crystallographic data from the closely related GIRK2 channel.

Findings obtained from this study are in line with others already published, confirming that mutation of KCNJ5 potassium channel are responsible of selectivity loss. In addition, our results for the first time demonstrate that not only mutation in the selectivity filter but also

near this region are involved in this pathological mechanism. They highlight the importance of KCNJ5 potassium channel in the pathophysiology of PA and specifically for its common sporadic form. The missense mutations and rare SNP variant we have reported in this study emphasize that the molecular genetics of PA is an unfinished story.

Despite the information that we currently have about the KCNJ5 activity and functionality, more informations have to be gained to establish whether other SNP variants in KCNJ5 also have a role to play in PA and what impact germline variation in *KCNJ5* might have on subjects with APAs carrying somatic mutations in the same gene. Furthermore, other studies have to be done to better clarify some molecular aspects that are still understood. It has been already demonstrated that KCNJ5 receptors, isolated from heart and pituitary gland, have been seen to be functionally modulated by G-protein subunits of G-coupled protein cholinergic M_2 and dopaminergic D_2 receptors respectively; thus, it could be reasonable to hypothesize the same behaviour in the adrenal gland. Indeed, from several line of evidences resulted that free DA is present in this area as well as D_2 and D_4 dopaminergic receptors have been isolated. However, there are no reports which prove this conclusively in the human adrenal glands. Another important issue that needs to be studied in deep is how KCNJ5 mutant forms modulate the Ca^{2+} ion flow and its cytoplasmic concentration. Furthermore, it is important to clarify the role that this important second messenger exerts on aldosterone release, cellular vitality and growing, as conflicting results have been already published.

7.6.2 Brisighella results and conclusion

In the similar way of the KCNJ5 study, to realize the second part of this work, we started from a population, constituted by 1546 randomized Caucasian volunteers, sampled for the Brisighella Heart Study between 2008-2009. We divided our population in two subgroups, non-hypertensive and hypertensive according to blood pressure values. The latter was in turn divided in further two subgroups, responder and non-responder, according to the antihypertensive therapy responsiveness. Since the population was randomly recruited we had a very heterogeneous phenotype with different biochemical parameters, and this represent an important limitation of the study. All the DNA samples were genotyped for the considered SNPs.

Firstly, we have investigated the allelic distribution of each gene, which can be present as homozygous WT, heterozygous, or homozygous SNPs. From this first analysis statistically significant *P-value* has been found in the only two genes, AGT (rs699) and CYP11B2(1799998), and quantified as 0.004 and 0.001 respectively.

As it is possible to see from the table 9, the allelic frequency of homozygous SNPs and heterozygous is much more represented in the not-hypertensive than the hypertensive subgroup, in both genes AGT and CYP11B2. These results indicate that the presence of at least one polymorphic allele has a protective effect against the pathological condition considered. Furthermore, taking in consideration the allelic frequency within each subgroup, for AGT it is possible to highlight that the homozygous SNP is much more abundant than the WT in both subgroups, whereas with regard to CYP11B2 the homozygous WT allele is more abundant than the heterozygous and homozygous SNP. This means that for the AGT gene the SNP could be associated with a non-pathological phenotype referring to the hypertension condition, whereas for the CYP11B2 gene, there is an opposite situation with a more diffused WT allele in hypertensive individuals.

Similarly to the allele frequency, also in the polymorphism distribution analysis it is possible to highlight a wider distribution of the polymorphic allele than the WT allele within the same group; furthermore, a greater presence of the polymorphic allele in non-hypertensive group than the hypertensive it has been observed. Taken together these results, indicate that in this population study the presence of at least one polymorphic allele, for the AGT(rs699), CYP11B2 (rs1799998) and KIF6 (rs9471077) genes, is not associated with an hypertensive phenotype. Thus, in light of these results it is possible to say that our results are not in accordance with the most part of studies already published. To date, it is well known that

AGT polymorphism (rs699) is responsible of a Cytosine (C) – Thymine (T) substitution in the position 699, that leads to a missense mutation M235T (Methionine – Threonine) in the protein structure. From several lines of evidence resulted that individuals homozygous for the T allele show a higher level of angiotensinogen than the homozygous M allele, and this AGT concentration may consistently affect the kinetic of angiotensin I generation.[150] However, conflicting results and data about the correlation of this SNP with hypertension and CAD are also present. Phenotypical heterogeneity, ethnic and racial differences might be responsible of these discordant results. Bohlender et al. investigated in detail the relationships between AGT concentrations and RAS cascade, focusing on the prominent role of AGT. They demonstrated that the enzymatic kinetics of renin are a function of AGT concentrations. This feature can be considered as an intermediate phenotype for cardiovascular disease.[151]

Similarly to the AGT results, also for the CYP11B2 we obtained controversial results comparing with other studies. Indeed, a wide body of evidences demonstrate that the considered SNP, rs1799998, leads to a -344 Cytosine/Tyrosine substitution at the putative binding site for the steroidogenic transcription factor (SF-1), influencing enzyme expression and protein levels. To date, it is well known that aldosterone is one of the most important hypertensive factor, involved in the blood pressure regulation, by handling the H_2O volume and the Na^+ concentration into the blood. Thus, any type of alteration, that leads to an increase of the circulating aldosterone concentration, has direct implication in the blood pressure values. Genetic polymorphisms in aldosterone synthase gene and in particular -344C/T variation have been largely investigated, because of their influences on the blood pressure values.[152,153] Indeed, Fontana et al. demonstrated that -344 C/T CYP11B2 polymorphism shows an additive effect on the aldosterone production in subject with resistant hypertension, with crescent levels in homozygous WT (CC), heterozygous (CT), and homozygous SNP (TT).[154] This result was also confirmed by another functional study, which showed that T allele binds to steroidogenic factor (SF-1) with lower affinity than C allele, and the binding of SF-1 to CYP11B2 inhibit the expression of the enzyme, supporting the increased aldosterone synthase activity in T allele.[155] Despite of a wide number of studies show a clear correlation between -344C/T polymorphism and the hypertensive phenotype, there are few studies that do not support this hypothesis.[156,157] However, we cannot exclude a false negative results for these studies because of a small number of individuals.

Finally, a possible correlation between genetic characteristics and antihypertensive response was investigated in our study. Gender, age, cholesterolemia, body mass index or alternatively waist circumference were used as covariates in addition to the genotype to

correct the final results. According our analysis no correlation was found between genotype and response to the antihypertensive therapy.

We could explain the lack of any type of correlation between the SNPs in the considered genes and the tested hypotheses because of different limitations occurring in the present study; the two most important of which are:

- Low number of individuals
- Unselected population for hypertensive phenotype

We used a randomized Italian rural population, and not a selected hypertensive patients; thus, selecting the hypertensive subgroups the population number lowered widely, and consequently the proportion between case and control was not adequate to the study. In particular the small sample size strongly affected the statistical power to detect any association between the investigated polymorphisms and both hypertensive susceptibility and anti-hypertensive response. Finally we didn't add any type of biochemical validation analysis, for example aldosterone/angiotensinogen quantification for the two genes AGT and CYP11B2, to confirm or exclude the genotyping results.

The low number of individuals and unselected population for the hypertensive phenotype have limited enormously our study, and biased the accuracy of our results. However, our findings, could lay the groundwork for further studies, as for example analysing others SNPs in the CYP11B2 gene, with the opportunity to identify an haplotype associated with the above described phenotype.

In conclusion we can say that from this case-control study, for the reasons above explained, we cannot withdraw any type of information that may be used to increase our knowledges about hypertensive susceptibility and personalized therapy.

8.0 Conclusion and future perspective

8.1 Aldosteronism, a curable form of secondary hypertension

PA is the cause of hypertension for a significant minority of patients. Its importance lies in the diagnosis offering the prospect of a curable form of hypertension. The molecular basis for rare syndromic forms of PA and the somatic mutations that may be driving sporadic adenoma formation is evolving rapidly. The role of *KCNJ5* for the commoner sporadic form of PA is less well understood. In a cohort of 251 patients with sporadic PA, we identified 2 cases with functional heterozygous missense mutations in *KCNJ5* (R52H and E246K). These mutations are distinct from the mutations previously identified in the selectivity filter of KCNJ5 in syndromic forms of PA or as somatic mutations in sporadic APAs. Another 12 patients were carriers for a rare functional SNP variant of the KCNJ5 gene, rs7102584, that produces an E282Q amino acid substitution in the channel. We have shown that despite their location in the channel the functional behaviour of these variants is similar to the selectivity mutants previously reported. These data suggest that mutant germline KCNJ5 channels are present in some apparently sporadic cases of PA. The clinical implications of this are unclear. It may be that they have a distinct phenotype or that PA patients with an adenoma and a germline mutation are less likely to have a long-term cure from unilateral adrenalectomy. Also the frequency of the functional SNP, rs7102584, could provide a germline basis for a significant minority of sporadic cases (≈5%). If it turns out that these germline mutations do affect the way we manage sporadic PA patients, then it will be necessary to routinely screen for them as part of their clinical workup.

Furthermore, a better understanding of how dopamine via D_2 and D_4 dopaminergic receptors modulates KCNJ5 potassium channel activity, and how the most important intracellular messenger, $Ca2^+$ ion, may regulate the aldosterone release, cellular growing and death, could be useful for an innovative therapeutic approach of PA.

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