# REVIEW OF LITERATURE

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## BLOOD PRESSURE AND SYSTEMIC HYPERTENSION: HEMODYNAMICS

By simple definition, pressure exerted by the flow of blood upon the walls of capillaries is termed as 'blood pressure'. The perception of pressure as the heart contracts against the resistance of blood vessels is in fact a simple application of the Ohm's law in physics which can be defined as "the product of the cardiac stroke volume, heart rate and systemic vascular resistance" where the product of cardiac stroke volume and heart rate is in fact, cardiac output (Guyton and Hall, 2006; Salvi, 2012). In this context, blood pressure is highest in the aorta and larger arteries and gradually falls down to zero as it reaches the larger veins and vena cava. The value of BP rises to as much as 120 mm Hg in a healthy adult individual during ventricular systole and falls to about 80 mm Hg during ventricular diastole. This pressure range is essential for maintaining an adequate perfusion rate and supply of nutrients to all the tissues of the body. Blood pressure is regulated within this range by several factors acting in concert with each other (Guyenet, 2006; Chopra *et al*, 2011). Traditionally, determinants of blood pressure have been known to be the renin-angiotensin-aldosterone-system, adrenergic stimuli to the blood vessels and the myocardium, baroreceptors, vagal responses, renal handling of salt & water and myogenic tone of vascular smooth muscles. All these systems are modulated in a fine-tuned manner by different modulators like renin, angiotensin II, ACE, $AT_1R$, noradrenaline, adrenoceptors, Ach, nitric oxide, L-type calcium channels, so on and so forth. It is these systems that regulate the blood pressure to ensure that none of the tissues in the body is deprived of the nutritional support achieved through blood. Based on our current understanding of blood pressure control, we still have a myopic view of the mechanisms involved in regulation of blood pressure. In spite of such a myriad assembly of factors maintaining normal blood pressure, individuals often tend to escape these control mechanisms and develop a condition of higher blood pressure termed as 'systemic hypertension'. Hypertension typically develops with an increase in systemic vascular resistance with or without increase in cardiac output (Kaplan, 2011). These two determinants are dependent upon kidney sodium excretion, baroreflexes and local autoregulation responses. Cardiac output may be modulated by extracellular fluid content which is actually given by total body sodium content (not plasma sodium concentration). This

parameter is entirely influenced by renal handling of sodium (Guyton and Hall, 2006; Vincent, 2008). Another factor modulating cardiac output is contractility & heart rate and these parameters may be in turn modulated by sympathetic tone and endogenous inotropic agents (Guyenet, 2006; Vincent, 2008). Modulation of systemic vascular resistance is more complex and involves an intricate balance between vasoconstrictor and vasodilatory mechanisms. These mechanisms may involve secretion of agents due to activation of the baroreceptor pathway, juxtaglomerular apparatus, local blood vessels and atria. Different factors like angiotensin II, noradrenaline, endothelin, thromboxane, prostaglandins, natriuretic peptides, bradykinin, excretion of $Na^+$ and nitric oxide among others are responsible for maintaining the intricate balance (Webb and Bohr, 1981; Umans and Levi, 1995; Carretero and Oparil, 2000; Oparil *et al*, 2003; Guyenet, 2006). Signals like blood pressure and blood viscosity lead to shear stress (Ku, 1997) thus influencing secretion of local effectors like endothelin or nitric oxide (Umans and Levi, 1995) for maintaining the appropriate cross-sectional diameter of the blood vessel.



## PRIMARY AND SECONDARY HYPERTENSION

Hypertension is most commonly classified as primary or secondary. Primary hypertension (also known as *essential hypertension*) is a condition in which an individual develops higher blood pressure due to some unidentified etiology (Carretero and Oparil, 2000; Beilin, 1988; Johnson *et al*, 2008). More than 90% of individuals suffering from hypertension belong to this class with the age of onset being above 50 years and with a strong family history. Thus, this form of hypertension is also termed as hereditary hypertension. Primary hypertension shows stronger correlations between family members and races (Johnson *et al*, 2008). Secondary hypertension is the development of hypertension due to some identifiable etiopathologic factor. Most common causes are renal diseases, stenosis of renal arteries, pheochromocytoma and hyperaldosteronism. The underlying pathophysiologic factor is commonly diagnosed upon medical examinations and in most of the cases is potentially curable (Akpunonu *et al*, 1996; Faselis *et al*, 2011).

## DEFINITIONS OF HYPERTENSION

Definitions of hypertension have metamorphosed over time owing to clinical studies in diverse populations and research elaborating the etiopathology of

hypertension. Different guidelines are elaborated by national agencies and these guidelines have minor differences based on the cohort studies in respective countries/ethnic groups. These differences are appreciated and taken into consideration while defining the intensity of therapy for any patient; however, due to its multifactorial etiopathology physicians are more likely to individualize diagnosis and therapeutic alternatives on a patient-to-patient basis. Guidelines published by the 'Joint National Committee (JNC)' set up by the American Medical Association (AMA) are the most commonly referred and cited set of guidelines (Chobanian *et al*, 2003) for defining hypertension. Other guidelines and their relative values are provided in the table below:

**Table1: Classification of hypertension based on different national guidelines[1]**

| Sr. No. | Name of Guideline | Country of Publication (Year) | Definition or term | Values (mm Hg) | |
|---|---|---|---|---|---|
| | | | | Systolic | Diastolic |
| 1 | Joint National Committee 8 (JNC 8) (James *et al*, 2014) | USA (2013) | Normal | < 120 | < 80 |
| | | | Prehypertension | 120-139 | 80-89 |
| | | | Stage 1 | 140-159 | 90-99 |
| | | | Stage 2 | ≥ 160 | ≥ 100 |
| 2 | World Health Organization/ International Society of Hypertension (WHO/ISH) (Weber *et al*, 2014) | UK (2013) | Grade1 | 140-159 | 90-99 |
| | | | Grade2 | 160-179 | 100-109 |
| | | | Grade3 | ≥ 180 | ≥ 110 |
| 3 | European Society of Cardiology/ European Society of Hypertension (ESC/ESH) (Mancia *et al*, 2013) | UK (2013) | Normal | 120-129 | 80-84 |
| | | | High-Normal | 130-139 | 85-89 |
| | | | Grade1 (mild) | 140-159 | 90-99 |
| | | | Grade2 (moderate) | 160-179 | 100-109 |
| | | | Grade3 (severe) | ≥ 180 | ≥ 110 |
| | | | Isolated systolic hypertension | ≥ 140 | < 90 |
| 4 | Canadian Hypertension Education Program (CHEP) | Canada (2013) | Grade A | < 130 | < 85 |
| | | | Grade B | 130-179 | 85-109 |
| | | | Grade C | 180-199 | 110-119 |

| | | | | | |
|---|---|---|---|---|---|
| | (Dasgupta *et al*, 2014) | | Grade D | ≥ 200 | ≥ 120 |
| 5 | British Hypertension Society IV (BHS-IV) (Williams, 2004) | UK (2004) | Normal | < 130 | < 85 |
| | | | High-Normal | 130-139 | 85-89 |
| | | | Grade1 (mild) | 140-159 | 90-99 |
| | | | Grade2 (moderate) | 160-179 | 100-109 |
| | | | Grade3 (severe) | ≥ 180 | ≥ 110 |
| | | | Isolated Systolic Hypertension-Grade1 | 140-159 | < 90 |
| | | | Isolated Systolic Hypertension-Grade2 | ≥ 160 | < 90 |
| 6 | Indian Guidelines on Hypertension-III (IGH-III) (Shah *et al*, 2013) | India (2013) | Normal | < 130 | < 85 |
| | | | High-Normal | 130-139 | 85-89 |
| | | | Grade1 (mild) | 140-159 | 90-99 |
| | | | Grade2 (moderate) | 160-179 | 100-109 |
| | | | Grade3 (severe) | ≥ 180 | ≥ 110 |
| | | | Isolated Systolic Hypertension-Grade1 | 140-159 | < 90 |
| | | | Isolated Systolic Hypertension-Grade2 | ≥ 160 | < 90 |



[1] While preparing this table, preference has been laid on guidelines most commonly referred in literature. Hence, chronological order is not followed

## EPIDEMIOLOGY OF HYPERTENSION IN INDIA

There has been no national survey to identify the epidemiology of hypertension in the subcontinent. However, several authors have accumulated data from WHO publications and other regional studies in India to generate a consensus on the trends of hypertension in the country. Based on these publications, it could be concluded that hypertension is the major cardiovascular disorder responsible for the highest mortality caused by non-communicable diseases in India. While data compiled a decade ago showed that prevalence of hypertension is low among the rural population (Gupta, 2004), recent data analysis revealed that affluence does not have any bearing upon the occurrence of hypertension in the Indian population and hypertension occurs in both the urban and rural classes, almost equally (Moser *et al*, 2014). Risk factors for the occurrence of hypertension resemble the global trend and include increasing age, smoking, alcohol intake, concomitant diabetes and high salt intake in the diet (Devi *et al*, 2013). Important gender differences could be observed regarding the prevalence of hypertension in India. It was found that though prevalence rates did not differ much (26% men v/s. 23% women), diagnosis rates did. Women, more than 70% of them, had their blood pressures assessed with some health services. Age related prevalence indicates that 40% of individuals crossing 70 years of age are likely to suffer from hypertension. Again, alcohol consumption at any time of life increased the likelihood of an individual suffering from hypertension (Anand, 2010; Moser *et al*, 2014). The major issue among the Indian populace remains the ignorance and lack of awareness regarding diagnosis and treatment of hypertension. It has been observed that detection of hypertension remains the major concern while managing hypertension in India and hence if early detection of this condition can be handled, it can be managed in a far better manner (Devi *et al*, 2013).

## ETIOPATHOGENESIS OF HYPERTENSION AND COMPENSATORY MECHANISMS

As mentioned above in *'blood pressure and systemic hypertension: hemodynamics'* section, blood pressure is the product of cardiac output and systemic vascular resistance. It is thus clear that the patient may either have increased systemic vascular resistance (which is often more common), increased cardiac output (which is less common) or both. The ultimate product of this situation is hypertension. It may be

observed that in most of the adult, middle aged and geriatric population systemic vascular resistance is increased. This population consists of more than 90% of the total patients suffering from hypertension in one or the other form (Kearney *et al*, 2004; Kumar *et al*, 2013). Increase in systemic vascular resistance is actually the increase in vascular tone and narrowing of lumen diameters in resistance vessels like arteries and arterioles. There are several etiologic factors responsible for increasing the vascular tone (Figure 1 below). This includes secretion of vasoconstrictive peptides like AngII and endothelin-I. Stimulation of $\alpha_1$-adrenergic receptors by norepinephrine is another mechanism responsible for constriction of blood vessels (Calhoun *et al*, 2008). All these mechanisms converge to a final pathway where there is a GPCR mediated increase in cytosolic calcium, $[Ca^{++}]_i$, in vascular smooth muscle cells that causes vasoconstriction of the blood vessel (Koenigsberger *et al*, 2004) in a radial manner leading to narrowing of lumen diameter and consequent increase in vascular tone. If increase in vascular tone is accompanied by increasing vascular stiffness (or reduced elasticity of blood vessels), there is an enhanced load bearing onus on the left ventricle which, if persistent or not corrected, can lead to ventricular hypertrophy followed by ventricular dysfunction (Carretero and Oparil, 2000).



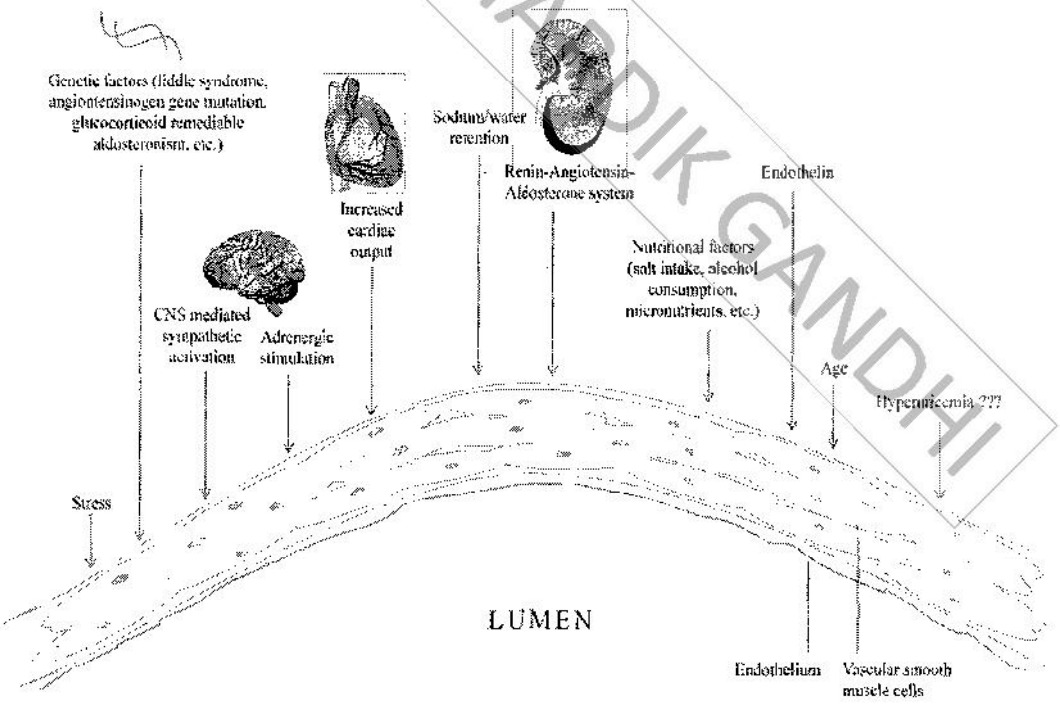



Figure 1: Etiopathological factors of hypertension [Redrawn from: Oparil *et al*, 2003]

The role of sympathetic nervous system in the pathogenesis of hypertension cannot be ignored. The major contribution provided by the sympathetic nervous system in the development of hypertension is in two forms: 1) increased release of norepinephrine which mediates direct constriction of blood vessels, and 2) enhanced sensitivity of norepinephrine in peripheral tissues (Mark, 1996; Brook and Julius, 2000) such that even traces of norepinephrine can shoot blood pressure values by 10-15 mm Hg. Environmental factors additionally play an important role in the pathogenesis of hypertension. Stress increases the peripheral sensitivity of circulating catecholamines thus predisposing the individual to increased vascular tone and heart rate (Esler, 2000). The compensatory mechanisms of the body responsible for control of blood pressure are dulled and thus elevations in blood pressure cannot be managed without pharmacotherapy. Abnormal handling of renal salt and water disturbs the natriuresis/volume/blood pressure balance (Chiolero *et al*, 2001), thus leading to hypertension. The renin-angiotensin-aldosterone-system is one of the key effectors of the etiopathogenesis of hypertension and is mostly involved in renovascular hypertension. Hypertension may occur independent of renin status, i.e. the patient may be classified as having low renin or high renin but the effector angII, is the main culprit responsible for all of the detrimental effects leading to hypertension and its complications (Gradman and Kad, 2008; Arnold *et al*, 2013; ).

Different regulatory mechanisms exist in the human physiology as a means of controlling unanticipated elevations in blood pressure (Figure 2). These include the baroreceptor mediated regulation which reduces sympathetic outflow from the brain whenever increased pressure is sensed at the aortic and/or carotid baroreceptors (Guyenet, 2006; Xie *et al*, 1991; Guo and Abboud, 1984). Spatial changes induced in the blood vessels due to narrowing of the lumen diameter lead to secretion of endothelium derived relaxing factor, chemically known as nitric oxide (NO), which causes cGMP-mediated relaxation of vascular smooth muscle cells (Cai and Harrison, 2000). Renal capsule has a general role in maintaining the fluid-balance in the body. Renal diuresis mechanisms govern the overall blood volume and increased diuresis can reduce blood volume which improves cardiac output by reducing venous return (Legrand and Payen, 2011). The macula densa region of the kidney senses perfusion pressure of the tubules and thus regulates the release of the peptidase 'renin' which cleaves angiotensinogen to form ang I, the precursor for AngII (Persson *et al*, 2004;

Peti-Peterdi and Harris, 2010). One very important facet in the development of hypertension is that there is partial or complete blunting of the compensatory mechanisms that regulate blood pressure towards normotensive values.

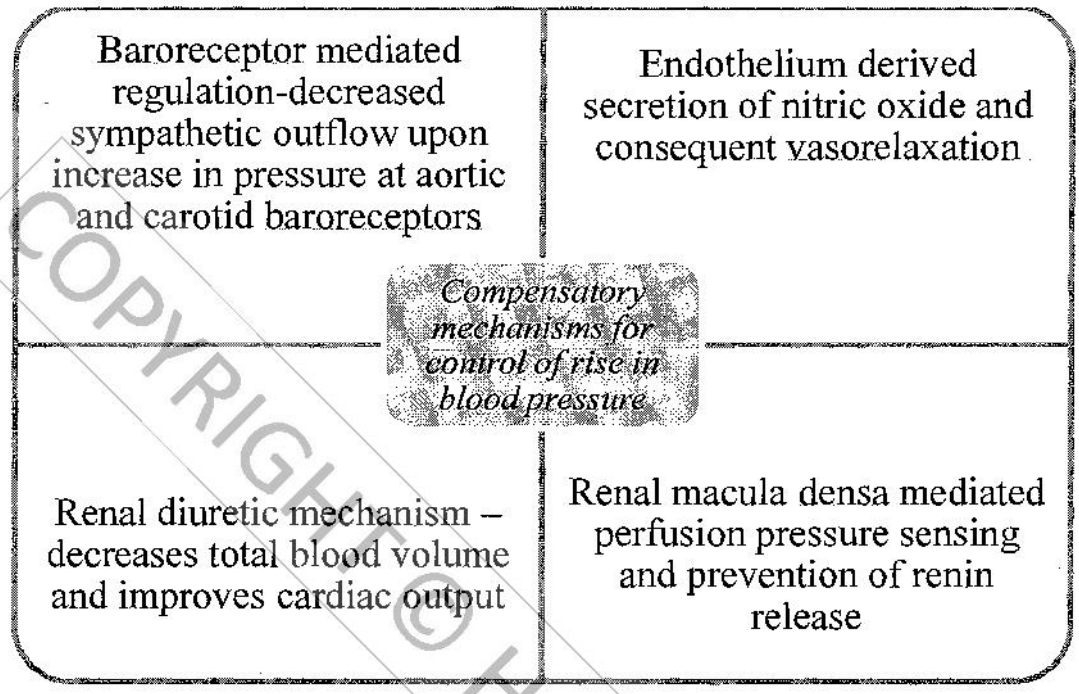



Figure 2: Different physiological mechanisms to prevent rise in abnormal blood pressure. These mechanisms operate through the brain, vasculature and kidneys.



***Sympathetic nervous system***

Several hemodynamic, rheologic and trophic changes in the cardiovascular system are maintained by a balance between the parasympathetic and sympathetic arms of the autonomic nervous system (Brook and Julius, 2000). Any imbalance in the autonomic system favouring the sympathetic arm, which is concomitant with reduced parasympathetic outflow, can lead to increased cardiovascular morbidity and mortality. Increased heart rate is usually normalized by increases in parasympathetic tone but in a situation of autonomic imbalance, reduced parasympathetic tone can lead to perpetual increases in heart rate and thus contributes to the pathological condition of hypertension (Brook and Julius, 2000; Oparil et al, 2003). Several population-based studies have concluded that increase in sympathetic tone is the ultimate cause of vascular remodeling which is preceded by vascular smooth muscle cell (VSMC) proliferation. Norepinephrine spillover studies, which afford the researchers with an index of norepinephrine release from sympathoeffector nerve terminals, are also in agreement

with the population-based studies and support the conclusion that one of the major contributors in the development of hypertension is elevated sympathetic tone (Esler, 2000).

The development of hypertension which is mediated through increased sympathetic tone is a complex network of different mechanisms. These involve alterations in the chemoreflex and baroreflex mechanisms, trophic changes in the heart and blood vessels and mainly renal sympathetic stimulation. There is central as well as peripheral resetting of baroreflexes under conditions of hypertension. In hypertensive patients, it could be observed that the arterial baroreceptors are reset to a higher pressure value, thus preventing the regulatory feedback. Also, there is a central resetting of the aortic baroreflexes (Xie *et al*, 1991; Guo and Abboud, 1984). Based on experimental data, it may be assumed that this resetting of the carotid and aortic baroreflexes is mediated, to a certain extent, by AngII (Xie *et al*, 1990; Li *et al*, 1996). Owing to its ability of 'presynaptic facilitatory modulation of norepinephrine release', AngII magnifies the peripheral responses to sympathetic stimuli (Abboud, 1974). Furthermore, reactive oxygen species and vascular factors like endothelin also cover out baroreceptor activity and contribute to the development of hypertension (Chapleau *et al*, 1992; Li *et al*, 1996). With respect to chemoreflex, stimuli such as hypoxia or apnea, lead to an overstated chemoreflex function leading to an obviously augmented sympathetic tone (Somers *et al*, 1995; Scultz *et al*, 2007). Sustained activation of the sympathetic arm of the autonomic nervous system leads to sustained release of norepinephrine and the cardiovascular system thus becomes a subject of the direct and indirect actions of norepinephrine (Louis *et al*, 1969; Goldstein, 1981). Norepinephrine also affects the release of various factors like FGF, TGF-β and IGF-1, all of which are trophic in nature (Grassi, 1998; Brook and Julius, 2000; Wakatsuki *et al*, 2004). Chronic sympathetic activation thus leads to development of left ventricular wall hypertrophy and consequent vascular remodeling ensues (Grassi *et al*, 1995; Oparil *et al*, 2003). As compared to normotensive individuals, hypertensive patients show an increased renal sympathetic tone. Direct stimulation of the renal nerves in animals has shown that there is an increased reabsorption of sodium-water and a fall in urinary output, both of which contribute to vascular volume expansion and thus cause increase in blood pressure *via* increase in cardiac output. Alternatively, renal denervation has been shown to reverse the raised blood pressure in experimental models of hypertension

(DiBona and Kopp, 1997; Kopp, 2011). This mode of therapy has also been found to be beneficial in the management of human hypertension and recently several regulatory authorities have added 'surgical renal denervation' as one of the major treatment alternatives for hypertensive patients (Schlaich *et al*, 2009; Krum *et al*, 2009). Precisely, these facts and results support the role of increased sympathetic activity in the etiopathogenesis of hypertension.

***Vascular reactivity***

Increased vascular reactivity is one of the facets of hypertension. It has been observed that hypertensive patients exhibit a higher vasoconstrictor tone in response to norepinephrine and other vasoconstrictive agents (Ziegler *et al*, 1991). Chronic high levels of circulating norepinephrine tend to cause down regulation of noradrenergic receptors but this does not occur in hypertensive states which cause an increase in peripheral resistance and rise in blood pressure (Oparil *et al*, 2003). Normotensive progeny born to hypertensive parents also show an increased sensitivity to the actions of norepinephrine. This suggests that vascular reactivity may be inheritable. Since offspring from parents without a history of hypertension do not show enhanced vascular reactivity, it may be concluded that hypersensitivity is of genetic origin and merely not a result of elevated blood pressure (Calhoun *et al*, 1993).

***Endothelial dysfunction***

NO is responsible for the maintenance of a continuous vasodilator tone in healthy individuals. NO, expressed by the normal vascular endothelium, is a potent vasodilator, inhibits platelet aggregation and adhesion and also prevents migration and proliferation of smooth muscles (Forstermann and Munzel, 2006; Pacher *et al*, 2007). NO may be released in response to stimuli like increased blood pressure, arterial stretch and/or increased shear stress. In case of human hypertension, macro- and micro-vessels of the periphery, heart and kidney undergo endothelial damage. Partial damage to the endothelium leads to dysfunction in the secretion of various factors that influence vascular tone and structure (Forstermann and Munzel, 2006). In case of hypertension, activation of an alternative cyclooxygenase pathway leads to generation of oxidative stress that reduces NO availability (Cai and Harrison, 2000; Puddu *et al*, 2000; Schulz *et al*, 2008). In the absence of NO, prostacyclin and an unidentified hyperpolarizing factor secreted from the endothelium maintain the relaxation. Other etiopathologic

factors like presence of concomitant hyperhomocysteinemia may further demoralize the compensatory actions of prostacyclin and the hyperpolarizing factor (Mitchell *et al*, 1992; Emsley *et al*, 1999; Matoba *et al*, 2000). Another factor secreted by the endothelium is endothelin-1 (ET-1) whose actions are opposite to that of NO, i.e. it is vasoconstrictive in nature. Under normal situations, ET-B receptor mediated NO elaboration keeps the ET-1 mediated vasoconstriction in check. However, under blunted NO release, ET-1 mediated vasoconstriction can overwhelm compensatory vasodilatory mechanisms and thus participates in increasing the blood pressure in patients with essential hypertension (Krum *et al*, 1998; Oparil *et al*, 2003).

***Pro-angiogenic factors***

Microvascular rarefaction is one of the discrete characteristics of hypertension (Goligorsky, 2010). It exemplifies a reduction in the parallel blood vessel circuits resulting in an alteration of microcirculation which ultimately contribute to end-organ damage. Impaired angiogenesis is one of the primary reasons for occurrence of rarefaction (Goligorsky, 2010; Feihl *et al*, 2006). Angiogenesis is a highly complex and regulated process which involves formation of vascular networks from microvessels. It is stimulated by hypoxic conditions (a compensatory mechanism to counteract ischemia) and is regulated by levels of NO. Lower than normal levels of NO mar the process of angiogenesis whereas normal or elevated levels support angiogenesis. Angiogenic factors like VEGF and FGF function only in presence of NO for promoting angiogenesis (Namikoshi et al, 2006; Goligorsky, 2010). Role of such growth factors is essential for angiogenesis to reduce target-organ damage. Such growth factors promote collateral vessel formation. Placenta inducible growth factor (PlGF) also acts by activating VEGF signaling (Carmeliet *et al*, 2001; Luttun *et al*, 2002). Lack of VEGF signaling can lead to limb ischemia or claudication as indicated by clinical trials (Baumgartner and Isner, 2000).

***Vascular remodeling***

Chronic elevated blood pressure leads to persistent systemic vascular resistance. This situation leads to an alteration in the structure of resistance vessels. These structural changes may be defined in terms of a ratio, media-to-lumen diameter (Rosei *et al*, 1995; Rizzoni *et al*, 2012). Thus, changes in media thickness and narrowing of the arterial lumen were initially perceived as means of vascular insufficiency but with the

observations made by Glagov *et al* (1987), it was acknowledged that in pathological conditions, arteries undergo several structural changes to preserve blood flow. This rearrangement of matrix and muscular material around the vessel's lumen, initially termed as vascular growth, was later dubbed as *vascular remodeling* by Mulvany (1993). Vascular remodeling may thus be defined as an active process that involves alterations in the cell growth, migration, survival and regulation of extracellular matrix, ultimately influencing vascular structure and function. Classification of different forms of vascular remodeling is given in figure 3 below:

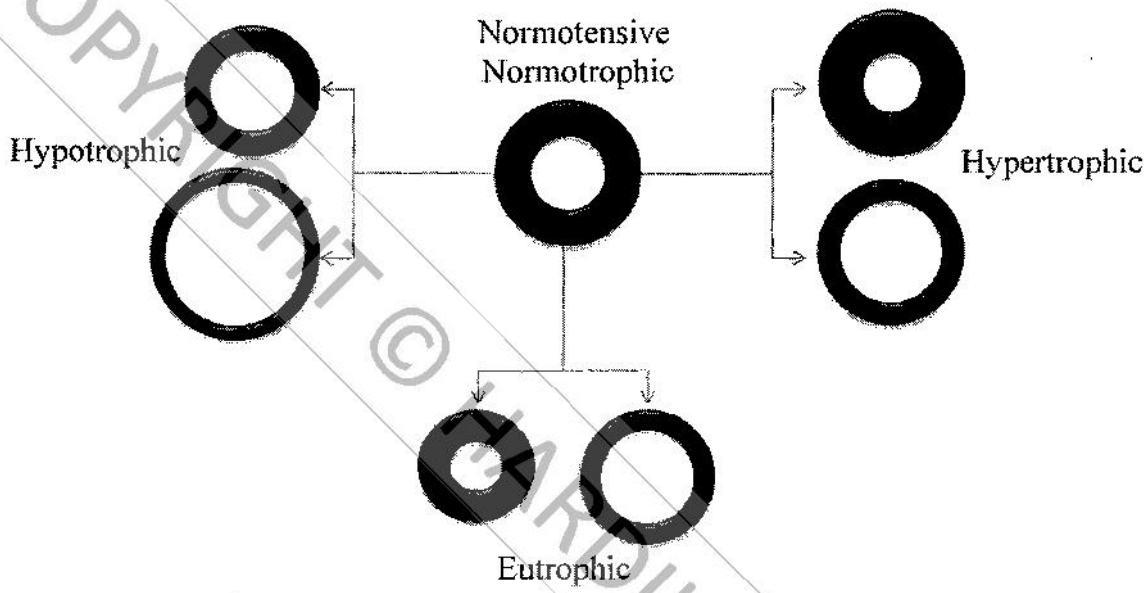



Figure 3: Remodeling may be hypertrophic (increased cross-sectional area as observed in severe hypertension), eutrophic (no change in cross-sectional area, mild-to-moderate hypertension) or hypotrophic (decreased cross-sectional area, following chronic antihypertensive therapy). As can be observed from the diagram, each form of remodeling may result either in increased lumen diameter (outward remodeling) or a decrease in lumen diameter (inward remodeling). Remodeling should not be confused with *rarefaction* where there is a decrease in the number of parallel blood vessel circuits as a result of increased peripheral vascular resistance.

Persistently elevated blood pressure stimulates arterial stretch leading to increased tensile forces in the arterial walls. This phenomenon further stimulates vessel wall thickening to normalize the changes in the tensile strength. Modifications in the structural content of the vessel walls result from several factors like growth, inflammation, apoptosis, fibrosis and cell-matrix interactions acting in concert with each other (Intengan and Schiffrin, 2001). While growth, inflammation and fibrosis result from secretion of several cytokines as a result of hypertensive pathophysiology,

apoptosis results from activation of programmed cell death cascades in blood vessels (Cho *et al*, 1995; Hamet *et al*, 1995). Inflammation triggers fibrosis to some extent and ultimately involves accumulation of remodeling components like collagen, fibronectin and other extra cellular matrix material. This kind of accumulation is further aggravated due to integrins (which are responsible for cell-matrix interactions) resulting in modulation of arterial structures. Other very important mechanism involved in remodeling is the imbalance between MMPs/TIMPs which may result in increased collagen turnover (Diez *et al*, 1995; Intengan and Schiffrin, 1999) and the balance may thus be tilted in favor of increased extracellular matrix remodeling (Galis *et al*, 1994). In case of chronic hypertension, the constricted vessel lumen is continuously enveloped in a gradually remodeling matrix. Thus the rearrangement of smooth muscles surrounding the lumen leads to inward remodeling. This type of process is actually compensatory in the beginning and behaves as an adaptive mechanism but later becomes maladaptive. It causes arterial stiffness and compromises end-organ function leading to complications of hypertension (Franklin, 2005; Payne *et al*, 2010).

***Uric acid - a proposed pathological factor in hypertension***

Hyperuricemia has been linked with human hypertension and CVD (Rich, 2000; Alderman *et al*, 1999) but there is no consensus regarding hyperuricemia whether it is a cause or effect of hypertension. Thus it remains to be classified either as a risk factor for hypertension or one of the markers of hypertension. Renal vasoconstriction and plasma renin activity, both relate positively to hyperuricemia, suggesting that uric acid may be an offshoot of the local RAAS in the kidney. Conclusions from major randomized trials like SHEP (Franse *et al*, 2000) did not find any correlation between uric acid levels of treated and untreated patients. Another major randomized trial, LIFE (Dahlof *et al*, 2002), suggested that higher baseline serum uric acid levels were associated with increased CVD risk. Mechanisms by which uric acid might be involved in the development of hypertension have been identified: 1) it stimulates renal arteriolopathy and tubulointerstitial inflammation, 2) it reduces NO excretion in the macula densa by reducing nNOS levels, 3) it is a marker for xanthine oxidase associated oxidative products which contribute to the development of hypertension and 4) it induced PDGF expression in VSMCs leading to smooth muscle proliferation (Johnson *et al*, 2003; Mazzali *et al*, 2010). One important aspect regarding experimental models used to study uric acid levels is that uric acid is metabolized to allantoin by an

enzyme uricase that is expressed in most experimental species except humans and other higher primates (Yeldandi *et al*, 1991; Szasz and Watts, 2010). This fact is important to note since canine and rat models are frequently used to study hyperuricemia where uric acid levels cannot be appreciated due to elaboration of uricase and thus interpretations should be done carefully if hyperuricemia is correlated to hypertension on the basis of such studies.

***Salt-sensitive hypertension and renal microvascular disease***

Evolutionary studies suggest that organisms evolved from salty environments of the ocean. This environment is analogous to extracellular fluid for mammalian cells which is rich in electrolytes. Further, the course of evolution offered remarkable challenges to the evolving organisms when they moved from salt-water to fresh-water (low in electrolytes) and ultimately to land. Regulatory systems developed in the human body to adjust with the low-salt environment on land so as to maintain homeostasis (Smith, 1959; Lifton *et al*, 2001; Fournier *et al*, 2012). Developing civilizations went through two major changes which contributed to increased salt intake by individuals: 1) They learnt to conserve food with the addition of salt and 2) daily nutritional intake in industrialized areas involved excessive salt in the diet. This led to sodium retention and ultimately water retention causing volume overload (Danziger, 2001; Lifton *et al*, 2001; Weder, 2007; Fournier *et al*, 2012). The renin-angiotensin-aldosterone system remains active *in continuum* to normalize this overload but has its detrimental effects on the kidney and cardiovascular system leading to hypertension. It has been argued that abnormal handling of salt/water by the kidney and concomitant renal vascular disease may be the primary cause of hypertension (Khalil, 2006; O'Shaughnessy and Karet, 2006). This argument has been the subject of exhaustive deliberations and has been supported by several researchers. It has been suggested that renal vasoconstriction for a prolonged period of time allows the development of renal arteriopathy, glomerulonephrosclerosis and tubulointerstitial disease which lead to sodium/water retention and thus contribute to the etiopathology of hypertension (Bidani and Griffin, 2002; Bidani and Griffin, 2004). Several stimuli like increased sympathetic tone and overactivity of the renin-angiotensin-aldosterone system lead to renal vasoconstriction. Renal handling of salt/water may be normal at this stage but chronic renal hypertension leads to initiation of renal injury which may involve renotubular ischemia, interstitial inflammation, local generation of AngII, decreased NO release, etc (Pohl, 1997;

Garovic and Textor, 2005). As mentioned earlier, renal arteries also undergo remodeling and lead to development of arterio- and arteriolo-pathy. This may be observed when kidneys of hypertensive animals show PAS-positive areas suggesting glomerulonephrosclerosis (Isaacson *et al*, 1991; Caetano *et al*, 2001; Marcantoni *et al*, 2002). All these factors result in increased renal vascular resistance wherein and single-nephron glomerular filtration rate is compromised. As an adaptive mechanism, the balance between vasoconstrictors and vasodilators shifts in favor of vasoconstrictors, and this kind of mechanism leads to sodium retention and increased systemic blood pressure (Bidani and Griffin, 2002). As a means for compensating rather than allowing maintenance of normal perfusion pressure, kidneys equilibrate at a higher blood pressure and thus sodium handling returns to normal but total systemic vascular resistance is increased as the pressure-natriuresis relations show a parallel rightward shift (Garovic and Textor, 2005).

***The renin-angiotensin-aldosterone system***

The physiology of the RAAS is a complex cascade of events which is the definitive blood volume and pressure regulation order of the human body and has important implications in the study of hypertension and related cardiovascular diseases (Carey and Siragy, 2003; Rosivall, 2009). The cascade starts with the release of renin, an aspartyl protease, from the juxtaglomerular glands of the kidney (**Figure 4**). This enzyme cleaves angiotensinogen, synthesized in the liver, which is the primary precursor of the RAAS to a decapeptide termed as angiotensin I (angI). Renin is the rate-limiting enzyme in this cascade and formation of ang I from angiotensinogen thus becomes the rate-limiting step. Stimuli for the release of renin include baroreflex response in the juxtaglomerular apparatus. The β-adrenergic sympathetic innervation increased chloride delivery at the macula densa division of the distal tubule. Renin also binds to a receptor termed the pro-renin receptor [(P)RR] which enhances the proteolytic activity of renin and also activates pro-renin, the precursor of renin (Nguyen et al, 2002; Nguyen, 2006; Nguyen, 2007). Activation of (P)RR has some effects which are independent of the RAAS pathway, yet have a bearing upon the pathophysiology of cardiovascular diseases. These include activation of the promyelocytic zinc finger (PLZF), activation of $PI_3$-Kinase and eventually MAPKs (Nguyen *et al*, 2004; Schefe *et al*, 2006). Activation of these factors result in increased proteosynthesis, proliferation and reduced apoptotic events (Nguyen *et al*, 2004).

AngI formed by the action of renin is majorly inactive and is cleaved to an octapeptide, AngII, by the action of angiotensin converting enzyme (ACE). ACE, a matrix metalloproteinase, may be available in circulation or may be expressed locally but majority of the AngII conversion takes place through ACE expressed by the pulmonary endothelial cells (Carey and Siragy, 2003; Rosivall, 2009). However, ACE is not the only enzyme with the ability to cleave angI to AngII. AngII formation may also occur due to the action of chymase, carboxypeptidase, cathepsin G or tonin (Clarke and Turner, 2012). ACE has the ability to hydrolyse bradykinin (Tom *et al*, 2003) and stimulate NO and prostacyclin, thus exhibiting vasodilatory properties. Increased angII levels, reduced baroreflex responses and sympathetic inhibition act as negative feedback for preventing/curtailing the release of renin (Harrison-Bernard, 2009; Rosivall, 2009). AngII formed through any of the aforementioned enzymes may eventually demonstrate different actions relevant to the pathophysiology of hypertension. AngII acts on the adrenal cortex, stimulating the release of aldosterone. Aldsoterone effects sodium reabsorption from the collecting ducts in the kidney thus contributing to reduced natriuresis (Mehta and Griendling, 2007). Aldosterone mediates its salt reabsorptive actions through the mineralocorticoid receptors. AngII itself has sodium reabsorption capacities and its activity in the brain leads to thirst stimulation and increased salt appetite (Fyhrquist *et al*, 1995). AngII also increases sympathetic outflow in an independent manner which is discussed below. Above all, the major mechanism by which the RAAS cascade is important in hypertension is the ability of angII to act directly on the vessel walls to mediate vasoconstriction and attendant rise in blood pressure. This vasoconstrictive action of angII is mediated through a *Gq* coupled GPCR, the angiotensin type 1 receptor ($AT_1R$). The $AT_1R$ arbitrates vasoconstriction, inflammation, vascular hypertrophy and fibrosis (Touyz and Schiffrin, 2000; Kanaide *et al*, 2003).

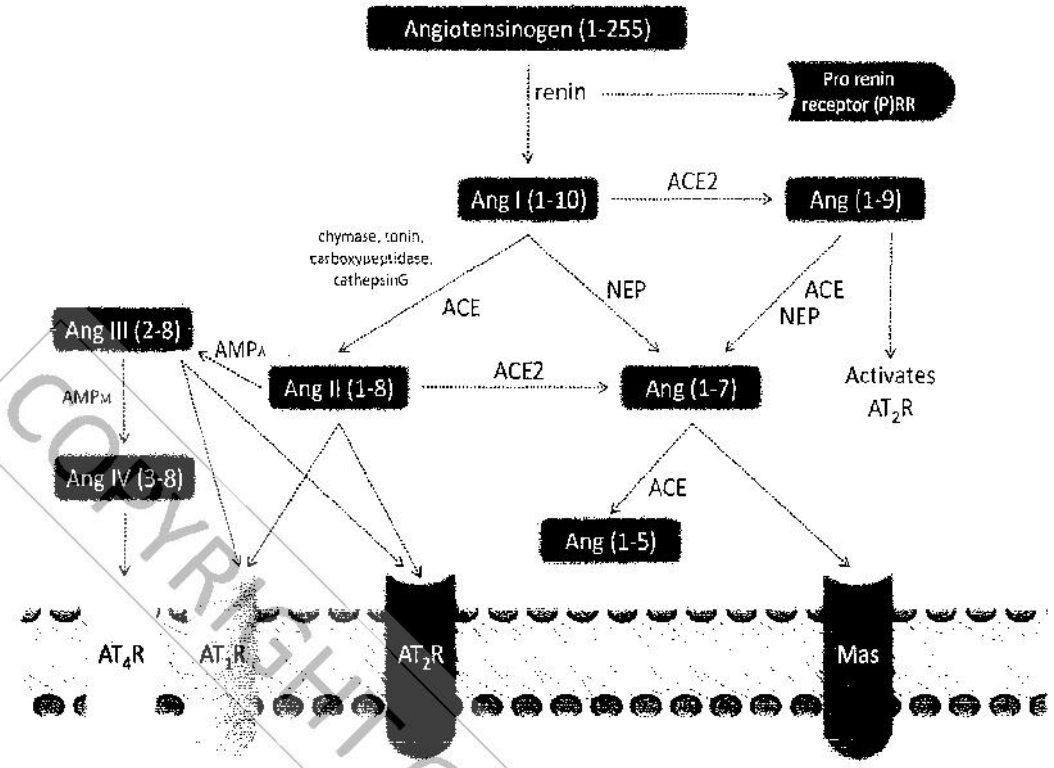



Figure 4: The complete renin-angiotensin cascade. $AT_1R$ stimulation causes vasoconstriction, aldosterone release, hypertrophy, fibrosis and vascular remodeling. The pathophysiological effects of $AT_1R$ stimulation are curtailed by $AT_2R$ activation which activates NO°/cGMP pathway and inhibits MAPKs among other actions causing anti-proliferative, anti-inflammatory and vasodilatory effects. *Mas* receptor stimulation is analogous to $AT_2R$ and promotes Akt phosphorylation, NO° release and vasodilation thus opposing the effects of $AT_1R$ activation. The much less explored $AT_4R$ stimulation by ang IV results in proinflammatory effects but its bearing upon the pathophysiology of human hypertension is yet to be known. ACE-Angiotensin Converting Enzyme; AMPA- Aminopeptidase M; AMPM-Aminopeptidase M; *Mas*- Meiosis activating sterol; NEP-Neutral Endopeptidase

Stimulation of the $AT_1R$ results in activation of several tyrosine kinases which in turn phosphorylate the tyrosine residues in several proteins thus activating or inhibiting them. This results in vasoconstriction, cell growth and proliferation in blood vessels (Marrero *et al*, 1997; Dostal *et al*, 1997; Griendling *et al*, 1997). Activation of various signaling molecules like the small G-proteins (Ras, Rho, Rac, etc.), GPCR, second messengers, protein kinases, etc. are activated by AngII through the $AT_1R$ (Robinson and Cobb, 1997) and contribute to vascular remodeling. Growth and migration of VSMCs in blood vessels is also mediated by AngII through *trans*-activation of EGFR (Greindling *et al*, 1994; Griendling *et al*, 2000). AngII mediated activation of the $AT_1R$ stimulates PI3Kγ which ultimately activates the LTCCs in the smooth muscle cells leading to a contractile response. Akt or protein kinase B is a

signal molecule activated downstream to PI3Kγ (Saward and Zahradka, 1997; Wymann and Pirola, 1998; Takahashi *et al*, 1999a). It prevents the degradation of pore-forming subunit of the LTCC and thus maintains the $Ca^{++}$-influx and resulting contractile state (Ushio-Fukai *et al*, 1999). Pathological actions of angII in VSMCs implicate Rho and its effector Rho-kinases whereby the Rho-kinases phosphorylate the myosin subunit of myosin-light-chain-phosphatase (MLCP) leading to inhibition of the enzyme and maintenance of the smooth muscles in a contractile state (Loirand, 2006). Additionally, Rho-kinases negatively impact protein kinase A mediated phosphorylation of eNOS preventing the release of NO. AngII negatively regulates the NO signaling pathway and thus contributes to endothelial dysfunction (Ming, 2002; Lee, 2004). AngII is also linked to upregulation of p22phox mRNA in the vasculature. p22phox is a subunit of the NOX [NAD(P)H oxidases] group which are responsible for superoxide generation in the vasculature and contribute to oxidative vessel wall stress (Griendling *et al*, 1994; Pagano, 1998). AngII-dependent NOX activation leads to enhanced formation of $O2^{\cdot-}$, which readily reacts with NO forming peroxynitrite ($ONOO^-$) (Meier, 1996; Fukui, 1997). This reduces the available NO and enhances vasoconstrictor response of angII.



## PHARMACOLOGICAL MANAGEMENT OF HYPERTENSION

Lifestyle modifications and dietary approaches to stop hypertension (DASH) (Sacks *et al*, 2001) are seldom effective in controlling blood pressure in hypertensive populations owing to non-compliance. It is almost always necessary to include drug-therapy as an appropriate measure to control blood pressure even in patients diagnosed with mild hypertension. Several agencies have drafted guidelines for the use of drugs in the management of hypertension and these guidelines are reinforced by data from landmark trials like the ALLHAT, UKPDS, INVEST and the HOPE trial among others (King *et al*, 1999; Sleight, 2000; Chrysant, 2003; Pepine *et al*, 2003). Although these reference guidelines may objectively provide information regarding the selection of appropriate class of agents from the vast therapeutic armamentarium, practical interpretations and patient-to- patient factors make pharmacologic therapy a subjective decision for the physicians and is ultimately individualized based on clinical judgment. An overview of the classes of medications typically utilized for the management of hypertension is given in Table 2. Diuretics, ACE-inhibitors and angiotensin receptor blockers form the first-line of therapy for any patient. Other agents may be added as required.

Several classes of novel antihypertensive agents have been approved in the recent past. Aliskiren, a rate-limiting inhibitor of renin in the RAAS, was approved in 2007 by the USFDA (Brown, 2008). It blocks the conversion of angiotensinogen to angI thus depleting the precursor for the formation of angII. However, the ALTITUDE trial (2011) concluded that aliskiren arm showed incidence of non-fatal stroke and renal impairment and thus appropriate contraindications were required to be added in the label of aliskiren (Parving *et al*, 2012). Other investigational renin inhibitors by Speedel Pharmaceuticals (Switzerland) and Vitae Pharmaceuticals (USA) are in different phases of clinical trials (Paulis and Unger, 2010). Clevidipine is a novel vasculo-selective calcium channel blocker indicated when oral therapy is not feasible (Ndefo *et al*, 2010). It was approved in 2008 and shows little effect on myocardial contractility. The ESCAPE clinical trials (ESCAPE-1 & 2) evaluated the efficacy of clevidipine in emergency settings where immediate reduction in blood pressure is necessary (Levy *et al*, 2007; Singla *et al*, 2008). The latest in class ARB, azilsartan medoxomil, was approved in 2011 for the treatment of essential hypertension (Jones *et al*, 2011). Levosimendan is an agent that has dual action, positive inotropy through calcium sensitization and vasodilatory action mediated by opening of ATP-sensitive potassium channels in vascular smooth muscles. Clinical studies have shown varying results with respect to its efficacy but it has been approved for use in patients with CVD accompanied by CHF (Antila *et al*, 2007; Packer *et al*, 2013). Another agent with a similar mechanism is nicorandil which has NO activation properties in addition to potassium channel opening action (Sakata *et al*, 2012; Wu *et al*, 2013). Other agents like cromakalim, rilmakalim and the likes were awaiting approvals from regulatory authorities (Gude, 2012). Rilmenidine, a preferential imidazoline receptor agonist, acts on the peripheral and medullary vasomotor structures thus mediating a sympatho-inhibitory effect and lowering blood pressure (Burke and Head, 2009).

**Table 2: Oral antihypertensive drugs[2]**

| Sr. No. | Class | Prototypical Drug | Focal Mechanism | Major Adverse Effects | Clinical trials |
|---|---|---|---|---|---|
| 1 | Diuretics | Hydrochlorthiazide | Inhibition of ion transport proteins; ECF volume reduction | Hypokalemia | ALLHAT, PROGRESS |
| 2 | β-Blockers | Propranolol | Reduction of cardiac output; decreased renin release from JGA | Bradycardia, rebound hypertension | COPERNICUS, CAPRICORN, BHAT, UKPDS |
| 3 | Calcium channel blockers | Amlodipine | Reduce intracellular availability of $Ca^{++}$ | Peripheral edema (DHP); Bradycardia (others) | INVEST, BEAT-HTN |
| 4 | ACE inhibitors | Captopril | Competitive inhibition of ACE and partial inhibition of AngII formation | Hyperkalemia, dysguesia, cough | SOLVD, HOPE, EUROPA, TRACE, UKPDS |
| 5 | Angiotensin receptor blockers | Losartan | Blockade of $AT_1$ receptor to prevent AngII mediated actions | Hyperkalemia | Val-HEFT, CHARM, LIFE, RENAAL |
| 6 | $\alpha_1$-Blockers | Prazosin | Sympathetic blockade through $\alpha_1$-blockade | Syncope, dizziness, palpitations | VHeFTI, ALLHAT |
| 7 | Aldosterone antagonists | Spironolactone | Prevent sodium/water retention | Hyperkalemia, gynecomastia | RALES, EPHESUS |
| 8 | Central $\alpha_2$-agonists | Methyldopa | Stimulate $\alpha_2$ receptors in the brain stem-decrease central sympathetic outflow | Transient sedation | POISE-2, ReHOT, BEAT-HTN |
| 9 | Direct vasodilators | Hydralazine | NO° mediated cGMP stimulation and/or $K^+$-channel activation | SLE | A-HeFT, BEAT-HTN |

[2] Each class may have its own further sub-classifications which are not shown here. The clinical trials mentioned in the last column are suggestive of the class and not individual drugs *per se*. A-HeFT, African-American Heart Failure trial; ALLHAT, Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial; BEAT-HTN, Black Education and Treatment of Hypertension study; BHAT, Beta-Blocker Heart Attack Trial; CAPRICORN, Carvedilol Post-Infarct Survival Control in Left Ventricular Dysfunction Trial; CHARM, Candesartan in Heart Failure Assessment of Reduction in Morbidity and Mortality Trial; COPERNICUS, Carvedilol Prospective Randomized Cumulative Survival Trial; EPHESUS, Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study; EUROPA, European Trial on Reduction of Cardiac Events with Perindopril in Stable Coronary Artery Disease Trial; HOPE, Heart Outcomes Prevention Evaluation Study; INVEST, International Verapamil-Trandolapril Study; LIFE, Losartan Intervention For Endpoint reduction in hypertension study; POISE-2, Perioperative Ischemic Evaluation study 2; PROGRESS, Perindopril Protection Against Recurrent Stroke Study; RALES, Randomized Aldactone Evaluation Study; ReHOT, Resistant Hypertension Optimal Treatment study; RENAAL, Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan study; SOLVD, Studies of Left Ventricular Dysfunction; TRACE, Trandolapril Cardiac Evaluation; UKPDS, UK Prospective Diabetes Study; Val-HeFT, Veterans Affairs Cooperative I study.

## NOVEL THERAPEUTIC TARGETS FOR MANAGEMENT OF HYPERTENSION

In spite of a gigantic armamentarium of anti-hypertensive drugs and new drugs emerging every year, the personalized therapy for controlling hypertension has remained elusive. Several molecular modulators of hypertension have been identified in the past decade. These modulators offer several targets for a precise control of blood pressure. Myriad targets are currently being pursued experimentally as well as clinically to reinforce the management of hypertension. ACE cleaves the octapeptide angiotensin II to form ang(1-7) and angiotensin I to ang(1-9) (Donoghue *et al*, 2000; Tipnis *et al*, 2000). Several studies have shown cardioprotective role of Ang (1-7). It has been shown that systemic injection of Ang (1-7) leads to systolic BP reduction in SHRs. It has been shown that Ang (1-7) exclusively acts on the *Mas* receptor and a possibility of Ang (1-7) acting on either $AT_1$ or $AT_2$ receptor has been excluded. Downstream effects of *Mas* receptor involve phosphorylation of eNOS suggesting the role of Ang (1-7) in endothelium-dependent relaxation. Several formulations of Ang (1-7) including HPβCD-Ang (1-7) (Lula *et al*, 2007), liposomal Ang (1-7) (Silva-Barcellos *et al*, 2004) and cyclised Ang (1-7) (Kluskens *et al*, 2009) have been reported to improve the efficacy of Ang (1-7) for clinical application. AVE 0991 was the first synthetic non-peptide compound found to have agonistic activity on the *Mas* receptor. Two novel peptides, CGEN-856 and CGEN-857, were discovered via computational drug discovery platform. These peptides are unrelated in structure to Ang (1-7) and have high specificity for the *Mas* receptor (Shemesh *et al*, 2008). These peptides show a dose-dependent blood pressure reduction and their activity is inhibited by *Mas* antagonist, A-779. 1-[[2-(Dimethylamino)ethyl]amino]-4-(hydroxymethyl)-7-[[(4-methylphenyl)sulfonyl]oxy]-*9H*-xanthen-9-one (commonly referred to as *'xanthenone'*) and resorcinonaphthalein have been reported to enhance the levels of endogenous ACE2. While acute administration reduced blood pressure in both SHR and normotensive rats, chronic administration of these compounds reduced blood pressure only in SHRs (Savergnini *et al*, 2010; Savergnini *et al*, 2013). Since aldosterone antagonists are fraught with adverse effects (Maron and Leopold, 2010), an alternative strategy would be the prevention of aldosterone formation itself. This requires inhibition of the enzyme, aldosterone synthase, also known as CYP11B2 (Azizi *et al*, 2013). Thus, analogous to the ACE/AngII pathway, it will prevent formation of aldosterone and its

consequent actions. However, in slight contrast to the ACE/AngII formation, aldosterone is not formed by alternative enzymes thus reactive increase in aldosterone levels upon AngII stimulation would not be possible. FAD286, an enantiomer of fadrozol (Novartis, Switzerland) was first shown to lower blood pressure in rats over-expressing renin and angiotensinogen through inhibition of aldosterone synthase. Other investigational molecules, SPP2745 (Speedel Pharmaceuticals, Switzerland) and LCI699 (Novartis, Switzerland), were also found to have positive effects on kidney and heart owing to aldosterone inhibition in experimental studies (Fiebeler *et al*, 2005; Schumacher, 2013; Lea *et al*, 2009; Bertagna *et al*, 2014). The major reason to pursue aldosterone synthase inhibitors is to find alternatives for preventing the effect of aldosterone on cardio-renal physiology so that the novel agents/targets are non-inferior in efficacy and better tolerated as compared to currently used aldosterone antagonists. Cortisol, a physiological steroid, also activates mineralocorticoid receptors in altered redox states. This effect stands in the way of efficacy of aldosterone synthase inhibitors. Transient receptor potential (TRP) channels are part of a superfamily of cation channels that are formed by tetramers of six transmembrane domain subunits (Firth *et al*, 2007). The vanilloid type 1-TRP channels (TRPV1) are activated by several chemical and physical stimuli. The cation permeable pore of TRPV1 senses activation of GPCRs like adrenoceptors or $AT_1R$ or stretch response (mechanotransduction) in the vascular smooth muscle cells, endothelial cells or pulmonary artery smooth muscle cells. This leads to increased influx of $Ca^{++}$ within the cell. This increased $[Ca^{++}]_i$ has two actions: 1) it causes a $Ca^{++}$ dependent increased activation of protein kinase A through phosphorylation and 2) it diminishes the inhibitory interaction between eNOS and caveolin-1. Displacement of caveolin-1 leads to binding of calmodulin with eNOS, which makes it a dock-site for Hsp90. Once Hsp90 binds to eNOS, the activated protein kinase phosphorylates the 1177 serine residue (1176 in mice) of human eNOS leading to its activation. This leads to a marked increase in formation of NO which causes guanylate cyclase mediated reduction of blood pressure (Sessa, 2010). TRPV1 channel is activated by capsaicin, a component of red-hot chilli peppers; rutaecarpine (a plant alkaloid) also activates TRPV1 (Deng and Li, 2005; Yang *et al*, 2010). Capsazepine, a TRPV1 channel blocker, opposes the action of TRPV1 activation (Yang *et al*, 2010).

RTX (resiniferatoxin) is an ultrapotent analog of capsaicin (structure shown below; Figure 5).

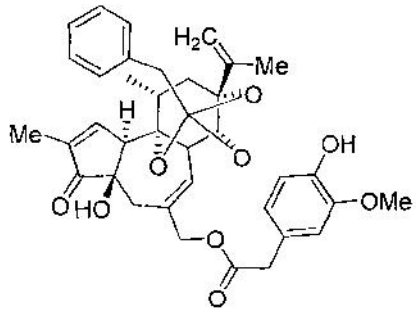



Figure 5: Resiniferatoxin (RTX) is a plant alkaloid obtained from the plant Euphorbia *resinifera*. It is an ultr-apotent analog of capsaicin and activator of TRPV1. It is an important pharmacological tool to study the effects of TRPV1 activation *in vivo*.

Iodinylated RTX, iRTX, is a potent blocker of TRPV1 channel (Sun *et al*, 2009). Based on previous studies it seems that activation of TRPV1 can prove to be useful in reducing the blood pressure. The angiotensin-independent effects of renin are mediated, in part, by binding to the pro-renin receptor, (P)RR (Nguyen *et al*, 2002). Activation of (P)RR mediates several effects that may not be favorable to the pathophysiology of hypertension like upregulation of PAI-1, TGFβ1, enhanced protein synthesis, cell proliferation and a reduction in apoptosis (Schefe, 2008; Huang *et al*, 2007). None of the currently available agents affecting RAAS is known to influence the (P)RR pathway. Investigational peptidic molecules that prevent binding of prorenin to (P)RR have indicated that blockade of (P)RR improves left ventricular function, reduces cardiac hypertrophy and fibrosis, and also prevents nephropathy (Ichihara *et al*, 2006; Susic *et al*, 2008). It is thus speculated that non-peptidic small molecule inhibitors of (P)RR may prevent the angiotensin-independent effects of renin and RAAS activation. Renalase, a flavoprotein, is known to be expressed strongly in the renal and cardiac tissue (Xu *et al*, 2005; Desir and Peixoto, 2014). It is an NAD(P)H oxidase, NOX, that effectively metabolizes catecholamines. Several studies in the recent past have demonstrated the involvement of NAD(P)H oxidase in different types of hypertension, renal and non-renal. The role played by renalase is depicted in Figure 6 below:

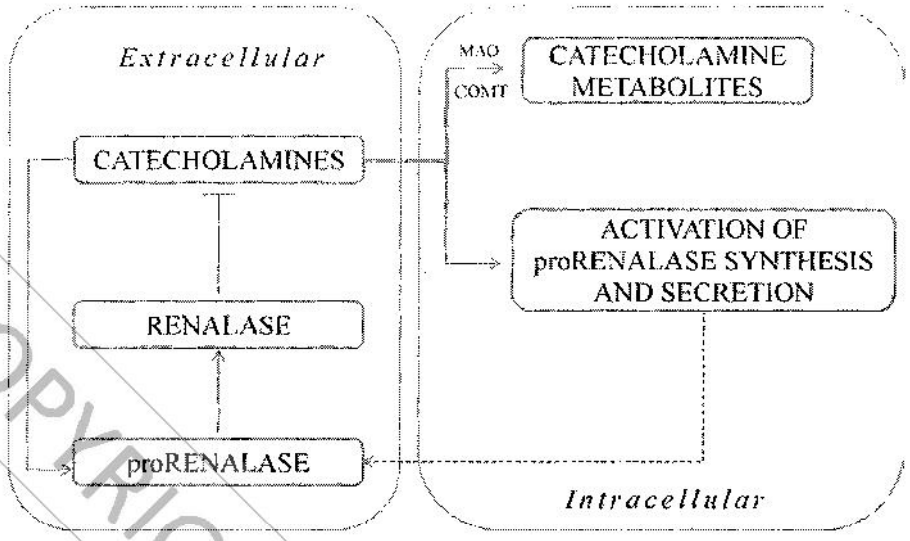



Figure 6: The physiology of renalase and its effect on catecholamine regulation. MAO, Monoamine Oxidase; COMT, Catechol-O-Methyl Transferase.

Renalase deficiency has been associated with intensified sympathetic tone and cardiovascular risk (Desir, 2009). It has been speculated that renalase-replacement therapy may improve cardiovascular outcome in patients suffering from hypertension. Vasopeptidase inhibitor is another very important target being pursued in the management of hypertension. Vasopeptidases, apart from ACE, involve other enzymes like the membrane-bound metalloproteinase, Neutral Endopeptidase (NEP or Neprilysin) and Endothelin-converting enzyme (ECE). Though NEP substrates might be vasodilatory or vasoconstrictive in nature, the prospect of successfully designing dual NEP/ACE- and triple NEP/ACE/ECE-inhibitors as a single molecule has motivated research in this direction (Gude, 2012). Ompatrilat, the prototypical NEP/ACE dual inhibitor, was shown to reduce blood pressure in experimental and clinical studies. Major trials like OCTAVE and OVERTURE showed that dual NEP/ACE inhibition might be effective in the management of hypertension (Packer *et al*, 2002; Kostis *et al*, 2004). Other agents like sampatrilat and fasidotril were also investigated. However, data from clinical studies raised concerns regarding angioedema. Safety profiles have remained an issue with these classes of agents. Instead of ACE inhibition, alternative strategy of combined NEP inhibition and $AT_1R$ blockade is being chased to improve the safety profile of NEP inhibitors (Jandeleit-Dahm, 2006). Agents like ilepatril (Sanofi-Aventis, France) and daglutril (Solvay,

Belgium) are a few agents currently undergoing clinical trials to study the efficacy of NEP inhibition with or without $AT_1R$ blockade (Paulis and Unger, 2010). NO donors and activators are being explored and newer alternatives keep emerging. These include soluble guanylate cyclase (sGC) activators like cinaciguat and riociguat, NO releasing agents like naproxcinod which is actually naproxen with NO releasing property (Merck, USA & Valbonne, France) and NCX-899 which is NO releasing enalapril (Valbonne, France) (Miller and Megson, 2007; Paulis and Unger, 2010). The tirade of biological therapeutics has penetrated in the management of hypertension as well, and as a result several biological products are being researched and tested upon for their potential as anti-hypertensive agents. CYT006-AngQb, a vaccine against AngII is technically made up of virus-like particles coupled to AngII (Tissot *et al*, 2008). Subcutaneous injection of this vaccine produces an immunological response equivalent to injection of foreign proteins in the systemic circulation and leads to production of antibodies against AngII. The preliminary idea behind this vaccine was to develop a safe and effective vaccine to reduce non-compliance in antihypertensive therapy (Brown, 2009; Maurer and Bachmann, 2010). However, another concern with this type of therapy is that the effects of the vaccine cannot be reversed or stopped easily. With these speculations, CYT006-AngQb underwent clinical trials in 2008 (Tissot *et al*, 2008) and showed mild reduction in blood pressure which was not comparable, infact, was inferior to ACE inhibitors and ARBs. It was also observed that blood pressure reduction was not reproducible across the dosing schemes that were detailed in the trial protocols and as a result progress of CYT006-AngQb to phase III was halted (Petrovsky, 2013). ATR12181 is another product which targets $AT_{1A}$ receptor (Ming *et al*, 2006; Zhu *et al*, 2006). Similar vaccines with modified immunogenicity and different adjuvants are being investigated as potential future therapies. This discussion is by no means exhaustive and with several new therapies being investigated day-in and day-out, different therapeutic strategies would have surfaced at the time when this text is circulated.



## $PI_3K$/Akt AS A POTENTIAL TARGET FOR ANTIHYPERTENSIVE THERAPY

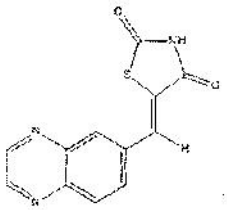
Constriction of vascular smooth muscle cells (VSMCs) as a result of increased myogenic tone, is one of the key elements that is responsible for maintenance of blood pressure (Guyton and Hall, 2006). This alteration of myogenic tone is not dependent on

neural mechanisms for blood pressure control and can be described due to the followings: 1) increased levels of intracellular calcium in the cell mediated through L-type calcium channels (LTCCs), 2) constriction in response to the increased levels of intracellular calcium and, 3) since the wall of the artery is unable to handle this tremendous pressure built within the VSMCs, there is forced dilation (Hill *et al*, 2006). Several pathways have been explored in the past to identify the agents or signals involved in the regulation of myogenic tone (Morello *et al*, 2009). Recently, phosphoinositide 3-kinase γ ($PI_3K\gamma$ for short) has been found to be a strategic regulator of vascular myogenic tone. $PI_3Ks$ are implicated in several pathways related to varied responses in a cell. $PI_3Ks$, being kinases, phosphorylate the 3'-OH group of inositol ring in the $PtdIns(4,5)P_2$ (phosphatidyl inositol bis phosphate) molecule. This phosphorylation results in the formation of $PtdIns(3,4,5)P_3$ (inositol tris phosphate) which can be metabolized further (Morello *et al*, 2009; Vanhaesebroeck *et al*, 2010). The $PI_3Ks$ have been classically divided into three classes: Class I, Class II and Class III (Katso *et al*, 2001, Carnevale *et al*, 2012a). The $PI_3K$ of our interest, $PI_3K\gamma$ (EC 2.7.1.153) belongs to Class IB and has been believed to be restricted to leukocytes. However, some recent evidence suggests that $PI_3K\gamma$ has avenues outside the immune system as well and is also present in the cardiovascular system in general and in cardiomyocytes, VSMCs, vascular endothelial cells and platelets in particular (Oudit, 2004; Morello *et al*, 2009).

$PI_3K\gamma$ is the kinase that is typically activated upon activation of GPCRs (Macrez *et al*, 2001; Carnevale *et al*, 2012a). As stated previously, since $PI_3K\gamma$ is present in vascular smooth muscle cells and it was found to be a regulator of myogenic tone, it would not be an exaggeration to suspect its role in maintaining a delicate balance between vasoconstriction and vasorelaxation. Such vasoactive phenomenon may be induced by multitude of GPCR agonists/antagonists. Different researchers have already shown the role of $PI_3K\gamma$ in increased smooth muscle contractility mediated through angII (Carnevale *et al*, 2012b). Thus $PI_3K\gamma$ mediates the downstream signaling effects of the $AT_1$ receptors, specifically vasoconstriction as the end-result. $PI_3K\gamma$, upon stimuli received by activation of $AT_1Rs$ (a GPCR coupled to $G_q$ type of G-Protein) excites the L-type calcium channels in smooth muscle cells, thereby increasing intracellular calcium to such high levels that they cross the contractile threshold and result in a contractile response (Macrez *et al*, 2001;

Viard *et al*, 2004). This effect was confirmed using specific monoclonal antibodies against $PI_3K\gamma$ where $PI_3K\gamma$ blockade in rat portal vein myocytes inhibited the ang-II mediated downstream $IP_3$ production and ensuing calcium influx (Quignard, 2001; Viard *et al*, 2004). Another important aspect of L-type channel function is its degradation. The pore-forming subunit of the LTCC ($Ca_v\alpha 1c$) is susceptible to proteolytic degradation in an unphosphorylated state. But one of the agents, acting further downstream to that of $PI_3K\gamma$, Akt (the name of this molecule bears no functional importance since it was derived from the T-cells of an Ak strain of mice, hence Akt), phosphorylates this subunit and thus protects it from proteolytic degradation (Quignard, 2001). All these findings found pillars of support when mice lacking $PI_3K\gamma$ showed protection against chronic angII exposure and angII evoked L-type $Ca^{++}$ influx (Carnevale *et al*, 2012b). Akt stands as a link between $PI_3K\gamma$ and LTCC in resistance arteries, regulating the increased myogenic tone upon stimulation (Shiojima and Walsh, 2002). Another very interesting effect mediated through $PI_3K\gamma$ signaling is that of "mechanotransduction". Patel *et al* have shown that even in the absence of angII, pressure exerted on the receptors may activate the $AT_1R$ and could activate $PI_3K\gamma$ through $\beta\gamma$ signaling. Thus, modulation of the myogenic response mediated by $PI_3K\gamma$ may proceed in a ligand-dependent or independent approach (Patel *et al*, 2010). A point of concern that needs address at this juncture is that whether ARBs (Angiotensin Receptor Blockers, "*sartans*") fail to achieve adequate control of blood pressure due to this effect? In light of these findings, inhibition of $PI_3K\gamma$ and its downstream Akt signaling offers lucrative targets to relieve increased myogenic tonus and cause relaxation (Yang and Raizada, 1999; Kippenberger *et al*, 2005; Li and Malik, 2005a). Inhibition of kinase-dependent $PI_3K\gamma$ signaling and its downstream Akt signaling were found to markedly impair the myogenic tone in isolated vessels (Kippenberger *et al*, 2005; Carnevale *et al*, 2012b). Another finding of high-repute in this regard is of the fact that inhibition of $PI_3K\gamma$ signaling impairs accessory subunit ($Ca_v\beta 2a$) phosphorylation and thus decreases the probability of opening of LTCC (Viard *et al*, 2004). In the past decade, several small-molecule inhibitors of $PI_3K\gamma$ have been developed and explored in cardiovascular studies. Two small molecules are available commercially, though not for therapeutic use (Carnevale *et al*, 2012b):

- AS605240 (Cayman chemical, Sigma aldrich, Biovision)
- GE21 (under patent with IRCCS Neuromed, Italy)



(AS605240)

The antihypertensive effect of these inhibitors is manifested as: reduction of total peripheral resistance and counter action of development of myogenic tone, thus relaxing resistance arteries (Morello *et al*, 2009; Carnevale *et al*, 2012b). Based on the information available on the subject of $PI_3K\gamma$ specific inhibition, we can certainly build up on the premise that $PI_3K\gamma$-inhibition is a promising strategy to treat hypertension (Takahashi *et al*, 1999a; Dugourd *et al*, 2003). Recent genetic evidence in humans supports the association of $PI_3K\gamma$ to blood pressure regulation (Carnevale *et al*, 2012a) and thus inhibition of this pathway may be considered as a promising tool against hypertension.

**THE ANGIOTENSIN RECEPTOR *TYPE 1***

The peptide angiotensin II (angII) mediates its effects through the angiotensin receptors in mammalian cells. Two types of receptor subtypes have been cloned and characterized: angiotensin receptor type 1, $AT_1$ and angiotensin receptor type 2, $AT_2$ (Murphy *et al*, 1991; Sasaki *et al*, 1991; Mukoyama *et al*, 1993). The $AT_3$ and $AT_4$ receptors are not fully characterized as of now (Wright and Harding, 1997; Krebs *et al*, 1996). All the subtypes, except $AT_4$, are known to bind angiotensin (Chaki and Inagami, 1992). Most of the perceptible actions of AngII are mediated through the type 1 receptor (IUPHAR Receptor Code ***2.1.Ang.01.000.00.00***). Experimental studies are yet dissecting the role of $AT_2$ receptors in different physiological functions (Dinh *et al*, 2001). The $AT_1$ receptor is coded by the human gene AGTR1 (a.k.a *at1*), located on chromosome 3q24. The gene sequence shares ~90 % sequence homology with rodent *agtr1* gene coding for the same protein [www.ensembl.org]. The human $AT_1$ receptor gene product consists of 359 amino acids with a molecular mass of about 41 kDa (Dinh *et al*, 2001). The $AT_1$ receptor belongs to the superfamily of GPCRs and consists of seven transmembrane units. Location of four cysteine residues in the extracellular domain results in formation of disulphide bridges which govern the tertiary structure of the receptor. The extracellular loops and the transmembrane domain (TMD) provide the site for binding of AngII. This receptor is

recognized by its ability to bind antagonistic ligands like losartan and the type, however, the antagonist binding site differs from the AngII-binding site (Dinh *et al*, 2001; Higuchi *et al*, 2007). Predominant expression of this receptor can be found in the liver, adrenal glands, kidney, vascular smooth muscles and lungs. Acutely increased levels of AngII are known to activate $AT_1R$, however, chronic exposure to AngII may lead to downregulation of the receptor (Lassegue *et al*, 1995; Griendling *et al*, 1996). While insulin and LDL are known to upregulate $AT_1R$ (Nickenig *et al*, 1997a; Nickenig *et al*, 1997b); AngII, estrogen, EGF and PDGF are known to downregulate it (Gunther *et al*, 1980; Nickenig *et al*, 1996; Takeda *et al*, 2000). The effects of AngII upon target tissues are found to be momentary; the reason for this is the endocytosis of this receptor within 10 mins of its activation. One-fourth of the endocytosed receptors are then recycled back to the plasma membrane while the remaining are degraded by the lysosomes (Gunther *et al*, 1980). Phosphorylation of serine and threonine residues present on the C-terminal end of the cytoplasmic surface of $AT_1R$ plays an important role in internalization of these receptors. This effect is mediated, in part, by caveola (Ishizaka *et al*, 1998). Intrinsic kinase activity is not present in any GPCR, but G-protein receptor kinases (GRKs) are responsible for phosphorylation of the serine and threonine residues (Oppermann *et al*, 1996). The $AT_1R$ is also phosphorylated at various tyrosine residues and various tyrosine kinases like JAK, FAK and Src family kinases are known to arbitrate such actions (Oppermann *et al*, 1996; Kim *et al*, 2005).

***Signaling pathways***

The $AT_1R$ being a GPCR, is coupled to the $G_{q/11}$ protein for effecting any downstream signal. This protein $G_{q/11}$ in turn mediates activation of second messenger systems like the $IP_3$/DAG pathway activated through phospholipase Cβ (Touyz and Schiffrin, 2000). Other non-G-protein related pathways are also activated upon $AT_1R$ stimulation. Phospholipase Cβ cleaves $PIP_2$ leading to the formation of $IP_3$ and DAG (Alexander, 1985; Griendling *et al*, 1989; Touyz and Schiffrin, 2000). $IP_3$ binds to its receptor present on the sarcoplasmic reticulum leading to release of calcium from intracellular stores. This calcium forms a complex with calmodulin to activate MLCK which phosphrolyates the light chain of myosin to enhance its interaction with actin filaments (Touyz and Schiffrin, 1997; Touyz *et al*, 1999). This effect leads to smooth

muscle contraction and is predominantly observed in vascular smooth muscle cells. Normally, this effect is terminated by MLCP, which dephosphorylates myosin and thus its interaction with actin is abolished but Rho kinase inhibits MLCP leading to increasing contractions. On the other hand, DAG activates protein kinase C (PKC), which phosphorylates the $Na^+/H^+$ pump and increases the cellular *p*H during contraction phase which helps in sustaining the effect. Increased alkalinization of the cell retains $[Na^+]_i$ and thus increases $[Ca^{++}]_i$, leading to sensitization of the contractile apparatus to $Ca^{++}$ and increased intracellular *p*H (Tepel *et al*, 1998; Touyz *et al*, 1999), potently stimulates DNA synthesis leading to cell growth and proliferation (Damron *et al*, 1998; Touyz *et al*, 1999). Thus by activating the $Na^+/H^+$ pump, $AT_1R$ activation ultimately modulates VSMC contraction and growth (Touyz and Schiffrin, 1997; Touyz *et al*, 1999). DAG also contributes in the Ras/Raf/MEK/ERK pathway and exhibits activation/inactivation of different proteins through phosphorylation, the downstream molecules of which contribute in vasoconstriction (Kusuhara, 1998; Touyz *et al*, 1999). Agonist binding at $AT_1R$ leads to activation of PLD which is responsible for hydrolysis of phosphatidylcholine to choline and phosphatidic acid. Phosphatidic acid is rapidly converted to DAG leading to activation of PKC and subsequent effects mediated through DAG (Alexander *et al*, 1985; Griendling *et al*, 1989). The $AT_1R$ also leads to phosphorylation and activation of $PLA_2$, which results in the formation of arachidonic acid. Metabolites of arachdonic acid are known to regulate vascular tone and NAD(P)H oxidation of VSMCs. $AT_1R$ activation also mediates stimulation of growth and migration related signaling, through a myriad of downstream proteins, which is above and beyond VSMC contraction (Griendling *et al*, 2000). The non-G-protein pathways include NAD(P)H and ROS signaling, MAP kinase activation, and stimulation of receptor & non-receptor tyrosine kinases (Griendling and Ushio-Fukai, 2000; Griendling *et al*, 2000).

***$AT_1R$ and $PI_3K$/Akt signaling***

Phosphoinositide-3-kinases ($PI_3Ks$), a family of intracellular signal transducers, is also activated through $AT_1$ receptors. In vascular smooth muscle cells and cardiomyocytes, AngII mediates the activation, phosphorylation and migration of $PI_3K$ through $AT_1R$ activation. It also induces the translocation of the p85 subunit of $PI_3K$ in various regions of

the cell from the perinuclear area to the foci, throughout the cytoplasm and to the cytoskeletal proteins (Saward and Zahradka, 1997). This action of AngII is reported to peak around 15 mins after binding at $AT_1R$ and then subsides to baseline levels at 30 mins. It has been shown that small molecule inhibitors of $PI_3K$, wortmannin and LY294002 block AngII mediated hyperplasia and contraction in smooth muscle cells. A couple of years ago, Akt (protein kinase B), was identified to be an important downstream molecular target of $PI_3K$ stimulation in VSMCs (Saward and Zahradka, 1997; Wyman and Pirola, 1998). It is not clear how exactly $AT_1R$ activation leads to $PI_3K$-dependent Akt activation but it is suggested that it may involve redox-sensitive pathways and c-Src (Ushio-fukai *et al*, 1999). Akt is associated with VSMC proliferation and growth. Akt is known to regulate protein synthesis through activation of p70S6 kinase (Eguchi *et al*, 1999) and stimulates $Ca^{++}$ currents in aortic smooth muscle cells, thus modulating AngII mediated contractions (Eguchi *et al*, 1999; Seki *et al*, 1999). AngII mediated activation of arachidonic acid catabolism generates metabolites like 5(S)- and 12(S)-hydroxyeicosatetraenoic acid through LOX, which activates Akt (Neeli *et al*, 2003; Moreno, 2009). PLD activation in CHO cells overexpressing endothelial differentiation receptor is known to activate Akt (Banno *et al*, 2001). However, it is not clear whether the same effect can be observed in VSMCs upon AngII mediated PLD activation. At present, it is speculated that AngII mediated Akt activation in VSMCs may be $PLA_2$- or PLD-dependent (Banno *et al*, 2001; Li and Malik, 2005). A peculiar effect observed with Akt is that it is known to inhibit caspases and influences Bcl-2 and c-myc expression, thus preventing apoptosis and promoting cell survival. Thus, phosphorylated and activated Akt is known to stimulate several downstream negotiators like Bad, GSK3, eNOS and mTOR among others which regulate events ranging from cell survival, cell cycle and protein synthesis to angiogenesis and vasomotor tone (Matsui *et al*, 2001; Shiojima and Walsh, 2002; Sata and Nagai, 2002; Downward, 2004).



## THE ALPHA1 ($\alpha_1$) ADRENERGIC RECEPTOR

The $\alpha_1$-adrenergic receptors belong to the superfamily of 7TM-GPCRs and are responsible for most of the actions of noradrenaline *in vivo* and several physiological actions of adrenaline as well (Garcia-Sainz, 1993; Cotecchia, 2010). Molecular cloning has

revealed that the $\alpha_1$-adrenergic receptor has three subtypes - $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$ in total (Schwinn *et al*, 1995; Graham *et al*, 1996) and all the three were found to have different amino acid sequences and consequently variable pharmacological actions (Table 3). All the receptors are known to bind to adrenaline and noradrenaline unequivocally, though they are also known to bind other synthetic agonists like phenylephrine and oxymetazoline selecti-

**Table 3: IUPHAR data for the different alpha1-adrenergic receptor subtypes[3]**

| Receptor subtype | Gene symbol | Chromosomal loci | IUPHAR Code | Amino acid sequence length | Preferential human tissue distribution |
|---|---|---|---|---|---|
| Alpha1a ($\alpha_{1A}$) | ADRA1A | 8p21-p11.2 | 2.1.ADR.A1A.000.00.00 | 466 | Prostate, urethra, heart, liver, vasculature, cerebellum and cerebral cortex |
| Alpha1b ($\alpha_{1B}$) | ADRA1B | 5q23-q32 | 2.1.ADR.A1B.000.00.00 | 519 | Prostate, cervix, uterus, umbilical vein, spleen and coronary endothelium |
| Alpha1d ($\alpha_{1D}$) | ADRA1D | 20p13 | 2.1.ADR.A1D.000.00.00 | 572 | Aorta, bladder, iliac and femoral arteries |



[3] Information retrieved from the family menu given at http://www.iuphar-db.org/DATABASE (Accessed on 27th Aug, 2014). This is the official website of the International Union of Basic & Clinical Pharmacology.

vely (Schwinn *et al*, 1991; 1988; Garcia-Sainz *et al*, 1992; Garcia-Sainz, 1993; Cotecchia *et al*, 2000). The hydrophobic transmembrane loops form the α-helices that are connected by alternating extracellular and intracellular hydrophilic loops. The hydrophobic regions form the ligand binding sites whereas the intracellular hydrophilic regions are responsible for interacting with G-proteins, signaling molecules and regulatory proteins (Wess, 1997; Bylund, 2007).

***Signaling and regulation of $\alpha_1$-adrenergic receptors***

For the most part, actions of ligands interacting with the $\alpha_1$-adrenergic receptors are elaborated through interaction with $G_{q/11}$ resulting in stimulation of phosphoplipase C activity and thus promote the hydrolysis of $PIP_2$ leading to the formation of $IP_3$ and DAG (Garcia-Sainz, 1993; Zhong and Minneman, 1999; Cotecchia, 2010). The details of this signaling are mentioned in *'The angiotensin receptor type 1'* section above. The three subtypes couple to phospholipase C with different levels of efficacy, with $\alpha_{1A}$ having the highest and $\alpha_{1D}$ the lowest (Theroux *et al*, 1996; Taguchi *et al*, 1998). The three subtypes also couple to other G-proteins at varied degrees and show myriad actions like induction of cardiac hypertrophy by interacting with $G_{12/13}$ (Hawrylyshyn *et al*, 2004). The resultant activation of proteins leads to mobilization of calcium from intracellular stores. In addition, these receptors also activate the influx of calcium through VDCCs and voltage-independent calcium channels (Muth *et al*, 1999; Petrashevskaya *et al*, 2004). $\alpha_1$-Adrenergic receptors also signal through the kinases of the MAPK family thus regulating the growth promoting effects of these kinases. MAPK signaling activated upon $\alpha_1$-adrenergic receptor stimulation contributes to increased DNA synthesis and cellular proliferation in human VSMCs (Hu *et al*, 1999; Chang and Karin, 2001). In fibroblasts, cell proliferation and growth mediated through different JNKs (46 and 54 KDa), p38 MAPK is also mediated by $\alpha_1$-adrenergic receptor stimulation (Alexandrov *et al*, 1999; Piascik and Perez, 2001; Clerk *et al*, 2001). It has also been shown that $\alpha_{1B}$ mediates cell-cycle progression while the other two subtypes mediate cell cycle arrest in fibroblasts. In hepatocytes, interleukin-6 signaling is inhibited through $\alpha_1$-adrenergic receptor mediated MAPK activation (Auer *et al*, 1998; Nguyen and Gao, 1999). Stimulation of predominant subtypes ($\alpha_{1A}$ and $\alpha_{1B}$) in cardiomyocytes *in vitro*, results in a hypertrophic response which in turn is accompanied by activation of

hypertrophic genes like *c-jun, c-fos* and *egr-1*(Iwaki *et al*, 1990; Knowlton *et al*, 1993). Also, there is an upregulation of contractile proteins like MLC-2 and reactivation of embryonic genes like ANF, myosin and actin (Iwaki *et al*, 1990; Knowlton *et al*, 1993; Cotecchia, 2010).

The regulation and desensitization of different $\alpha_1$-adrenergic receptor subtypes follow contradictory patterns. The regulation of $\alpha_{1A}$ receptor subtypes is quite reticent and it undergoes, if at all, very modest agonist-induced endocytosis and recycling. In stark contrast, the $\alpha_{1B}$ receptor undergoes rapid phosphorylation by GRKs followed by desensitization and endocytosis when stimulated by an agonist (Diviani *et al*, 1996; Diviani *et al*, 1997; Stanasila *et al*, 2008). With the $\alpha_{1D}$ receptor, noradrenaline and protein kinase C mediated phosphorylation is connected to the desensitization of the receptor (Garcia-Sainz and Villalobos-Molina, 2004). β-Arrestin also plays a significant role in downregulating the effects of $\alpha_{1A}$ receptor, while $\alpha_{1B}$ receptor shows only weak interaction with β-arrestin (Stanasila *et al*, 2008).

***Correlation of $\alpha_1$ adrenoceptor subtypes with VSMC contraction***

Previously, it was shown that $\alpha_1$ receptor mRNA and receptor proteins were expressed on the peripheral arteries of the test species (rats or humans). After the subdivision of the $\alpha_1$ adrenoceptor subtypes, question arose as to which vascular bed houses which kind of $\alpha_1$ adrenoceptor subtype? Based on several studies, it was demonstrated that each vascular bed is host to one dominant $\alpha_1$ adrenoceptor that mediates contraction (Yamada *et al*, 1980; Jones *et al*, 1985). Though there is a dearth of acutely specific receptor-subtype ligands, experiments utilizing different tissue preparations have been utilized to coherently demonstrate that the $\alpha_{1A}$ adrenoceptor mediates contraction of caudal and renal arteries whereas the $\alpha_{1D}$ adrenoceptor mediates aortic smooth muscles, mesenteric, femoral and iliac arteries (Piascik *et al*, 1995; Piascik *et al*, 1997; Hrometz *et al*, 1999). The $\alpha_{1B}$ adrenoceptor subtype is least involved in mediating contraction of any kind of vasculature. This is also evident from the study where phenylephrine (pan-specific $\alpha_1$ adrenoceptor agonist) was shown not to have any pressor response in $\alpha_{1B}$ adrenoceptor knock out mice (Cavalli *et al*, 1997). Alternatively, overexpression of the $\alpha_{1B}$ adrenoceptor did not result in an increased systemic blood pressure thus confirming the paucity of this

subtype in regulating blood pressure (Zuscik *et al*, 2001). Similar studies were performed to evaluate the effects of such molecular and genetic manipulations on blood pressure. Rokosh and Simpson (2002) reported that $\alpha_{1A}$ adrenoceptor knock out mice show lower MAPs as compared to controls and their responses to phenylephrine are also understated. Similarly, $\alpha_{1D}$ adrenoceptor knock out mice did not show any changes in basal blood pressure values but the vasopressor response was impaired (Piascik and Perez, 2001). Overall, it may be concluded that in case of vascular contractility the dominance can be given as follows: $\alpha_{1A} > \alpha_{1D} >>> \alpha_{1B}$ (Piascik and Perez, 2001). The predominance of the $\alpha_{1D}$ subtype in major arteries suggests that this is the most significant receptor involved in maintenance of vascular tone. By using selective antagonists of this subtype, it was shown that pressor responses induced by neurogenic stimulation or by $\alpha_1$ adrenergic agonists can be blocked, suggesting the functional role of $\alpha_{1D}$ receptors in development of vascular resistance (Tanoue *et al*, 2002). Catecholamines are known to induce proliferation, apart from contraction, in vascular smooth muscle cells. RASM cells show expression of different $\alpha_1$ adrenoceptor subtypes involved in smooth muscle proliferation (Yu et al, 1996; Ulu *et al*, 2010). Prolonged stimulation of the $\alpha_1$ adrenoceptors (by non-catecholamines as well), is known to increase the mRNA expression of α-actin, signifying the augmentation of the contractile phenotype of these cells. $\alpha_{1D}$ and $\alpha_{1B}$ receptors are supposedly the mediators of such a response. Chronic exposure to $\alpha_1$ adrenergic agonists induces a hypertrophic response in these vascular smooth muscles with a concomitant rise in the volume and accumulation of contractile proteins (Bishopric *et al*, 1987; Long *et al*, 1989). $\alpha_{1A}$ Adrenoceptors are known to activate signaling pathways that are responsible for increasing the size of murine ventricular myocytes (Papay *et al*, 2013). Signaling is mediated through different kinases like PKC, PI3K, MAPK and the Ras proteins acting in concert with each other. Some signaling events of the different adrenoceptor subtypes may be contradictory to each other thus resulting in a cross talk; like activation of $\alpha_{1B}$ subtype inhibits signaling mediated through the $\alpha_{1A}$ subtype (Rossier *et al*, 1999). These phenomena are important in relation to hypertension since increase in blood pressure as well as upholding of increased blood pressure in SHRs involve hypersensitivity of vascular smooth muscles to $\alpha_1$ adrenoceptors. Stimulation may either lead to increase in proportion of receptors that are able to respond to increased levels of catecholamines or there may be an

increase in the post-receptor events that mediate $\alpha_1$ adrenoceptor responses in hypertension. It has been suggested that expression of $\alpha_{1D}$ receptor is increased in organs harvested from SHR or animals from other models of hypertension (Villalobos-Molina *et al*, 1999; Villalobos-Molina *et al*, 2008), however, radioligand binding studies have failed to detect these receptors (Hosoda *et al*, 2005). This paradoxical result raises doubts regarding the functional role of the $\alpha_{1D}$ subtype in development and maintenance of hypertension, yet the abundance of these receptors found from rat and human aortic smooth muscle cell membranes definitely point towards the prevalent role of the $\alpha_{1D}$ subtype in hypertension (Garcia-Sainz and Villalobos-Molina, 2004). α-Adrenergic responses in the aortic, carotid and mesenteric arteries of the normotensive as well as the hypertensive rats have been shown to be mediated through the $\alpha_{1D}$ adrenoceptors. In fact, it was also shown that the carotid and aortic rings from hypertensive rats were more reactive to noradrenaline as compared to those from normotensive animals (Tanoue *et al*, 2002). Another interesting observation is that $\alpha_{1D}$ adrenoceptors show an age-dependent rise in their expression (Ibarra *et al*, 1997). This can be correlated directly with hypertension as the population curve tends to show an increased occurrence of hypertension with increase in age. Thus it may be strongly suggested that $\alpha_{1D}$ adrenoceptors are involved in the pathogenesis and maintenance of hypertension.

***$\alpha_1$ Adrenoceptors and $PI_3K$/Akt signaling***

$PI_3K$ has been implicated in mediating the effects of several receptors including the pertussis-toxin insensitive GPCRs, of which $\alpha_1$ adrenoceptors form the subset. Since these receptors are heterotrimeric in nature it is possible that any of the monomer units might be responsible for activation of $PI_3K$ (Molkentin and Dorn, 2001). The p85 subunit of $PI_3K$ is activated by activation of $G_{\beta\gamma}$ subunit of Gq receptors in platelets (Geltz and Augustine, 1998). It has been demonstrated that $\alpha_1$ adrenoceptor mediated mitogenesis is a result of $PI_3K$ activation in human VSMCs (Biesen *et al*, 2013). Specific information regarding the subtype involvement in $PI_3K$ activation is scarce however it may be believed, based on the available evidence, that the three subtypes overlap in different tissues with regard to $PI_3K$ activation. One study has reported that $\alpha_{1A}$ and $\alpha_{1B}$ but not the $\alpha_{1D}$ subtype are involved in $PI_3K$ activation and downstream Akt signaling (Xiao *et al*, 2006). Norepinephrine mediated

the activation of $PI_3K$ in stably transfected cell lines expressing the $\alpha_{1A}$ and $\alpha_{1B}$ subtypes but not in those expressing $\alpha_{1D}$ (Ballou *et al*, 2003). The authors concluded that $\alpha_{1A}$ receptors mediate $PI_3K$ activation via the $G_\alpha$ subunits (Mohl *et al*, 2012) and $\alpha_{1B}$ receptors mediate $PI_3K$ activation through $G_{\beta\gamma}$ subunits (Garcia-Sainz *et al*, 2011). Conversely, it was also shown that sequestration of the βγ subunits from the GPCR did not result in any change in $PI_3K$ activation in $\alpha_{1B}$ transfected cells (Hu *et al*, 1999a). Another study has reported that $\alpha_1$ adrenoceptor mediated activation of $PI_3K$ is associated with activation of Akt and Ras proteins. Initially there is PDK mediated phosphorylation of Akt (Thr 308) (Kuo *et al*, 2008) followed by rise in substrate specificity through subsequent phosphorylation (Ser 473) (Bayascas and Alessi, 2005). Full activation of this protein requires phosphorylation at both the residues. Yamboliev and colleagues (2005) have shown that noradrenaline induces $PI_3K$ mediated membrane depolarization in canine mesenteric vein rings. They showed that norepinephrine leads to an increase in phosphorylated $PI_3K$ and Akt upon adrenergic stimulation with noradrenaline. Budzyn *et al* (2005) also reported that wortmannin, a $PI_3K$ inhibitor, attenuates contractile responses to phenylephrine in arterial preparations.

## CROSS TALKS BETWEEN RAAS AND THE α-ADRENERGIC SYSTEM

Several clinical and preclinical studies have suggested that RAAS and the α-adrenergic system are involved in the homeostasis of blood pressure. Experimental evidence from several studies has implied that these two systems are not totally independent; in fact, they are intertwined through some common pathways and act in concert for the management of blood pressure. A liaison of sorts exists between the two for accomplishing the regulation of cardiovascular functions. The interactions between the adrenergic arm of the sympathetic nervous system and RAAS have physiological as well as pathophysiological consequences. The systems are known for mutually fortifying the responses to increases or decreases in blood pressure but may occasionally lose control over the response machinery leading to development of hypertension. In such situations, it becomes imperative that the entire compensatory system may be shut to prevent damage to vital organs caused by pressure overload. Several experimental studies have tried to delineate the occurrence of cross talks amongst the adrenergic system and RAAS.

**Van Zwieten and de Jonge (1986)** indicated that prolonged activation of the sympathetic nervous system and the RAAS are detrimental for the cardiovascular system. Renin release from the JG cells of the kidney is mediated through SNS stimulation while the anti-natriuretic and vasoconstrictor activity observed upon renal nerve stimulation is mediated by $\alpha_1$ adrenoceptors. The root of the vasoconstrictor activity of AngII lies not only in the activation of post-synaptic $AT_1$ receptors but also in the activation of several processes related to the sympathetic nervous system like enhanced release and reduced uptake of noradrenaline, promotion of noradrenaline release through presynaptic AngII receptors and sensitization of peripheral $\alpha_1$ adrenoceptors.

**Seidelin *et al* (1987)** initially interrogated whether the interaction between noradrenaline and angII in humans, if any, was presynaptic or postsynaptic? The authors studied the hemodynamic effects of noradrenaline released physiologically upon exogenous angII infusion and experimental SNS stimulation and they found that plasma noradrenaline responses were not enhanced by angII. Upon simultaneous infusion of angII and noradrenaline against angII only and control, it was observed that angII acts at some postsynaptic site to mediate the augmentation of noradrenaline mediated rise in systolic blood pressure.

**Lang *et al* (1992)** in a clinical study showed that AngII is able to induce sodium reabsorption in the proximal tubule and distal segment of the nephrons thereby exhibiting anti-natriuretic action without affecting the glomerular filtration rate. When non-depressor doses of prazosin were administered to animals the effects of AngII were blunted suggesting that there exists a renal interaction between the $\alpha_1$ adrenoceptors and AngII.

**Farivar *et al* (1995)** evaluated the effects of losartan in phenylephrine mediated fibrosis. Phenylephrine is known to mediate fibroproliferative responses in cardiac fibroblasts. Since this effect is known to be mediated through the $\alpha_1$ adrenoceptor, it may be assumed that prazosin might block these events. The authors studied the effects of losartan as well as prazosin in phenylephrine treated animals. Prazosin treated animals showed a normal histopathology. Interestingly cardiac histopathological sections from losartan-treated animals were also free from signs of fibrosis. The results of this study

suggest that $AT_1$ receptors are also involved in the fibroproliferative responses to phenylephrine.

**Maeso *et al* (1996)** utilized a functional antagonism assay on SHR aortic rings to demonstrate that AngII potentiates the contractile response of aortic rings to phenylephrine. Losartan was used as an antagonist to affect the contractile response produced by phenylephrine. It was observed that losartan did reduce the contractile response produced by phenylephrine without affecting the relaxation produced by acetylcholine or sodium nitroprusside, indicating a receptor mediated role. However, this attenuation was not observed in endothelium-denuded and *L*-NAME treated aortic rings suggesting a probable role of NO in causing this effect.

**Li *et al* (1997)** studied the effects of AngII stimulation on $\alpha_1$ adrenoceptor subtype expression in ventricular myocytes. They found that AngII stimulation has no effect on $\alpha_{1B}$/ $\alpha_{1D}$ receptor expression but $\alpha_{1A}$ receptor mRNA was found to be downregulated and this effect was mediated through the $AT_1$ type of angiotensin receptors. Transcription inhibitor studies found no changes in the transcription levels of $\alpha_{1A}$ receptor mRNA in AngII treated cells vs control cells and it was concluded that downregulation of the $\alpha_{1A}$ receptor mRNA was primarily a result of AngII mediated reduction in $\alpha_{1A}$ receptor mRNA stability.

**Barki-Harrington *et al* (2003)** showed that there is a physiological interaction between the β-adrenoceptors and the $AT_1$ receptors. This is the first report indicating a direct interaction between any 2 GPCRS. They showed that valsartan (an $AT_1$ antagonist) reduces isoproterenol-mediated elevation in heart rate in mice and selective blockade of β-adrenoceptors prevented angII-mediated contractility. They also demonstrated that a single antagonist, either valsartan or propranolol, may be able to inhibit the signaling by both the receptors in question. This mechanism was termed as 'transinhibition' since one molecule blocks its own receptors and downregulates the signaling pathways of the reciprocal receptor.

**Abdullah *et al* (2011)** studied the effects of carvedilol in intact rats and its response to AngII. The study involved injection of AngII in rats that may/may not be treated with carvedilol. This kind of adrenergic blockade did show an inhibitory effect on the vascular

responses of AngII suggesting the interactions between the adrenergic system and RAAS in normotensive animals.

**Barrett-O'Keefe *et al* (2013)** studied the effects of age related cross-talks between AngII and $\alpha_1$ adrenoceptor mediated vasoconstriction. The study was based on the hypothesis that AngII mediated vasoconstriction would be more in the elderly as compared to young individuals owing to cross talks with $\alpha_1$ adrenoceptors and this effect may subside in the presence of $\alpha_1$ antagonism. The results of the study demonstrated that increased sensitivity to AngII mediated vasoconstriction may be attributed, in part, to potentiation of $\alpha_1$ adrenoceptor mediated vasoconstriction caused by AngII. They also suggested that this consideration may be clinically applied to design optimally the therapy for patients of hypertension and heart failure.

**Vittorio *et al* (2014)** retrospectively reviewed the interactions observed between the adrenergic system and RAAS in major clinical trials like Val-HeFT and CHARM-added. The authors suggest that $\alpha_1$ adrenoceptor and $AT_1$ receptor cross talk occurs at two levels: at the molecular receptor and the second messenger levels. Heterodimerization between the $\alpha_{1D}$ adrenoceptor and $AT_1R$ has been observed in preeclamptic pregnant rats. Further, since both receptors are coupled to Gq subunit, the subsequent signaling mechanisms overlap significantly and result in second messenger level regulation.

Information on the renin-angiotensin-sympathetic interactions has also been extended to the possible sites of these interactions-stimulation of the sympathetic nervous system leads to renin secretion and AngII formation (DiBona, 1989b); released norepinephrine negatively regulates AngII receptors in cultured brain neurons (Mancia *et al*., 1995) and in vascular tissue through its interactions with $\alpha_1$-ARs (Du *et al*., 1997). Evidence has also been provided that angII triggers a sympathetically mediated blood pressure rise associated with systemic vasoconstriction when dosed intracerebrally. It suggested a central facilitatory effect of AngII on sympathetic outflow (Wolff *et al*., 1984; Hall, 2004; Zimmerman, 1984). AngII plays a facilitatory role on the neuroadrenergic transmission across sympathetic ganglia (Zimmerman, 1984; Reid, 1992; Reit, 1972) and potentiates norepinephrine release from sympathetic nerve terminals via stimulation of presynaptic angiotensinergic receptors (Zimmerman, 1984; Reid, 1992; Starke, 1977).

AngII amplifies the α-receptor mediated vasoconstrictor responses to exogenously administered or endogenously produced norepinephrine. Furthermore, AngII has been shown to exert inhibitory effects on baroreceptor reflex control of heart rate and sympathetic nerve traffic (Zimmerman, 1984; Reid, 1992). In light of all these observations, blockade of a single receptor seems futile while simultaneous blockade of the $\alpha_1$ adrenoceptor and $AT_1$ receptor seems a prudent strategy for the management of hypertension and related cardiovascular disorders.

## COMBINATION THERAPY - THE CONCEPT OF DESIGNED MULTIPLE LIGANDS

Earlier research used to be centered on the one-target-one-disease paradigm. It was believed that desired therapeutic effect may be produced through selective manipulation of a single target and this would also prevent off-target effects. Hence, the concept of monodrug therapy was followed throughout the healthcare network but the professionals were not too late in realizing that intrinsic biological networks have a lot of cross-talks amongst them and hence modulation of a single target definitely produces a signal which is recognized by some other coherent network and results in a compensatory action (Wermuth, 2004; Morphy *et al*, 2004). This machinery was identified to be the cause of failure of different compounds in the clinic. Moreover, with increased understanding of the etiopathologies of different medical conditions, it was realized that many diseases are a result of multifactorial orchestra ultimately manifesting the symptoms and hence a single drug might not be sufficient to manage the entire situation. It was thus envisaged that a parallel modulation of different pathways by combining multiple therapeutic mechanisms would be a prudent strategy for the management of multifactorial disorders (Morphy *et al*, 2004; Morphy and Rankovic, 2005). This remains true for hypertension as well where several factors are acting in concert leading to rise in basal blood pressure. Hence combination therapy is usually preferred by clinicians over monodrug therapy for the management of hypertension (Gradman *et al*, 2011). Major clinical trials have also outlined the importance of modulating more than one target so as to achieve optimal blood pressure. The advantages of such a therapy would be increased efficacy in such a way that target BP is achieved more easily within a predicted time frame and a reduction in dose, as two- or

more drugs are being combined, leading to reduced side effects. The aspect of combination therapy initially involved polypharmacy where the patient was supposed to take a couple of pills, capsules or any other dosage form simultaneoulsy which frequently led to non-compliance. This problem was overcome with the advent of fixed-dose combinations (FDCs). FDCs involve formulation of two- or more pharmaceutically compatible drugs into a single tablet at required doses so that dosing regimen may be simplified and the patient is relieved from the burden of taking more than one pill (Gautam and Saha, 2008; Gupta *et al*, 2010; Huffman, 2014). This improves patient compliance especially in geriatric class of patients in whom swallowing pills is a common problem. Fixed dose combinations have ruled the roost since quite a few decades and newer combinations are coming up every year (Table 3). However, the concept of FDCs is also wrought with its own limitations. Though the drugs being combined may be pharmaceutically compatible but they do present with complex pharmacokinetic-pharmacodynamic relationships requiring special formulation techniques, less flexibility in dose adjustments and there is also a potential risk towards drug-drug interactions which could mar the basis of any FDC (Gautam and Saha, 2008; Hennekens, 2008).

The alternative to an FDC is development of a new chemical moiety that simultaneously modulates multiple pharmacological targets (Schyler and Horuk, 2006; Zimmerman *et al*, 2007). Any such drug available in the market today was not specifically designed to be multiply-targeted but was serendipitously discovered to be so. Labetalol and carvedilol are agents of this category with a dual antagonistic activity on adrenergic $\alpha_1$ and $\beta$ (pan) receptors (Rahn, 1992). Morphy *et al* (2004), first proposed the rational designing of molecules that may affect two- or more targets. They suggested that evenhanded modulation of multiple targets with a single chemical moiety can be deliberately designed to provide improved efficacy coupled to desirable xenobiotic behavior and minimal side effects.

**Table 4: Novel fixed dose combinations being investigated for arterial hypertension**[4]

| Sr. No. | Compounds | Company | Mechanism |
|---|---|---|---|
| 1. | Olmesartan medoxomil + Amlodipine + Hydrochlorthiazide | Daiichi-Sankyo, Japan | ARB + CCB + Diuretic |
| 2. | Azilsartan kaemodoxomil + Chlorthalidone | Takeda Pharmaceuticals, Japan | ARB + Diuretic |
| 3. | Aliskiren + Amlodipine | Novartis, Switzerland | Renin Inhibitor + CCB |
| 4. | Aliskiren + Amlodipine + Hydrochlorthiazide | Novartis, Switzerland | Renin Inhibitor + CCB + Diuretic |
| 5. | Lisinopril + Pyridoxal phosphate | Medicure, Canada | ACEI + cardioprotective vitamin |



The major challenge in designing such compounds is not achieving target function, rather the challenge remains in accomplishing a balanced modulation of all the targets in question (Morphy and Rankovic, 2006; Costantino and Barlocco, 2012). The authors have coined the term '*designed multiple ligands (DMLs)*' and deliberated the two approaches that may be followed for designing such ligands (Morphy *et al*, 2004; Morphy and Rankovic, 2009). The first and more rational approach is the Pharmacophore-combination approach. In this approach, targets for desired actions are identified, pharmacophores are addressed from selective ligands acting on individual targets and then the pharmacophores are joined *in silico* through a cleavable or non-cleavable linker. This allows the medicinal

[4] This table is based on the clinically investigated compounds listed in PhRMA as on July, 2014. ACEI, angiotensin converting enzyme inhibitor; ARB, Angiotensin receptor blocker; CCB, Calcium channel blocker

chemist to understand the physicochemical and dynamic interactions following such pharmacophoric combinations (Morphy *et al*, 2004; Di Napoli and Papa, 2003; Buijsman *et al*, 1999; Murugesan *et al*, 2002). Alternatively, non-pharmacophoric structures, if at all present mutually in all prototypical structures being combined, may be overlapped and studied further. However, the onus ultimately remains on the lab chemist who synthesizes such compounds and the biologist who screens it to show final activity on the said targets (Morphy and Rankovic, 2007). Another approach is the Screening-approach. This involves screening of compound libraries for potential target activities. Such an approach has been attempted at various levels of success by different researchers (Walsh *et al*, 1995; Ryckmans *et al*, 2002). In this case absence of non-specific activities have to be ruled out and multiple screens are required to be conducted which raises logistical concerns since large compound libraries may not be located at the place where screening is carried out.

## DUAL BLOCKERS INVOLVING $AT_1R$ OR $\alpha_1$-ADRENOCEPTOR BLOCKADE

### *$\alpha_1$ and ß receptor blockers*

A simultaneous reduction in peripheral resistance and cardiac output can prove beneficial in the therapy of hypertension. Labetalol and carvedilol provide the best examples of this strategy and have been used in the management of hypertension since a very long time with good efficacy (Rahn, 1992). Other agents of this class include bucindolol, primidolol, etc. Clinical data regarding the efficacy of these compounds is quite strong and suggests that carvedilol and labetalol improve the hemodynamic profile of patients suffering from hypertension (Tomlinson *et al*, 1987; Cubeddu *et al*, 1987). Improvement in endothelial dysfunction is also observed and ejection fraction is significantly improved in geriatric patients (Katholi and Couri, 2011). Labetalol is also safe for use in pregnant patients.

### *$\alpha_1$ and calcium channel blockers*

Though none of the agents of this class is available clinically, experimental molecule S-2150 exerted vasorelaxation of rat aortic rings with an $IC_{50}$ value of 190 nM. A clear hypotensive effect was observed in different models of hypertension including the

SHRs and two-kidney-one-clip rats (Iwaki *et al*, 1997). A similar effect was also observed in normotensive rats indicating the premise of calcium channel blockade.

***Dual inhibition of $AT_1R$ and Neprilysin***

Neprilysin (*aka* neutral endopeptidase) inactivates several endogenous peptides including bradykinin. It is thus one of the regulators of the kinin-kallikrein system involved in control of blood pressure (Turner *et al*, 2001). Preventing the inactivation of vasodilatory bradykinin, when combined with $AT_1R$ blockade can result in synergistic effect since control of blood pressure rise can be effected through two different systems. LCZ696, an investigational molecule from Novartis, Switzerland, is undergoing clinical trials and has previously shown blood pressure reduction to the tune of valsartan (Solomon *et al*, 2012). In one study, LCZ696 was found to be superior to placebo for treating patients suffering from mild-to-moderate hypertension (Solomon *et al*, 2012; Jhund *et al*, 2014). Other molecules having the same mechanism, daglutril and VNP489, are also in various phases of clinical development (Paulis and Unger, 2010).

***Dual inhibition of $AT_1R$ and ET receptor***

The concept of dual $AT_1R$ and ET receptor blocker surfaced when losartan and $ET_A/ET_B$ receptor antagonist (SB 290670) produced additive reductions in blood pressure as compared to individual therapy (Kowala *et al*, 2004). This resulted in increased interest of pharmaceutical majors towards the development of dual $AT_1R$ and ET receptor blockers. One investigational molecule, PS433540, showed good binding affinities for both receptors in radioligand binding assays (0.8 nM for $AT_1$ and 9.3 nM for $ET_A$). It has also been reported to be effective, safe and well tolerated and is currently in the final stages of clinical trials. Another molecule is BMS346567, which also shows good binding affinities for both the receptors (2 nM for $AT_1$ and 14 nM for $ET_A$) (Murugesan *et al*, 2005).

***Dual $AT_1R$ blockade and PPARγ agonism***

Some $AT_1R$ blockers are known to possess partial agonism of peroxisome proliferator-activated receptor gamma (PPARγ) receptor. It has been reported that telmisartan inhibits $AT_1$ receptor gene expression through PPARγ activation (Imayama *et*

*al*, 2006). The dual inhibition of angiotensin II function by telmisartan-$AT_1$ receptor blockade and downregulation would contribute to more complete inhibition of the RAAS. Telmisartan, an $AT_1R$ blocker and a partial agonist of PPARγ, may be quite useful for the treatment of patients with hypertension with complications such as diabetes and atherosclerosis. A multitargeted ligand may be more useful for microalbuminuria reduction as compared to an $AT_1R$ blocker with no PPARγ agonistic action. Telmisartan achieved more microalbuminuria reduction than other $AT_1R$ blockers lacking PPARγ agonism, possibly through suppression of the inflammatory state in metabolic hypertensive patients (Miura *et al*, 2005; Yano *et al*, 2007). Two more molecules azilsartan and PF-03838135 are reported to possess $AT_1$ receptor antagonism and a partial agonism of PPARγ (Paulis and Unger, 2010).

***Dual $AT_1R$ and calcium channel blockade***

Hadizadeh *et al* (2010) reported the synthesis and evaluation of novel dihydropyridines prepared by connecting the imidazole nucleus of losartan to the dihydropyridine rings. Two compounds from their series were reported to have calcium channel blocking properties with parallel AngII antagonism observed on rat aortic rings. The authors have reported that the test compounds *Dimethyl 4-[2-butyl-1-(2'-carboxybiphenyl-4-yl)methylimidazol-4-yl]-1, 4-dihydro-2, 6-dimethylpyridine-3, 5-dicarboxylate* and *Diethyl 4-[2-butyl-1-(2'-carboxybiphenyl-4-yl) methylimidazol-4-yl]-1, 4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate* are $10^3$ and $10^5$ times more potent as compared to losartan respectively.

Cholesterol is just one of the substrates as ACAT utilizes other oxysterols, plant sterols and various other sterols for enzymatic catalysis, but plant sterols are poor substrates (Liu *et al*, 2005). Apropos cholesterol homeostasis, ACAT plays an important role in lipoprotein assembly, dietary cholesterol absorption and intracellular cholesterol metabolism (Buhman *et al*, 2000a). Meiner *et al* (1996), disrupted the mouse ACAT gene and, contrary to popular belief, showed that ACAT activity still retained in the liver and intestine insinuating the likelihood of other isoforms of ACAT. The cloning of ACAT1 gene thus escorted the cloning of the other isoform and taking cue from the work of Meiner *et al* (1996), cDNA for human ACAT2 was cloned in 1998 by three independent research groups (Anderson *et al*, 1998; Cases *et al*, 1998; Oelkers *et al*, 1998). Two isoforms of ACAT have thus been discovered till present day in mammalian species, ACAT1 and ACAT2, both of which are encoded by different genes (Farese, 2006). ACAT1 is ubiquitously expressed whereas expression of ACAT2 is restricted to liver and enterocytes. Both these isoforms have unique functions and belong to the *Acact* gene family. The *Acact* gene family also encodes an enzyme termed the acyl CoA: diacylglycerol transferase-1 (DGAT1) and its orthologues (Burnett *et al*, 1999; Buhman *et al*, 2000a). These three enzymes namely ACAT1, ACAT2 and DGAT form the initial members of the family of enzymes termed as membrane bound O-acyltransferase (MBOAT) (Chang *et al*, 2011). The enzymes of this family form the multispan membrane-bound enzymes that employ fatty acyl-CoAs (long or medium chain) and another hydrophobic substance like cholesterol as their substrate. This results in the formation of a neutral hydrophobic ester like cholesteryl oleate and coenzyme A. An important characteristic of the enzymes of the MBOAT family is the presence of an invariant histidine and a near-invariant asparagine within a long stretch of hydrophobic and moderately hydrophilic residue (Chang *et al*, 2011). Above and beyond cholesterol homeostasis, ACAT1 aids the progression of atherosclerosis via accumulation of cholesteryl esters in macrophages leading to conversion of smooth muscle cells to foam cells, ultimately leading to plaque initiation and subsequent events (Fazio *et al*, 2001; Linton and Fazio, 2003). ACAT2 functions in a more dedicated manner to facilitate cholesterol absorption and lipoprotein secretion (Shelness and Sellers,

2001; Nguyen *et al*, 2012). In lieu of these findings, ACAT inhibitors have remained attractive targets, though elusive, for pharmaceutical companies to prevent plaque development and cholesterol absorption through intestine. An attempt has been made here to recapitulate the cellular and tissue localization, biochemical regulation, active site and catalytic residues as well as attempts towards therapeutic inhibition of the ACAT isoforms. Significant events in the history of ACAT are listed in Table1.

**Table 1: Significant events in the history of ACAT**

| SR. NO. | EVENT | YEAR | REFERENCE |
|---|---|---|---|
| i. | Cholesterol esters first identified as components of blood | 1895 | Hurthle, 1895 |
| ii. | Cholesterol esters were shown to be present in arterial atheromatous plaques | 1910 | Windaus, 1910 |
| iii. | Cholesterol esters are formed in blood | 1935 | Sperry, 1935 |
| iv. | Rat liver homogenates can esterify cholesterol with palmitic acid | 1958 | Mukherjee *et al*, 1958 |
| v. | A liver enzyme was found to be responsible for esterification using cholesterol and fatty acyl coA as substrates and identified as ACAT | 1964 | Goodman *et al*, 1964 |
| vi. | LCAT catalyses the esterification of cholesterol in blood | 1968 | Glomset, 1968 |
| vii. | ACAT activity was identified in macrophages apart from other tissues | 1979 | Brown *et al*, 1979 |
| viii. | Unsuccessful attempt to purify ACAT | 1982 | Doolittle and Chang, 1982 |
| ix. | Brown and Goldstein discovered that cholesteryl esters in OxLDL particles taken up by macrophages are first | 1983 | Brown and Goldstein, |

| | | | |
|---|---|---|---|
| | hydrolyzed in lysosomes and then re-esterifed prior to storage as lipid droplets | | 1983 |
| **x.** | ACAT1 cDNA was cloned after a decade of efforts using the complementation of a mutant CHO cell line devoid of ACAT activity | 1993 | Chang *et al*, 1993 |
| **xi.** | Disruption of mouse ACAT gene revealed that esterification activity is still retained in the liver and intestine suggesting the presence of another isoforms | 1996 | Meiner *et al*, 1996 |
| **xii.** | cDNA for human ACAT2 was cloned | 1998 | Anderson *et al*, 1998; Cases *et al*, 1998; Oelkers *et al*, 1998 |
| **xiii.** | Eflucimibe, Phase I trial concluded and molecule enhanced to Phase II | 2002 | Burnett, 2003 |
| **xiv.** | Avasimibe & Progression of Lesions on Ultrasound (A-PLUS) trial halted as a result of lesion progression and suggested lack of activity | 2003 | Tardif *et al*, 2004 |
| **xv.** | Pactimibe trial, ACAT Intravascular Atherosclerosis Treatment Evaluation (ACTIVATE) suspended | 2006 | Nissen *et al*, 2006 |
| **xvi.** | Liver specific inhibition of ACAT2 using antisense oligonucleotides | 2006 | Bell *et al*, 2006 |
| **xvii.** | CAPTIVATE randomized trial prematurely terminated | 2009 | Meuwese *et al*, 2009 |



**ACAT GENE AND PROTEIN STRUCTURE**

The human ACAT1 gene (*SOAT1*-Sterol O-Acyltransferase 1) is located on chromosome 1q25, the approximate molecular mass of the gene product being 65 KDa (Chang *et al*, 1994). However, it functions as a homotetramer (Yu *et al*, 1999) and the

molecular mass of the active molecule was actually found to be 263 KDa. Human ACAT2 gene (*SOAT2*-Sterol O-Acyltransferase 2) is located on chromosome 12 and the region of interest has a locus affecting the response of plasma LDL and VLDL to high-fat or high-cholesterol intake in the diet (Cases *et al*, 1998). The human ACAT2 gene encodes a single 46 KDa protein. Corresponding genes for ACAT1 and ACAT2 in mice are found on chromosomes 1 and 15 respectively (Uelmen *et al*, 1995; Cases *et al*, 1998). Predominance regarding the tissue distribution of any of the isoforms is controversial and is detailed in the succeeding section. Efforts by different groups of researchers have identified four mRNA transcripts for human ACAT1 (7, 4.3, 3.6 and 2.8 Kb) (Chang *et al*, 1993; Pape *et al*, 1995; Matsuda *et al*, 1996; Wang *et al*, 1996). All these transcripts have the same open reading frame but differ in the length of their untranslated regions (Li *et al*, 1999) ultimately responsible for formation of the homotetramer. The 4.3 Kb transcript originates from an atypical RNA recombination which involves trans-splicing of two precursor RNAs from chromosomes 1 and 7 (Li *et al*, 1999). A single mRNA transcript (2.2 Kb) is encoded by the human ACAT2 gene (Buhman *et al*, 2001). Both the isoforms share a lot of sequence identity near the -COOH terminus and are nearly 40 % identical in their gene products (Anderson *et al*, 1998; Cases *et al*, 1998; Oelkers *et al*, 1998).

Amino acids 403-409 of human ACAT1 form a highly conserved sequence (the FY*X*DWWN motif) which might be involved in binding to the acyl-CoA during the catalytic process (Buhman *et al*, 2001). This sequence is a communal occurrence in members of the *Acact* gene family. The MK*XX*SF motif (amino acids 265-270 of human ACAT1) is another conserved motif in the family members and the serine residue present in this motif is essential for ACAT activity (Cao *et al*, 1996; Joyce *et al*, 2000; Buhman *et al*, 2001). Other potential motifs common to the family members of *Acact* include a tyrosine phosphorylation motif and an N-linked glycosylation site. The N-terminal of both the isoforms contains a leucine-zipper motif but it is not known whether this motif has any role in formation of homotetramers (Chang *et al*, 1993; Buhman *et al*, 2000a). An interesting point in the structural biochemistry of ACAT proteins is that ACAT1 and ACAT2 do not form hetero-oligomeric complexes with each other (Chang *et al*, 2000).

ACAT1 contains 1 disulfide linkage and 7 free cysteines (Guo *et al*, 2005a) none of which are required for the activity of the enzyme but the disulfide linkage is essential for its structural stability.

Several investigations have been performed to study the membrane topology of human ACAT1 albeit without reaching a consensus. Lin and colleagues first studied the membrane topography of ACAT1 and suggested that ACAT1 contains at least 7-TMDs (Lin *et al*, 1999). This proposition was revised by the work of Guo and colleagues who proposed a 9-TMD model (Guo *et al*, 2005b). Again, Joyce *et al* (2000) proposed a 5-TMD model with 4 TMDs lying near the -$NH_2$ terminal and 1 TMD near the -COOH terminal. The method used by Lin *et al* (1999) as well as Joyce *et al* (2000), suffered from a common flaw that the actual membrane topography of the enzyme might be altered in the detection process (Guo *et al*, 2005b). Joyce and colleagues employed successive –COOH terminal truncations which could alter the structural stability of the protein in question (Joyce *et al*, 2000). Further, they have not shown whether ACAT activity was retained in each truncated subunit (Joyce *et al*, 2000). Lin and co-workers used the method of epitope insertion and double cytoimmunofluorescence to discern their topological model (Lin *et al*, 1999). Though the authors did report that each tagged protein was partially active, we do not have concrete evidence to believe that structural conformation remained unchanged in the entire protocol. Guo *et al* (2005b), were able to show that modest truncations near the -COOH terminal (the method used by Joyce *et al* (2000) for topographical studies) led to a near complete loss of enzymatic activity for ACAT1. Hence the model proposed by Joyce and colleagues seems unacceptable (Joyce *et al*, 2000). The topographical model suggested by Lin and colleagues indicated that ACAT2, being an integral membrane protein, contained two detectable TMDs (Lin *et al*, 2003). The authors asserted that other transmembrane domains may be found in the lipid bilayer but were not reported by them. On the contrary, Joyce and co-workers determined the topography of ACAT2 and reported that ACAT2 has five and not two TMDs (Joyce *et al*, 2000). However, data regarding ACAT2 TMDs is not concrete, as Chang and co-workers have reviewed (Chang *et al*, 2009). The authors argue that as was the case with ACAT1, the approaches used by Joyce *et al* (2000) and Lin *et al*

(1999), have an apparent possibility that the actual membrane topography of ACAT2 is altered during the process. The transfection procedure used by both the groups has a propensity to produce leaky cells and this might have definitely been stained by antibodies (Chang *et al*, 2009). This may not be acceptable because the results are rendered non-specific due to the permeable or dying cells.

**CELLULAR LOCALIZATION AND TISSUE DISTRIBUTION**

ACAT1 is normally found to be present in a variety of tissues including liver, sebaceous glands, adrenal glands, macrophages, fibroblasts and monocytes (Lee *et al*, 1998; Chang *et al*, 2000; Sakashita *et al*, 2000) while ACAT2 is found in intestinal mucosa and liver microsomal fraction (Joyce *et al*, 1999; Smith *et al*, 2004). Figure 2 indicates the location and physiological functions of ACAT isoforms of major importance. In humans, ACAT2 is the prime ACAT isoform found in the small intestine while ACAT1 protein expression is high in adrenals, macrophages and sebaceous glands. In the liver, ACAT activity is present not only in hepatocytes but also in *Kupffer* cells which are nothing but stellate macrophages (Buhman *et al*, 2001). In terms of cellular localization, expression of ACAT1 mRNA is ubiquitous in mammalian tissues (Chang *et al*, 1993; Uelmen *et al*, 1995; Meiner *et al*, 1997). ACAT1 is chiefly found to be present in the ER, spanning the ER membrane 5 or 7 times (Lin *et al*, 1999; Joyce *et al*, 2000), however such localization may change with different cell conditions like cholesterol loading. For example, cholesterol loaded human macrophages produce more numbers of ER-derived vesicles that are rich in ACAT1 (Temel *et al*, 2003; Liu *et al*, 2005; Sakashita *et al*, 2010). Some expression of the ACAT1 mRNA in mouse macrophages has been reported near trans-Golgi network (Khelef *et al*, 1998). As mentioned above, ACAT2 is expressed in the microsomes of liver and small intestine exclusively. More specifically, ACAT2 has been found to be present in the apical region of the intestinal villi (Chang *et al*, 2000; Lin *et al*, 2003) whereas ACAT1 is uniformly distributed over the villus-crypt axis (Chang *et al*, 2000), albeit in a lower amount. Immunological methods have helped in determining the predominance of the two isoforms in given tissues. Sakashita and colleagues demonstrated the relative prevalence of

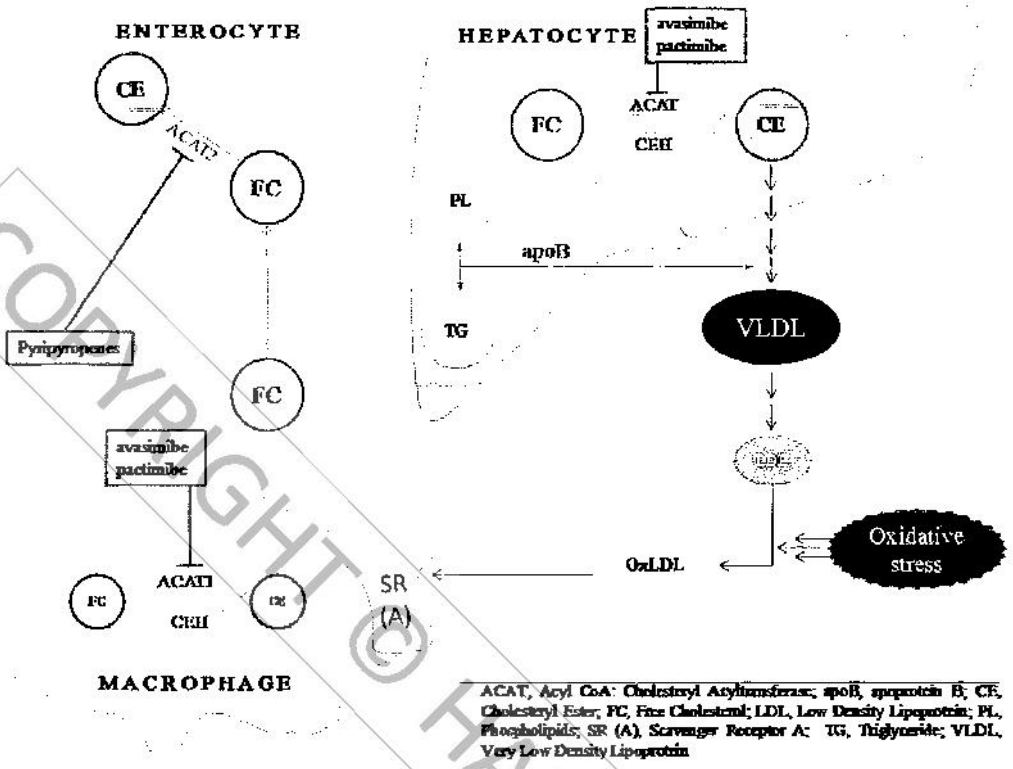



Figure 2: Physiological functions of ACAT. ACAT reduces the toxicity of free cholesterol by converting it from a polar derivative to a non-toxic ester form which can be stored in the cells in the form of lipid droplets. Apart from being stored lipid reservoirs, the cholesteryl esters formed as a result of this esterification process are utilized in the assembly of lipoproteins. In the macrophages, these cholesteryl esters combine with oxidised LDL leading to formation of the foam cell. The catalysing function of the ACAT isoforms can be inhibited (blunt arrows) by non-specific inhibitors like avasimibe and pactimibe or isoform-specific agents like pyripyropenes (boxes).

ACAT1 in normal human tissues using immunohistochemical detection methods (Sakashita *et al*, 2000). The authors report that prevalence of ACAT1 is highest in Kupffer cells, adrenal cortex & alveolar macrophages to be specific and macrophages of the liver, spleen and kidney in general (Sakashita *et al*, 2000). Occurrence of ACAT1 is also substantial in hepatocytes, oesophagal and fundic glands, mucosal epithelial cells of the large intestine, transitional epithelial cells of the urinary tract, epithelial cells of proximal and distal tubules in the kidney, myentric glia, neurons, bronchiolar and alveolar epithelia

(Sakashita *et al*, 2000). They have specifically utilized an anti-ACAT1 antibody, DM10, specifically immunoreactive against ACAT1 (Chang *et al*, 1995; Miyazaki *et al*, 1998) for immunodetection of ACAT1. The samples used for this study were all autopsy cases. The exact orientation of ACAT2 in the ER is unknown but data supporting the alignment of active site towards cytosol have been reported (Lin *et al*, 2003). Rudel and colleagues have inferred that active sites of ACAT1 and ACAT2 are, in fact, located on opposite sides of the ER membrane (Rudel *et al*, 2000). Thus, contradictory to previous reports, the active site of ACAT1 is located facing the cytosolic side of the ER whereas the active site of ACAT2 faces the lumen of ER (Joyce *et al*, 2000; Lada *et al*, 2004). It is hypothesized that since fatty acyl-CoA is not permeable to the ER membrane, the most plausible alignment of the active sites of ACAT is towards the cytosol (Coleman and Bell, 1983; Lin *et al*, 1999). Overall, it has been postulated that ACAT1 is present wherever cholesterol is required to be esterified for storage whereas ACAT2 is present in tissues involved in esterification of cholesterol for lipoprotein assembly and secretion (Anderson *et al*, 1998).



**ACTIVE SITE RESIDUES AND CATALYTIC ACTIVITY**

Both the isoforms of ACAT are homologous in their amino acid sequence near their C-termini but they are quite different in their intracellular functions due to their distinct nucleotide sequences (Das *et al*, 2008). A few studies have identified different residues required for ACAT activity. Kinnunen and colleagues showed that histidine residue(s) are required for ACAT activity (Kinnunen *et al*, 1988). This is quite plausible because histidine residues are frequently involved in enzymatic catalysis due to the reactive imidazolium nitrogen which may function as hydrogen bond acceptor/donor (An *et al*, 2006). Other groups of researchers have supported this finding and suggested that a histidine residue at C-terminal of ACAT is one of the active site residues (Lin *et al*, 2003; Guo *et al*, 2005b). Further, Sojin An and colleagues demonstrated that the histidine residues required for activity in ACAT1 are different from those required for activity of ACAT2 (An *et al*, 2006). They have shown that H386 and H460 are essential for ACAT1 catalysis whereas for ACAT2, the corresponding counterparts are H360 and H399 (An *et*

*al*, 2006). A vital step in the cholesterol esterification process is the cleavage of the thioester bond in the acyl-CoA molecule. The energy released during this process propels the esterification process. ACAT enzymes possess intrinsic thioesterase activity and the crystal structure of this thioesterase domain reveals a triplet residue, serine-histidine-aspartic acid, as the active site of the enzyme (Chakravarty *et al*, 2004; Das *et al*, 2008). Much of the work regarding identification of presumed active sites came from the work of Das and co-workers (2008). After several chemical modifications and site-directed mutagenesis studies, they reported several important residues required for ACAT activity (Das *et al*, 2008). Their results are consistent with previous reports indicating the role of the amino acid triad, serine-histidine-aspartic acid, for activity of ACAT (Guo *et al*, 2001). Das & colleagues have specifically identified that serine at position 456, histidine at 460 and aspartic acid at 400 is necessary for activity of ACAT1(Das *et al*, 2008). Additionally, aspartic acid residue at 400$^{th}$ position is also essential for appropriate folding and structural stability of the protein (Das *et al*, 2008). Moreover, full enzymatic activity of ACAT2 is dependent upon a histidine residue at 438$^{th}$ position (Das *et al*, 2008). Studies with tyrosine mutant enzymes revealed the importance of a tyrosine residue at 404$^{th}$ position for ACAT1 and at 382$^{nd}$ position for ACAT2. Mutation led to about 20 % decrease in cholesterol esterification activity (Das *et al*, 2008). Further to this, ACAT1 has a highly conserved sequence from amino acids 265 through 270 which contains a serine residue that is fundamental to the cholesterol esterification process. With regard to the highly conserved sequence, human ACAT1 has a conserved serine residue, Ser-269, which was found to be essential for activity because replacement of this serine residue with leucine resulted in loss of activity (Joyce *et al*, 2000). A corresponding serine residue is also present in the active site of ACAT2 (Joyce *et al*, 2000). Thus it was assumed that Ser-269 forms a part of the active site of ACAT1 (and for that matter ACAT2 as well). However, this may be considered only as a timid suggestion since there is a possibility that a serine-to-leucine mutation causes the mutated enzyme to be degraded at a faster rate as compared to the wild variety of the enzyme thus leading to a loss of activity not because of the structural modification but as a result of altered physiological metabolism (Chang *et al*, 2001). A

very interesting hypothesis proposed by Chang and colleagues states that the catalytic function of ACAT may be accomplished within the plane of the ER membrane (Chang *et al*, 2001). The authors argue that both the isoforms are able to form neutral lipid droplets that are found in the cytoplasm (earlier attributed exclusively to ACAT1) as well as contribute to the assembly of lipoproteins (attributed to ACAT2), a process which occurs in the ER lumen (Chang *et al*, 2001; Temel *et al*, 2003). The substrates for the reaction, fatty acyl-CoA and cholesterol are readily available from the ER membrane. Fatty acyl-CoA being impermeable, partitions on the cytoplasmic side of the ER membrane (Boylan and Hamilton, 1992), while cholesterol may equilibrate on either side of the ER membrane. Along these lines, it can be assumed that both fatty acyl-CoA and cholesterol diffuse laterally along the ER membrane, where at some point, they encounter ACAT, bind to it, leading to a conformational change in the enzyme so that the catalytically active sites are optimally exposed to the substrates for enzymatic catalysis to occur (Chang *et al*, 2001). Since these arguments have not been corroborated at biochemical or cell-biology levels, they are open to contemplative criticisms. A point worth noting in this observation is that if the catalytic function happens within the plane of the membrane, then CE generated in the lipid bilayer may leave the cytoplasmic layer to form lipid droplets or the CE may be recruited to form VLDL particles and thus the hypothesis proposed by the authors, that both isoforms are involved in neutral lipid and VLDL formation, will stand true.

**BIOCHEMICAL REGULATION**

The catalytic activities of ACAT1 and ACAT2 are similar in lot respects, but their biochemical activity may not be considered as identical to each other. Potential substrates subject to ACAT-mediated catalysis are listed in table 2. In addition to cholesterol, ACAT1 esterifies numerous other oxysterols (Cases *et al*, 1998; Buhman *et al*, 2001) suggesting a pivotal role in regulating bile acid and cellular cholesterol metabolism (Cases *et al*, 1998; Buhman *et al*, 2001). A variety of long chain fatty acyl-CoAs are also substrates to ACAT1 mediated catalysis (Table 2) (Yang *et al*, 1997; Cases *et al*, 1998). This broad substrate specificity is also present in murine ACAT1 (Cases *et al*, 1998).

**Table 2: Potential ACAT substrates**

| Sterols | Fatty acyl-CoAs |
|---|---|
| Cholesterol<br>27-Hydroxycholesterol<br>25-Hydroxycholesterol<br>24(*S*)-Hydroxy cholesterol<br>7α-Hydroxy cholesterol<br>24(*S*),25-Epoxycholesterol<br>Cholestanol | Arachidonyl CoA<br>Palmitoyl CoA<br>Linoleoyl CoA<br>Oleoyl CoA |



ACAT1 happens to be regulated at both, transcriptional and post-translational levels (Chang *et al*, 1997; Yang *et al*, 2001). *In vivo*, diet rich in fats, cholesterol (Pape *et al*, 1995; Uelmen *et al*, 1995) or dexamethasone treatment (Cheng *et al*, 1995b; Yang *et al*, 2004) led to increased ACAT1 activity. *In vitro*, cholesterol-loading, differentiation of monocytes to macrophages (Wang *et al*, 1996) and treatment with free fatty acids (Seo *et al*, 2001) are reported to increase ACAT1 activity. TGF-β (Hori *et al*, 2004) and TNF-α (Lei *et al*, 2009) are implicated in mediating the increased ACAT1 activity in differentiating monocytes thus leading to the formation of cells loaded with cholesteryl esters whereas adiponectin, an adipocytokine (Gandhi *et al*, 2010), shows a negative effect in this case (Furukawa *et al*, 2004). ACAT1 responds to cholesterol as its sterol substrate in a sigmoid habit (Chang *et al*, 1998), with a certain predilection over other oxysterols (Chang *et al*, 2009). Kinetic evidence has suggested that there is presence of a sterol-substrate site and a sterol-activator site in the structure of ACAT1 (Chang *et al*, 2009). Cholesterol increases the activity of ACAT1 several folds and it can be clearly seen that ACAT1 holds a typical bias towards cholesterol (Chang *et al*, 2009) as its substrate when compared with other variety of sterols having a 3β-OH configuration (Zhang *et al*, 2003; Liu *et al*, 2005). It has been discussed that cholesterol stimulates ACAT activity not only

by increasing substrate availability but also by acting as an allosteric activator of ACAT. The superiority of cholesterol to act as an allosteric activator of ACAT1 has been demonstrated against other sterols (Zhang *et al*, 2003; Liu *et al*, 2005) like 7-ketocholesterol and 7α-hydroxycholesterol. Liu and coworkers (2005) have reported that the stereochemistry of the 3-OH in the A ring is critical towards determining the ability of any sterol to behave as a substrate for ACAT1 (Liu *et al*, 2005). The authors further asserted that the axial orientation of the 3α-OH thus prevents the binding of epicholesterol, making it a poor substrate for ACAT1 (Liu *et al*, 2005). However, studies with *ent*-cholesterol, the mirror image of cholesterol with an equatorial orientation of the 3α-OH (same as that of 3β-OH in cholesterol), have revealed that it is the overall shape of the sterol molecule that is recognized by the substrate-binding site and not just the axial- or equatorial-orientation of the 3-OH group (Westover and Covey, 2004; Liu *et al*, 2005). As mentioned earlier, ACAT1 exists as a homotetramer. There is a dimer-forming motif (one which results in the formation of a dimer from a dimer, a tetramer) near the N-terminus, deletion of which converts the tetrameric enzyme to a functionally active dimeric form (Yu *et al*, 2002).

The *SOAT2* promoter contains *cis* elements [those regions within the same DNA strand that can regulate gene (*here SOAT2*) expression] for two transcription factors, Cdx2 and HNF1α, both of which are required for expression of the gene product ACAT2 (Song *et al*, 2006). While HNF1α is expressed in tissues like kidney, stomach, intestinal epithelium and hepatocytes, Cdx2 is constrained to the differentiated cells of the intestinal microvilli (Song *et al*, 2006; Chang *et al*, 2009).

ACAT2 also utilizes various oxysterols as substrates (Cheng *et al*, 1995a). It recognizes cholesterol with a higher specificity over ACAT1 and 25-OH cholesterol is also utilized more efficiently in comparison to ACAT1. With respect to fatty acyl-CoAs, ACAT2 has preference for linoleoyl-CoA or palmitoyl-CoA over oleoyl-CoA (Cases *et al*, 1998; Buhman *et al*, 2000a). Activity of ACAT2 is lowest with arachidonyl-CoA.

When the substrate specificities of ACAT1 and ACAT2 were put head-to-head, it was found that ACAT2 exhibits more specificity towards cholesterol in presence of plant sterols like sitosterol as compared to ACAT1 (Temel *et al*, 2003). Also, the rate of esterification of cholesterol and that of oleate incorporation into sterol esters is many times greater for ACAT2 as compared to ACAT1 (Temel *et al*, 2003). Reasons for such higher specificity and activity may be cited that on a mole-to-mole basis ACAT2 is more efficient than ACAT1 but with near 40% sequence homology none of the currently available methods can determine the specific activity of the two isoforms. Another likely explanation is the metabolic half-life of ACAT1 which is fairly shorter than that of ACAT2 (30 min v/s 6 hr), indicating that the rapid rate of ACAT1 turnover makes it less stable as a protein in comparison to ACAT2 leading to consequential decline in specific activity (Temel *et al*, 2003).

When ACAT activity was compared in overnight-fasted and -fed rats, it was found that the corresponding activity was two-fold higher in the fasted animals (Helgerud *et al*, 1982). Thus, nutritional status is yet another factor involved in the regulation of catalytic activity of ACAT isoforms.

A couple of studies in the past have deliberated upon the circadian regulation of ACAT activity. Since cholesterol biosynthesis follows a diurnal pattern in mammalian physiology, it was only prudent to check the effect of this circadian variation on activity of ACAT. It was found that the enzymatic activity of ACAT hits a higher point paralleling to that of the peaking cholesterol biosynthesis in mammalian systems. Thus, the circadian peak of cholesterol biosynthesis coincides with the physiological peak of ACAT catalysis (Erickson *et al*, 1980a). On the other hand, the circadian peak of adrenal corticosteroid was also found to be correlated to cholesterol ester levels and hence ACAT activity. Civen and co-workers showed that with circadian variation in adrenal corticosteroid levels, the ratio of esterified: free cholesterol was affected (Civen *et al*, 1982). Incidentally, they showed that the ratio of esterified: free cholesterol was lowest at the peak of corticosterone secretion. One of the major observations in this study was that as the levels of cholesteryl

esters begin to decline, ACAT activity was reduced to its minimum value. Another important observation put forward by Civen and colleagues was that the circadian change in ACAT activity is biphasic. ACAT activity was found to be at the maximum as the corticosterone levels begin to rise but reach a minimum as corticosterone levels peak (Civen *et al*, 1982). This suggested that as ACAT catalysis proceeded the amount of free cholesterol went down, leading to an increased requirement of the steroid precursor. Recent studies on diurnal variation of ACAT isoforms are lacking.

## ACAT - A THERAPEUTIC TARGET?

### *ACAT in Alzheimer's Disease*

Alzheimer's disease (AD) is the most common form of dementia characterized by abnormal deposits of β-amyloid (Aβ) in the brain and progressive neurodegeneration (Echavarri *et al*, 2011). It has been shown that cholesterol turnover in the brain can affect the development of AD (Shobab *et al*, 2005; Hirsch-Reinshagen *et al*, 2009) by influencing Aβ catabolism. Puglielli and colleagues (Puglielli *et al*, 2003) have associated the intracellular distribution of cholesterol with regulation of Aβ generation. Analysis of genetic data from AD patients has suggested an association of the *ACAT1* gene to that of AD risk (Bertram *et al*, 2007). Esterification of neuronal cholesterol has been implicated in the pathogenesis of AD (Chang *et al*, 2010). Further genetic evidence has also shown that lowered or nil activity of ACAT can confer protection against AD (Wollmer *et al*, 2003). Using specific ACAT1 inhibitors and $ACAT^{-/-}$ mice, it has been shown that ACAT plays an important role in processing of APP and generation of APP C-terminal fragments, Aβ40 & Aβ42 (Colell *et al*, 2009; Huttunen *et al*, 2009; Maulik *et al*, 2013). Thus, ACAT plays a modulatory role in promoting β- and γ-secretase activities for generation of Aβ fragments. Furthermore, ACAT increases the availability of APP from its early secretory pathway thus facilitating the activities of β- and γ-secretase (Puglielli *et al*, 2001; Hutter-Paier *et al*, 2004). However, these mechanisms are not well defined as of now and researchers are searching for other possibilities like the effect of ACAT in APP processing in the lipid rafts. Altered lipid droplet formation by modulating the activity of ACAT can also affect

APP trafficking and hence its processing. Also, patients undergoing cholesterol lowering therapies have been found to be among the lower risk groups for acquiring AD. This made ACAT a lucrative target among pharmaceutical companies pursuing novel strategies for treatment of AD.

***ACAT & Atherosclerosis***

Several reports have shown that the advent of foam cells rich in cholesteryl esters within the intima of arteries is an early event mediating atherosclerosis (Ross, 1999). Additionally, an alarming fact is that lipid-rich plaques are more prone to rupture as compared to lipid-poor plaques (Falk *et al*, 1995; Libby and Aikawa, 2002). A normal LDL particle is usually very slowly taken up by the monocytes or macrophages but oxidative stress can generate a special type of LDL particle, oxidized LDL (*OxLDL*), which can be very efficiently and rapidly taken up by the scavenger receptors of the macrophages (Itabe *et al*, 2011). After this oxidative modification, the uptake of OxLDL in a macrophage proceeds in an unregulated and unhindered manner. Upon uptake, the cholesteryl esters present in the lipoprotein are hydrolyzed and immediately re-esterified by ACAT1 and "the cholesteryl ester cycle" (Brown and Goldstein, 1983) begins. In macrophages, which form an important part of the pathophysiology of atherosclerosis, ACAT1 regulates the allocation of intracellular cholesterol to esterified- and free-cholesterol pools (Akopian and Medh, 2006). This is a very important event for any cell, as esterification of cholesterol sequesters free cholesterol in the form of esterified lipid droplets by making it unavailable for ABCA1-mediated efflux (Akopian and Medh, 2006; Voloshyna and Reiss, 2011; Sorci-Thomas and Thomas, 2012). This massive accumulation of cholesteryl esters in the form of lipid droplets leads to the formation of lipid-laden foam cells (Webb and Moore, 2007; Ouimet and Marcel, 2012). Cholesterol ester accumulation in vascular smooth muscle cells is also controlled by ACAT1 (Yagyu *et al*, 2000; Rong *et al*, 2005; Rong *et al*, 2013). Both of these events contribute significantly to the development of the lipid-rich core of the plaque.

Since ACAT plays a noteworthy role in the development of atherosclerotic plaque, it has been a potential pharmacological target for researchers. Pan-specific or non-specific ACAT inhibition has been tried by different researchers with varying levels of success. The following section describes the successes and failures of ACAT inhibitors.

***ACAT inhibitors as potential therapeutic agents for atherosclerosis***

Several studies identifying potential ACAT inhibitors for the management of hyperlipidemia and atherosclerosis have been reviewed (Pal *et al*, 2012). The past 2 decades have seen a generous number of publications on the subject of ACAT inhibitors (Figure 3). More than 150 patents have been filed suggesting a keen interest amongst researchers and the commercial arena regarding ACAT inhibition as a potential therapeutic strategy for atherosclerosis and AD. The development of several synthetic, herbal or microbial origin ACAT inhibitors has allowed the researchers to understand the role of ACAT in cholesterol turnover. However, at the same time the therapeutic potential of ACAT depletion has been questioned and marred by several unfavorable reports. A major hiccup in the future of ACAT inhibitors as potential drugs is the failure of almost all known compounds in clinical trials with the exception of melinamide. Melinamide, a non-competitive ACAT inhibitor, has been the only compound to be approved for treatment in Japan (Natori *et al*, 1986). Apart from this, all other compounds have failed miserably in advanced stages of clinical trials with avasimibe leading the brigade. A two-year long study [The A-PLUS trial (Tardif *et al*, 2004)] where patients were administered avasimibe on a daily basis failed to show any reduction in atherosclerotic plaques. Apart from that, avasimibe also raised the levels of LDL by about 9 percent generating chaos about the beneficial effects in atherosclerosis. The study with pactimibe [The ACTIVATE trial, (Nissen *et al*, 2006)] was more upsetting where patients with atherosclerosis were treated for 18 months with pactimibe not only failing to show any reduction in the atherosclerotic plaques but secondary endpoints of the study actually demonstrated a worsening in their condition. There were two major concerns: 1) the drug was non-specific with respect to

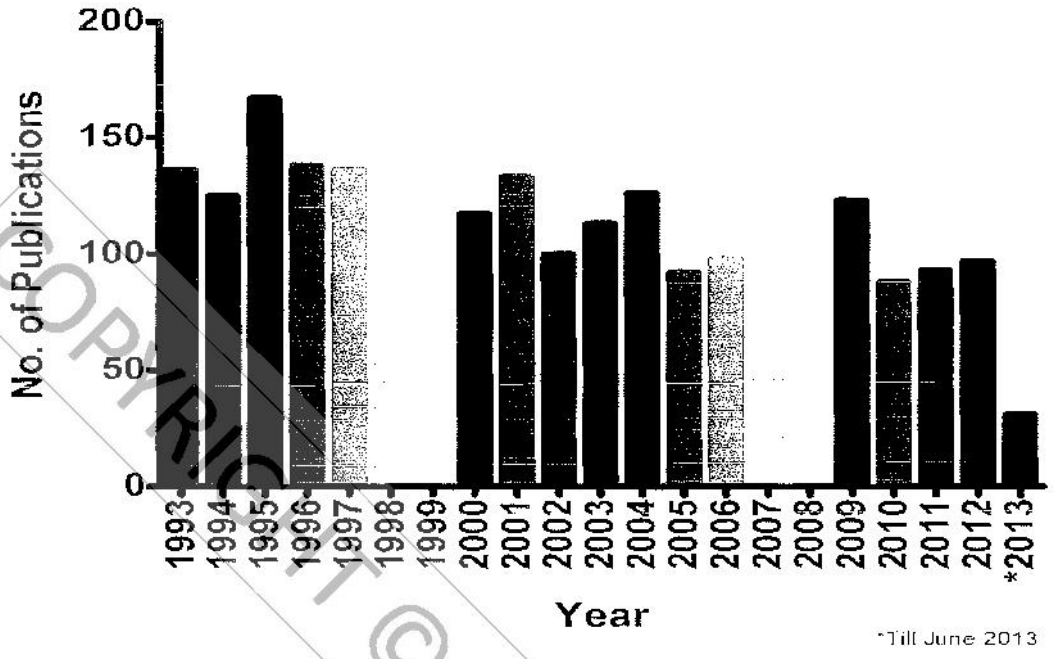



Figure 3: Trends of publication on ACAT. The graph indicates the no. of publications arisen after the discovery of ACAT1 cDNA by Chang & Colleagues (1993). A steady no. of publications shows that the interest of researchers has waxed and waned over a period of time. (Based on data retrieved from PUBMED using keywords like ACAT, acyltransferase and acyl coA: cholesterol acyltransferase. No. of publications for each keyword were noted for the indicated time period and the repetitions were eliminated to avoid duplication of data.)



ACAT inhibition, 2) plasma cholesterol levels remained unchanged, suggesting that inhibition of liver and intestinal ACAT2 is unsatisfactory (Nissen *et al*, 2006). The publication of the results of this trial was very unsettling and nearly put the concept of ACAT inhibition for atherosclerosis on a full stop. In lieu of the conclusions drawn from the ACTIVATE trial, the CAPTIVATE study was prematurely terminated (Meuwese *et al*, 2009). This seemed to be a prudent move from the sponsors as the results from the 15-month study in the CAPTIVATE trial had shown no major benefit with the administration of pactimibe as compared to placebo and in fact had resulted in an increase in the carotid intima media thickness (CIMT). This was accompanied by a rise in LDL cholesterol levels

and the pactimibe group was associated with a higher occurrence of major cardiovascular events like myocardial infarction and stroke in comparison to the placebo group (Meuwese *et al*, 2009). F12511 (generic name *eflucimibe*), chemically an anilide derivative, is a non-specific ACAT inhibitor developed by Eli Lilly in collaboration with French research group Pierre Fabre (Burnett, 2003). Eflucimibe has been previously reported to inhibit ACAT1 ($IC_{50}$ 39 nM) over ACAT2 ($IC_{50}$ 110 nM) with a certain predilection (Chang *et al*, 2000). Eflucimibe has been found to have favorable effects on serum cholesterol in cholesterol-fed animals (Junquero *et al*, 2001a; Junquero *et al*, 2001b; Rival *et al*, 2002). Eflucimibe cleared Phase I trials and was advanced to phase II of clinical trials in 2002 (Burnett, 2003). However, no further reports are available regarding the results of this phase II trial or progression to phase III or any randomized trials.

In experimental studies most of the ACAT inhibitors administered *in vivo* had shown two major manifestations: adrenotoxicity and cutaneous xanthomatosis [reviewed in (Buhman *et al*, 2000a)]. Both the effects are a direct result of inhibition of cholesterol esterification in the adrenal gland and skin respectively, leading to accumulation of toxic free cholesterol. Cholesterol crystal deposits have also been found in the brains of animals administered ACAT inhibitors [reviewed in (Chang *et al*, 2006a)]. Mutant mice lacking the ACAT1 gene ($ACAT1^{-/-}$) also showed these effects when fed with a high cholesterol diet (Fazio *et al*, 2001). An additional observation which was very useful in identifying the $ACAT1^{-/-}$ mice was the atrophy of meibomian glands. This led to narrowing of the eye openings by the age of 3-4 weeks in mice (Yagyu *et al*, 2000). With these effects becoming apparent in mice being treated with ACAT inhibitors, the question of safety also arose for the individuals participating in the clinical trials.

The identification of two isoforms of ACAT and their involvement in the pathogenesis of different conditions raised speculations about selective pharmacological inhibition of ACAT isoforms as a viable therapeutic option. Studies with $ACAT1^{-/-}$ and/or $ACAT2^{-/-}$ mice have provided useful insights regarding the depletion of specific isoforms and their role in conditions like atherosclerosis and AD. Some studies showed that ACAT

depletion in macrophages is actually pro-atherogenic due to the cytotoxicity of accumulated free cholesterol whereas aortic smooth muscle cells are relatively resistant to cytotoxic cholesterol accumulation and ACAT inhibition (Rong *et al*, 2005). In light of these findings, a very common observation was made suggesting that specific inhibition of ACAT1 can actually be detrimental due to accumulation of free polar cholesterol within the cells. Specific inhibition of macrophage ACAT1 can lead to increase in aortic lesion size and consequent detrimental effects. ACAT1$^{-/-}$ mice showed deposits of unesterified cholesterol on the skin, and brain histopathology showed similar deposits of cholesterol crystals in various regions of the brain. Alternatively, specific inhibition of intestinal ACAT2 was found to be beneficial in preventing diet-induced hypercholesterolemia and gall-stone formation (Kusunoki *et al*, 1995; Buhman *et al*, 2000b). Since ACAT2 is the predominant isoform present in the intestinal villi, inhibition of this isoform can lead to reduced esterification of dietary cholesterol marring its absorption. This shall ultimately reduce the production of apoB containing lipoprotein like cholesterol ester rich LDL. Another important observation made in this study was that ACAT2$^{-/-}$ mice were resistant to development of atherosclerosis when fed with a high-cholesterol diet. This again raised a speculation about the usefulness of ACAT2 specific inhibitors for the treatment of atherosclerosis and interestingly selective ACAT2 depletion showed induction of ABCA1 mediated free cholesterol efflux (Zhang *et al*, 2012). Selective ACAT2 inhibition increases the secretion of triglycerides into the newly forming VLDL particles leading to hypertriglyceridemia (Lee *et al*, 2004; Bell *et al*, 2006). Thus, selective inhibition of the ACAT isoforms is a deceitful path at present which should be trodden incisively. The research on ACAT2-specific inhibitors breathed a new life with pyripyropene-A, an ACAT2-specific inhibitor of fungal origin, exhibiting 2000-fold higher selectivity for ACAT2 over ACAT1 (Ohshiro *et al*, 2011; Ohtawa *et al*, 2013a; Ohtawa *et al*, 2013b). This molecule has shown very good results *in vitro* and in rodent models of hypercholesterolemia and atherosclerosis. Alternatively, pan-specific inhibitors of ACAT have shown very good results in models of diet-induced atherosclerosis with characteristic regression of aortic lesions. These molecules have also shown favorable effects on Aβ

turnover suggesting an advantageous role in AD-like condition. Liver-specific ACAT2 inhibition using antisense oligonucleotides has its own implications and is discussed in the subsequent sections.

***Is it sensible to use anti-sense oligonucleotides for ACAT inhibition?***

Among dwindling hopes and failing clinical trials, a study by Bell and colleagues smeared a ray of hope by utilizing antisense oligonucleotides (ASOs) for ACAT inhibition (Bell *et al*, 2006). The authors observed that ASOs against hepatic ACAT2 do reduce hepatic ACAT2 mRNA levels and activity without affecting the activity of ACAT1 in liver or affecting ACAT isoform activity in other tissues. Many endpoints, important to the pathophysiology of atherosclerosis were improved following biweekly ASO therapy to $LDLr^{-/-}$ mice for 16 weeks. Consequently, ASO treatment reduced hepatic cholesteryl ester content, plasma and LDL cholesterol and most importantly shifted plasma cholesteryl ester content from a saturated or monounsaturated to a principally polyunsaturated form. All these factors collectively resulted in a reduction in aortic cholesteryl ester content illustrating a reduction in the acuteness of atherosclerosis. In spite of these encouraging results, two important findings in this study featured against the use of liver-specific ASOs for treatment. One, liver specific ACAT2 inhibition led to increased triglycerides in nascent VLDL particles. This effect is a compensatory mechanism leading to increased plasma triglyceride levels (Lee *et al*, 2004; Bell *et al*, 2006). It is disturbing because hypertriglyceridemia can have its own implications in relation to hyperlipidemia or metabolic syndrome. Two, one of the ASOs used in the study led to elevation of transaminases, reflecting a possible hepatotoxic potential of these kind of agents. When ASO therapy has not yet received full clinical support such reports can certainly have a negative impact on the liability that ASOs might put forward when such agents are advanced towards clinical trials. Another point of concern in this study was whether ASO treatment was initiated after establishment of hypercholesterolemia or not. Because, even though the authors have used adequate controls for their experiments, amelioration of hypercholesterolemia after it has been ascertained for a few weeks can prove daunting.

Given these advantages and drawbacks of ASO therapy, it would be prudent to compare and contrast pharmacological ACAT inhibition *vis-à-vis* ASO-mediated ACAT inhibition. Most of the pharmacological ACAT inhibitors used in clinical trials have been non-selective to the ACAT isoforms ultimately leading to adrenal toxicity as one of the major concerns. Additionally, agents like avasimibe and pactimibe have failed to show efficacy in randomized clinical trials. However, non-selective inhibition has one clear advantage that since pharmacological inhibition is incomplete by nature, it leads to retention of ABCA1 activity. Thus free cholesterol efflux might counter the accumulation of free cholesterol alleviating the possibility of xanthomatosis or adrenotoxicity as observed in ACAT1$^{-/-}$ mice. Furthermore, non-selective inhibition can alter the composition of chylomicrons and VLDL decreasing the atherogenicity of these lipoproteins (Akopian and Medh, 2006). But clearly, as these agents have failed clinical trials, it is very difficult to say at this point that these advantages actually matter or not. On the other hand, ASO therapy is relatively free of side effects and animal studies show promising endpoints in relation of atherosclerosis progression. However, the risk of hepatic damage and hypertriglyceridemia may prove to be hurdles in the progress of ASOs as therapeutic alternatives. The relative lack of experience related to ASOs in human can also affect the relative sentiments regarding acceptance of this novel form of therapy. At present, it is unclear whether tissue-specific antisense inhibition or ACAT-inhibitory pharmacotherapy would offer better prognosis in experimental models and clinical cases of atherosclerosis.

***ACAT2-selective inhibition***

With encouraging results from the studies of Bell and coolleagues it became reasonably important for researchers to identify ACAT2-specific inhibitors that could be studied *in vivo* (Bell *et al*, 2006). The atheroprotection strategy with ACAT2-specific inhibition would have wider acceptability if it could be demonstrated; using an ACAT2-specific inhibitor that apart from ASO mediated ACAT2 inhibition, pharmacological inhibition of ACAT2 is also possible. Pyripyropenes are known ACAT inhibitors of fungal origin. Pyripyropene-A is a highly selective and potent inhibitor of ACAT2 ($IC_{50}$ 70 nM)

(Ohshiro *et al*, 2011). Ohshiro and colleagues evaluated this orally active ACAT2-specific inhibitor in a mouse model of atherosclerosis and found that ACAT2-selective inhibition can be beneficial for atherosclerosis (Ohshiro *et al*, 2011). Atherogenic animals treated with pyripyropene-A showed reduced intestinal cholesterol absorption and ultimately reduced accumulation of cholesterol and cholesteryl esters in LDL and VLDL suggesting a protective effect against development of atherosclerotic lesions. Thus orally active inhibitors of ACAT2 hold latent anti-atherogenic ability. When more research groups show interest in this matter and when more ACAT2-selective agents are discovered, the picture might become clearer. Further studies in robust animal models can direct the progression of ACAT2-selective compounds towards clinical trials.

## CURRENTLY USED METHODS FOR SCREENING OF ACAT INHIBITORS

Specific and non-specific inhibition of ACAT isoforms has been attempted by several researchers for the control of atherosclerosis with varying levels of success. All of these studies involve determination of the effect of inhibitors on the quantum of cholesteryl oleate formed by ACAT catalysis. Different methods have been employed for the estimation of cholesteryl esters in general and cholesteryl oleate in particular. By far the most commonly employed method is the one involving the use of radioactive substrates like [$^{3}$H] cholesterol or [$^{14}$C]oleoyl CoA, for the ACAT assay. This leads to production of radioactive cholesteryl oleate which is quantified by liquid scintigraphy (Erickson *et al*, 1980b; Chang *et al*, 1998; Temel *et al*, 2003). Hashimoto *et al* (1973) and Largis *et al* (1989) used a similar method for the determination of cholesteryl palmitate. In both the assays either cell homogenates or microsomes were used as a source of ACAT enzyme. Lada and co-workers developed a rapid and high-throughput cell-based assay for determination of cholesterol esters. This assay exploits the mimicry of fluorescent NBD-cholesterol (22-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-23,24-bisnor-5-cholen-3-ol) to that of native cholesterol. NBD-cholesterol shows relatively higher fluorescence in a non-polar milieu. Thus, esters of NBD-cholesterol are strongly fluorescent and this fluorescence can be measured with the help of fluorescence microscopy, HPTLC (High

Performance Thin Layer Chromatography) or fluorescent ACAT assay (Lada *et al*, 2004). Mizoguchi and associates have proposed a direct measurement method for the enzymatic determination of cholesterol esters by either colorimetry or fluorimetry. Cholesteryl esters are converted to free cholesterol with the help of cholesterol esterase and the liberated cholesterol is decomposed by $H_2O_2$, which on further treatment with 4-aminoantipyrine or amplex red produces a product which can be estimated by colorimetry or fluorimetry respectively. The inherent drawback of this method involves the need to isolate cholesteryl ester products from the reaction mixture using an efficient method like HPTLC (Mizoguchi *et al*, 2004). All these methods are laborious, time-consuming and/or expensive. Moreover, these methods possess a limitation of not being able to directly estimate cholesteryl ester levels in plasma.