Identification of Critical Promoter CpG Sites for Expression of APLN Transcription in Females by DNA de-Methylation

Rowyda N. Al-Harithy*, Alaa' A. Al-Ansari* and Alsharefarayya Alharthi**

*Department of Biochemistry, King Abdul-Aziz University, Jeddah, Saudi Arabia ** Nottingham University Hospitals NHS Trust, Nottingham, U.K.
dr.alharithy@gmail.com

Abstract: Modification of DNA by methylation of cytosines at CpG dinucleotides is a widespread phenomenon that leads to changes in gene expression. Other studies uncovered associations between the degree of CpG methylation at specific sites and gender. However, it is unclear whether apelin (APLN) gene promoter DNA methylation contributes to gene secretion. Therefore, this study aims to analyze the methylation pattern of BstUI and AvaII sites at the APLN gene promoter and investigate the impact of gender and BMI on APLN promoter DNA methylation pattern and gene expression. Genomic DNA was extracted from whole blood of 40 Saudi volunteers (mean age, 49.4±12.8 years; 20 females, 20 males). Methylation at the promoter region of the APLN gene was examined by specific-restriction enzyme digestion (BstUI and AvaII). Followed by polymerase chain reaction (PCR) and gel electrophoresis on 1% agarose gel. Nonselective Apelin-12 Enzyme Immunoassay kit was used to study protein expression in blood serum. Gene expression elevated significantly in the females harbor de-methylation at BstUI and AvaII sites compared with the females harbor methylation at both sites (P=0.008) and nearly significant to the males harbor de-methylation at both sites (P=0.06). A significant positive correlation was observed between de-methylation and gene expression in the females (r=0.7, P=0.008). The research suggests that there exists a mechanism to regulate the APLN transcription through de-methylation of APLN promoter at BstUI and AvaII sites, especially among females.

[Rowyda N. Al-Harithy, Alaa A. Al-Ansari and Alsharefarayya M. Al-Harthi. **Identification of Critical Promoter CpG Sites for Expression of APLN Transcription in Females by DNA de-Methylation.** *Life Sci J* 2017;14(9):88-93]. ISSN: 1097-8135 (Print) / ISSN: 2372-613X (Online). http://www.lifesciencesite.com. 8. doi:10.7537/marslsj140917.08.

Keywords: DNA Methylation, APLN gene, Promoter CpG sites, Gene expression, Gender

1. Introduction

DNA methylation is a valuable mark of epigenetic modifications, which influences the transcriptional machinery. Evidence has indicated that DNA methylation pattern can be influenced by environmental factors creating a source of epigenetic variability. Well-known, nutrient status differentially modulates DNA methylation in several metabolic genes. The amounts and distribution of DNA methylation is uneven among different human tissues. There are about 28,000 CpG islands (CGIs) in the human genome. About 70% of gene promoters are associated with CGIs (Maunakea *et al*., 2010; Deaton *et al*., 2011; Harrow *et al*., 2012). There are also data suggesting that differential DNA methylation mainly occurs at CpG shores, and not in CGIs (Irizarry *et al*., 2009; Ronn *et al*., 2013). The majority of promoters CGIs are un-methylated in somatic cells, and this is an important feature of gene promoters and gene expression control. DNA methylation is involved in many important biological phenomena, such as X chromosome inactivation, genomic imprinting and in controlling tissue-specific expression in adult somatic tissues (Robertson and Wolffe, 2000; Matarazzo *et al*., 2002; Scarano *et al*., 2005; Jones, 2012).

Apelin is a peptide of renin-angiotension-aldosterone system (RAAS) and is the endogenous ligand of the human G-protein coupled apelin receptor (APJ). Apelin plays a crucial role in regulating several physiological and metabolic functions, including cell proliferation, blood pressure, angiogenesis, glucose cellular uptake, intestinal glucose absorption, mitochondrial biogenesis and fatty acid oxidation. It also inhibit lipolysis as well as insulin secretion through different signaling pathways (Boucher *et al*., 2005; Sorli *et al*., 2006; Salcedo *et al*., 2007; Castan-Laurell *et al*., 2008; Sonmez *et al*., 2010; Kursunluoglu-Akcillar *et al*., 2014; Bertrand *et al*., 2015). Apelin is encoded by APLN gene in humans. The gene is located on chromosome Xq25-26.1 and encodes 77-amino acid pre-propeptide divided by shorter mature pepetide such as apelin-36, apelin-17, apelin-13 and apelin-12 (Japp and Newby, 2008).

Recently, the role of DNA methylation has gained a vast interest among scientists all over the world. Indeed, the methylation status of the APLN promoter, which is CpG-rich, determines its gene expression. The aim of the present study was to analyze the methylation pattern of two sites on the APLN gene promoter, the changes in gene expression

and the presence of a gender specific of methylation on APLN promoter.

2. Material and Methods

Subjects

The present study included 40 Saudi volunteers (mean age, 49.4±12.8 years; 20 females, 20 males). The subjects were selected from persons visited King Abdul Aziz University Hospital in Jeddah, KSA, for routine medical check up. They had normal blood pressure, blood sugar level, and were not under any treatment course. All participants gave their informed written consent before enrollment in the study. The research plan was approved by the Ethics Committee of KAU University, Jeddah, KSA.

Anthropometric measurements

Standard methods were used to measure height, weight, waist circumferences (WC), and hip circumferences (HC). Body weight was measured with light clothing on, with up to 0.1kg precision. Height was measured up to 0.1cm precision. Body mass index (BMI) was calculated as weight (kg) divided by height in meters squared (m^2). Waist-to-hip ratio (WHR) was also calculated as WC divided by HC.

Genomic DNA extraction

Genomic DNA was extracted using QIAamp DNA Blood Mini kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. DNA concentrations and purity was determined using Nano-Drop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Serum apelin level determination

Serum apelin concentration was determined using Nonselective Apelin-12 Enzyme Immunoassay kit (Phoenix Pharmaceuticals, Inc., USA) according to the manufacturer's instructions. This test kit is effective in the range of 0.0 to 100 ng/mL. Duplicate measurements were performed in a single experiment.

DNA methylation analysis

Genomic DNA extracted from blood of the volunteers were digested with methylation-sensitive restriction enzymes (BstUI and AvaII) (Thermo Scientific Company, Lithuania) that recognize and cut un-methylated CCGG and CCAGG motifs; respectively. The digested DNA samples were amplified with primers flanking the CCGG and CCAGG motifs in promoter region of APLN gene. Primers sequence was designed by the Primer-3 software and made by Macrogen Company (Rockville, MD, USA). The following primers were used in the PCR reaction includes: Forward primer, 5′-ACTGAGTCCTTCCCTAAGCG–3′ and reverse primer, 5′ –CCAGGGGCTGCATTTTGTAG- 3′. The thermal cycling protocol was: 5 minutes at 94°C, followed by 1:30 minutes at 94°C, for 40 cycles, annealing for 1 minutes at 60.5°C and 3:30 minutes of final extension at 72°C. PCR products were analyzed by 1% agarose gel electrophoresis.

Statistical analysis

Data are presented as means ± S.D. Comparisons between two groups were made by the Student's *t*-test of independence. The correlation of serum apelin level with DNA methylation state was analyzed by Pearson's rank order correlation. To analyze the association between DNA methylation pattern, gender and BMI, Pearson's Chi-square test of Independence was used. For all analysis, *P*-value <0.05 was considered as statistically significant. All statistical analyses were carried out using the SPSS for Windows V22.0 (SPSS Inc., Chicago, IL, USA).

3. Results

General parameters of the subjects

The characteristics are displayed in Tables 1 and 2. The female group had no significant difference compared to the male group in regards to their age, weight, BMI, waist, hip measurements, and apelin level. Height and WHR were significantly higher in the male group compare to the female group. There was no significant difference between lean and obese groups in each gender in terms of their age, WHR and apelin levels. In two female subjects, waist and hip circumference were significantly higher in the obese females compared to the lean females. By design, the weight and BMI were significantly higher in the obese group compared with the lean group in the females and the males.

Table 1. General parameters comparison between the females and the males.

Parameters	Females n=20	Males n=20	*P*-value
Age (yrs)	48.8±10.9	50.0±14.6	0.8
Height (cm)	158.6±11.4	168.5±6.7	0.004**
Weight (cm)	71.2±17.7	80.8±19.4	0. 1
BMI (kg/m^2)	28.7±8.1	28.4±6.4	0.9
Waist (cm)	81.1±34.5	73.8±34.9	0.7
Hip (cm)	91.4±36.4	77.6±40.2	0.3
WHR	0.95±0.5	0.99±0.2	0.04*
Apelin (ng/ml)	0.39±0.3	0.32±0.2	0.4

Abbreviation: BMI, body mass index; WHR, weight-hip ratio; n, number of subjects. Data are presented as mean±SD. Analyzed by Student's *t*-test of independence. *P*-value is significant when it is ≤0.05;*significant at the 0.01; **highly significant.

Table 2. General parameters comparison between the lean and the obese females and males.

Parameters	Females (n=20)			Males (n=20)		
	Lean (n=9)	Obese (n=11)	*p*-values	Lean (n=9)	Obese (n=11)	*P*-values
Age (years)	46.6±8.7	50.8±12.7	0.5	48.7±13.9	51.4±16.1	0.7
Height (cm)	163.9±11.2	153.8±9.7	0.06	167.7±3.8	169.3±9.1	0.6
Weight (cm)	58.2±8.8	82.8±15.4	0.001**	66.7±7.3	96.4±16.2	0.0001*
BMI (kg/m2)	21.6±2.7	35.0±5.6	0.0001**	23.7±2.2	33.6±5.2	0.0001*
Waist (cm)	59.9±34.1	99.9±22.8	0.02*	64.5±28.5	84.1±40.2	0.09
Hip (cm)	75.9±29.6	105.1±37.9	0.02*	68.7±31.2	87.4±48.3	0.2
WHR	0.8±0.3	1.1±0.6	0.1	1.0±0.1	1.0±0.3	0.3
Apelin (ng/ml)	0.5±0.3	0.3±0.2	0.1	0.3±0.2	0.3±0.3	0.9

Abbreviation: BMI, body mass index; WHR, weight-hip ratio; n, number of subjects.
Data are presented as mean±SD. Analyzed by Student's *t*-test of independence.
P-value is significant when it is ≤0.05;*significant at the 0.01; **highly significant.

Methylation status of the APLN promoter

Specific-restriction enzyme digestion technique identified methylation of APLN promoter region at BstUI and AvaII sites. Fortunately the methylation analysis for the volunteers showed that the two sites together were either methylated or de-methylated in the females and the males. To analyze the levels of methylation and de-methylation in the two groups, the analysis observed that the level of methylation at both sites in the female group was 80% and in the male group was 33.3%. On the other hand, the level of de-methylation at both sites in the females was 20% and in the males was 66.7% (fig1).

To analyze the association between DNA methylation pattern, gender and BMI, Pearson's Chi-square test of Independence was used. The result showed an association between DNA methylation pattern and gender (X^2=4.2, P=0.04), which indicates a gender-specific pattern. In regard to BMI, the results showed no association between DNA methylation pattern and BMI in the female and the male groups (X^2=0.6, P=0.4, X^2=0.4, P=0.5; respectively) (fig.2).

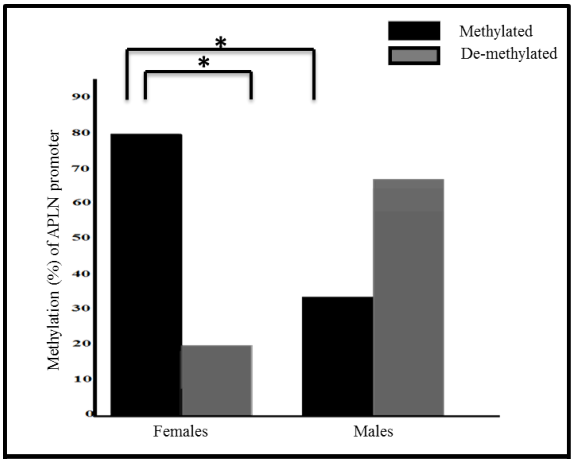

Figure 1. Association result between DNA methylation and gender. The bars represent the methylation level in different states (methylated and de-methylated) of APLN promoter sites. Analysis was done using Pearson's Chi-square test of Independence. The relationship is dependent, where X^2 = 4.2, df=1 and *P*-value= 0.04 . *P*-value is *significant when it is ≤0.05

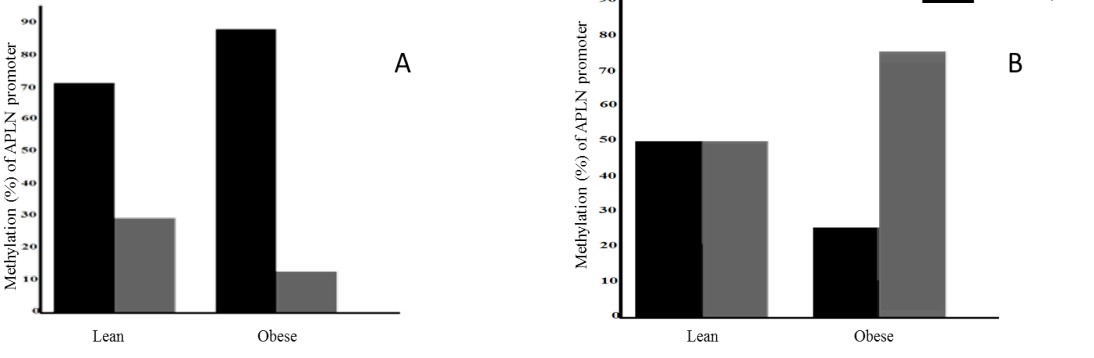

Figure 2. Association results between DNA methylation and BMI (A) in the females and (B) in the males. The bars represent the methylation level in different states (methylated and de-methylated) of APLN promoter sites. Analysis was done using Pearson's Chi-square test of Independence. (A) Independent relationship, where X^2 = 0.6, df=1 and *P*-value= 0.4 (B) Independent relationship, where X^2= 0.4, df=1 and *P*-value= 0.5. *P*-value is significant when it is ≤0.05.

Levels of APLN protein in the females and the males

To compare the result of APLN expression in the females harbor methylation and de-methylation at both sites to the males, *t*-test of independence was used. The analysis showed that gene expression elevated significantly in the females harbor de-methylation at both sites compared with the females harbor methylation at both sites (*P*=0.008) and nearly significant to the males harbor de-methylation at both sites (*P*=0.06) (Table 3). This result determines which pattern and gender effect gene expression. To analyze the relationship between DNA methylation pattern and gene expression, Pearson's Correlation was used. The analysis observed a significant positive correlation between the females harbor de-methylation and gene expression (r=0.7, *P*=0.008) (Table 4).

Table 3. Comparing APLN expression between the females and the males in different states of promoter methylation.

Promoter sites		Females (n=20)	Males (n=20)	*P*-values
Methylated	BstUI AvaII	0.31±0.21	0.39±0.22	0.6
de-methylated	BstUI AvaII	0.72±0.11	0.31±0.29	0.06

Data are presented as mean±SD. Analyzed by Student's *t*-test of independence.
P-value is significant when it is ≤0.05

Table 4. Comparing APLN expression of the methylated and de-methylated promoter sites in the females and the males.

Promoter sites		Females (n=20)	*P*-value	Males (n=20)	*P*-value
Methylated	BstUI AvaII	0.31±0.21	0.008**	0.39±0.23	0.8
de-methylated	BstUI AvaII	0.72±0.11		0.31±0.29	

Data are presented as mean±SD. Analyzed by Student's *t*-test of independence.
P-value is **highly significant at the 0.01

4. Discussions

To our knowledge, this is the first study that investigates DNA methylation pattern of APLN promoter region at BstUI and AvaII sites and apelin expression level in gender groups. It is well known that gene promoter methylation is an important mechanism for gene transcriptional inactivation. Recent studies have been directed towards utilization of the level of DNA methylation, methylation content and methylation pattern of gene promoters as biomarkers associated with the expression of the corresponding genes. The relationship between DNA methylation and transcription is more comprehend than the scientists realized at first. Therefore, further investigating factors that might affect DNA methylation pattern such as gender, age and BMI is necessary for understanding epigenetic codes, which can be observed in diseases.

Gender differences at the DNA methylation on the autosomal chromosomes have been studied in some human tissues and cell types and revealed no, few or small changes (Sarter *et al*., 2005; Eckhardt *et al*., 2006; Boks *et al*., 2009; Cotton *et al*., 2011). Hellman and Chess published their work on the gene body-specific methylation on the active X chromosome and conclude that CpG islands within promoter regions revealed higher methylation levels in the inactive compared with the active female X chromosomes (Hellman and Chass, 2007). Another study found that DNA methylation pattern in females had higher average methylation levels compared with males for the shore regions as well as the CpG islands, while males had higher average methylation levels than females in the open sea and shelve region. Since it is known that individual CpG sites exhibit differences in DNA methylation between genders on X chromosome, we tested if the degree of DNA methylation of BstUI and AvaII sites in APLN promoter differed in females compared to males. The analysis provided evidence of strong association between DNA methylation pattern and gender (ϕ=0.5, *P*=0.04). This suggests the presence of a gender-

specific methylation pattern on the APLN promoter at BstuI and AvaII sites. The pattern showed higher percentages of methylation in females compared with the males; which confirms previous findings of Hall and his team (Hall *el al*., 2014). Other association analysis was performed between DNA methylation pattern and BMI. In male group, the result showed no association (X^2=0.4, ϕ =0.3, *P*=0.5). However, in the female group, there was a reversible association (X^2=0.6, ϕ =-0.2, *P*=0.4). The result suggests that the obese females have a low apelin expression than the lean females and gender differences.

In the present study, the protein expression profile of APLN in relation to methylation of the APLN gene promoter at BstUI and AvaII sites was also studied. The result showed an increase of APLN expression accompanied by de-methylation of the two sites located inside CpG islands of the APLN promoter from the female group. Although the male group had higher levels of de-methylation at both sites, gene expression was much lower than the females harbor de-methylation at both sites. Further analysis observed a significant positive correlation between the females harbor de-methylation and gene expression (r=0.7, *P*=0.008) in comparing to the males harbor de-methylation (r=-0.2, *P*=0.8). This investigation determined the DNA methylation pattern and the gender that effect gene expression. Also provides evidence that APLN exhibited a higher expression in females indicating that males and females differ in gene expression. Further studies are warranted to determine the causal direction of this relationship, especially among females. The methylation of APLN promoter at BstUI and AvaII sites may be important biomarkers that promote the APLN expression, especially in females. Our result confirms previous studies that investigated the influence of sex on genome wide methylation (Liu *et al*., 2010; Hall *et al*., 2014). Liu and his team found that DNA methylation level in female saliva cells is higher in both the X chromosome as well as the autosomes. Hall and his colleques examined the impact of sex on the genome-wide DNA methylation pattern in human pancreatic islets and gene expression. They identified a number of genes including APLN on the X chromosome with differences in DNA methylation and gene expression between females and males.

This informs the potential use of the methylation in BstuI and AvaII sites of the APLN promoter in blood as powerful non-invasive molecular markers. As APLN plays a role in the regulation of glucose homeostasis and may contribute to the link between increased adipose tissue mass and obesity related metabolic diseases. These potential biomarkers might be used as tools to predict and follow up the physiological alterations associated with the development of the metabolic syndrome.

Collectively, we expect that accumulating knowledge from understanding individualized epigenetic profiles will provide valuable information in the future. There are several strengths to this study. First, by focusing on adults at a pre-disease stage. Second, the two groups were recruited from the same area. Limitations of our study include small sample size.

Conclusion

APLN promoter de-methylation at BstUI and AvaII sites may represent a valuable and important asset to be considered in further studies. Also the implementation of blood screening program for DNA methylation patterns should aid, in times to come, in reducing diseases incidence and mortality rates.

References

1. Bertrand C, Valet P, Castan-Laurell I. Apelin and energy metabolism. Front Physiol 2015; 6: 115.
2. Boks MP, Derks EM, Weisenberger DJ, Strengman E, Janson E, Sommer IE, *et al*. The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. PLoS One 2009; 4: e6767.
3. Boucher J, Masri B, Daviaud D, Gesta S, Guigné C, Mazzucotelli A, *et al*. Apelin, a newly identified adipokine up-regulated by insulin and obesity. Endocrinology 2005; 146(4): 1764-71.
4. Castan-Laurell I, Vítkova M, Daviaud D, Dray C, Kováciková M, Kovacova Z. Effect of hypocaloric diet-induced weight loss in obese women on plasma apelin and adipose tissue expression of apelin and APJ. Eur J Endocrinol 2008; 158(6): 905-910.
5. Cotton AM, Lam L, Affleck JG, Wilson IM, Penaherrera MS, McFadden DE, *et al*. Chromosome-wide DNA methylation analysis predicts human tissue-specific X inactivation. Hum Genet. 2011, 130: 187-201.
6. Deaton A, Webb S, Kerr A, Illingworth R, Guy J, Andrews R, *et al*. Cell type-specific DNA methylation at intragenic CpG islands in the immune system. Genome Res 2011; 21(7): 1074–1086.
7. Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, Burger M, *et al*. DNA methylation profiling of human chromosomes 6, 20 and 22. Nat Genet 2006; 38: 1378-1385.
8. Hall E, Volkov P, Dayeh T, Esguerra J, Salö S, Eliasson L, *et al*. Sex differences in the genome-wide DNA methylation pattern and impact on gene expression, microRNA levels and insulin

secretion in human pancreatic islets. Genome Biology 2014; 15:522.
9. Harrow J, Frankish A, Gonzalez J, Tapanari E, Diekhans M, Kokocinski F, *et al*. GENCODE: the reference human genome annotation for The ENCODE Project. Genome Res 2012; 22(9): 1760-74.
10. Hellman A, Chess A. Gene body-specific methylation on the active X chromosome. Science 2007; 315: 1141–1143.
11. Irizarry R, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, *et al*. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 2009; 41(2): 178-86.
12. Japp A, Newby D. The apelin-APJ system in heart failure: pathophysiologic relevance and therapeutic potential. Biochem Pharmacol 2008; 75(10): 1882-92.
13. Jones P. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet 2012; 13(7): 484-92.
14. Kursunluoglu-Akcilar R, Kilic-Toprak E, Kilic-Erkek O, Turgut S, Bor-Kucukatay M. Apelin-induced hemorheological alterations in DOCA-salt hypertensive rats. Clin Hemorheol Microcirc 2014; 56(1): 75-82.
15. Liu J, Morgan M, Hutchison K, Calhoun VD. A study of the influence of sex on genome wide methylation. PLoS One 2010; 5: e10028.
16. Matarazzo M, De Bonis M, Gregory R, Vacca M, Hansen R, Mercadante G, *et al*. Allelic inactivation of the pseudoautosomal gene SYBL1 is controlled by epigenetic mechanisms common to the X and Y chromosomes. Hum Mol Genet 2002; 11:3191–3198.
17. Maunakea A, Nagarajan R, Bilenky M, Ballinger T, D'Souza C, Fouse S, *et al*. Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 2010; 466(7303): 253–257.
18. Robertson K, Wolffe A. DNA methylation in health and disease. Nat Rev Genet 2000; 1(1): 11-9.
19. Rönn T, Volkov P, Davegårdh C, Dayeh T, Hall E, Olsson A, *et al*. A six months exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue. PLoS Genet 2013; 9(6): e1003572.
20. Salcedo A, Garijo J, Monge L, Fernández N, García-Villalón A, Sánchez Turrión V. Apelin effects in human splanchnic arteries. Role of nitric oxide and prostanoids. Regul Pept 2007; 144: 50–55.
21. Sarter B, Long TI, Tsong WH, Koh WP, Yu MC, Laird PW. Sex differential in methylation patterns of selected genes in Singapore Chinese. Hum Genet 2005; 117: 402-403.
22. Scarano M, Strazzullo M, Matarazzo M, D'Esposito M. DNA methylation 40 years later: its role in human health and disease. J Cell Physiol 2005; 204: 21–35.
23. Sonmez A, Celebi G, Erdem G, Tapan S, Genc H, Tasci I, *et al*. Plasma apelin and ADMA Levels in patients with essential hypertension. Clin Exp Hypertens 2010; 32(3): 179-83.
24. Sorli S, van den Berghe L, Masri B, Knibiehler B, Audigier Y. Therapeutic potential of interfering with apelin signalling. Drug Discov Today 2006; 11(23-24): 1100-6.

9/24/2017