Canadian Journal of Physiology and Pharmacology

Acute and chronic metabolic effects of carvedilol in high-fructose, high fat diet-fed mice: Implication of β -arrestin2 pathway

Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2021-0299.R1
Manuscript Type:	Article
Date Submitted by the Author:	03-Aug-2021
Complete List of Authors:	Ahmed, Hoda M. S.; Zagazig University, Department of Pharmacology and Toxicology; Ministry of Health Mohamed, Samar G.; Zagazig University, Department of Pharmacology and Toxicology; Ministry of Justice, Department of Toxic and Narcotic Drugs, Forensic Medicine Ibrahim, Wael S.; Zagazig University, Department of Pharmacology and Toxicology; Badr University, Department of Pharmacology Rezk, Asmaa M.; Zagazig University, Department of Pharmacology and Toxicology; Benha University Hospitals, Department of Pharmacy Mahmoud, Amr; Zagazig University Faculty of Pharmacy, Department of Pharmacology Mahmoud, Mona F.; Zagazig Univ Ibrahim, Islam; Zagazig University, Department of Pharmacology and Toxicology
Is the invited manuscript for consideration in a Special Issue:	Not applicable (regular submission)
Keyword:	β -Arrestins, Biased agonists, Carvedilol, Insulin resistance, Glucose tolerance

SCHOLARONE™
Manuscripts

1 Acute and chronic metabolic effects of carvedilol in high-fructose, high fat diet-fed
2 mice: Implication of β -arrestin2 pathway

3 Hoda M. S. Ahmed^{1,2}, Samar G. Mohamed^{1,3}, Wael S. Ibrahim⁴, Asmaa M. Rezk^{1,5}, Amr
4 A. A. Mahmoud^{1,6}, Mona F. Mahmoud¹, Islam A. A. E.-H. Ibrahim^{1*}

5 ¹ Department of Pharmacology and Toxicology, Faculty of Pharmacy, Zagazig
6 University, Egypt.

7 ² Medical Supply Chain, Abo-Hammad Health Administration, Ministry of Health,
8 Egypt.

9 ³ Department of Toxic and Narcotic Drugs, Forensic Medicine, Cairo Laboratory,
10 Medicolegal Organization, Ministry of Justice, Cairo, Egypt.

11 ⁴ Department of Pharmacology and Toxicology, School of Pharmacy, Badr University in
12 Cairo (BUC), Cairo, Egypt.

13 ⁵ Department of Pharmacy, Benha University Hospitals, Benha, Egypt.

14 ⁶ Present address: Department of Pharmacology, Pharmacy Program, Oman College of
15 Health Sciences, Muscat 114, Oman

16 *Corresponding author:

17 Islam A. A. E.-H. Ibrahim, Department of Pharmacology and Toxicology, Faculty of
18 Pharmacy, Zagazig University, 44519 Egypt.

19 E-mail: eaebrahiem@pharmacy.zu.edu.eg Tel/Fax: +200552303266

20 Orcid ID: 0000-0002-8931-465X

21 Short running title: Metabolic effects of carvedilol

22

23

24

Abstract

We aimed to investigate the acute and chronic effects of carvedilol on insulin resistance in high-fructose, high-fat diet (HFrHFD)-fed mice and the implication of β -arrestin2 pathway. The acute effect of carvedilol (10 mg/kg, i.p.) on glucose tolerance and hepatic lipid signaling in normal and insulin resistant mice was investigated. Then, the chronic effect of carvedilol on insulin resistance and dyslipidemia in HFrHFD-fed mice was examined. Changes in β -arrestin2 and its down-stream signals in liver, skeletal muscle, and adipose tissue were measured. This involved measuring phosphatidylinositol 4,5-bisphosphate (PIP2) and diacylglycerol (DAG) levels and protein kinase B (Akt)-activity. Carvedilol acutely reduced fasting blood glucose levels in both normal and insulin resistant mice without significantly affecting the glucose tolerance. These acute effects were associated with increased hepatic PIP2 but decreased hepatic DAG levels. Chronic administration of carvedilol significantly ameliorated insulin resistance and dyslipidemia in HFrHFD-fed mice. These chronic effects were associated with increased β -arrestin2, PIP2 and Akt activity levels but decreased DAG levels in the classical insulin target tissues. In conclusion, carvedilol acutely maintains glucose homeostasis and chronically ameliorates insulin resistance and dyslipidemia in HFrHFD-fed mice. The insulin sensitizing effects of carvedilol are highly correlated with the upregulation of β -arrestin2 pathway.

Keywords

Amlexanox; β -Arrestins; Biased agonists; Carvedilol; insulin resistance; Paroxetine

48

49 **Introduction**

50 Insulin resistance and type 2 diabetes are growing health problems worldwide. Indeed,
51 sedentary lifestyles and high-calorie diets are the main triggers of these disorders (**Muoio**
52 **and Newgard, 2006**). In addition, cardiovascular complications of insulin resistance and
53 type 2 diabetes, such as hypertension, atherosclerosis and cardiac remodeling, may be life
54 threatening (**Fox et al., 2004; Gast et al., 2012**). Current guidelines recommend the
55 avoidance of β -blockers as a therapeutic option for cardiovascular complications
56 associated with these disorders due to their acute hyperglycemic effects secondary to
57 inhibition of insulin secretion (**Kjekshus et al., 1990**). However, several clinical studies
58 have shown that long-term administration of carvedilol in type 2 diabetic patients may
59 improve insulin resistance, maintain glycemic control, and reduce hyperlipidemia in
60 contrast to the administration of other β -blockers (**Sica, 2005; Basat et al., 2006**).

61 Carvedilol is a third generation, nonselective β -blocker (**Frishman, 1998**) that blocks
62 β_1 , β_2 , and α_1 -adrenergic receptors (**Li et al., 2016**). Moreover, carvedilol exerts
63 antioxidant effects (**Malig et al., 2017**). Recent studies have shown that carvedilol is a β -
64 arrestin-biased agonist and can confer high levels of cardiac protection compared to other
65 β -blockers (**Wisler et al., 2007**).

66 The β -arrestin pathway is a G protein-coupled receptor downstream pathway that
67 mediates the desensitization of G protein-coupled receptors by uncoupling G proteins
68 (**Lefkowitz, 1998**). It has been shown that β -arrestins can activate their own downstream
69 signals independent of G proteins (**DeWire et al., 2007**). β -Arrestins act as scaffolding

proteins by recruiting several intracellular proteins to promote their interactions

(McDonald et al., 2000).

Phosphatidylinositol 4,5-bisphosphate (PIP₂) is a downstream signal for both G proteins and β -arrestins. G α q and G $\beta\gamma$ proteins, through their effects on phospholipase C (PLC), mediate the breakdown of PIP₂ into diacylglycerol (DAG) and inositol triphosphate (IP₃) (Wu Dianqing et al., 1993; Lee and Rhee, 1995). DAG promotes the activation of protein kinase C (PKC), which in turn deactivates insulin receptor substrate 1, leading to insulin resistance (Holland et al., 2007). On the other hand, β -arrestins promote the production of PIP₂ by uncoupling G proteins (Lefkowitz, 1998) and mediating the interactions between phosphoinositides and phosphatidylinositol 4-phosphate 5-kinase Ia (PIP5KI α) (Nelson et al., 2008; Ibrahim et al., 2013).

Previous studies have reported that β -arrestins can enhance insulin signaling by recruiting protein kinase B (Akt) and Src to promote the phosphorylation of Akt (Luan et al., 2009), which in turn activates glucose transporters (GLUTs) to mediate glucose uptake (Ueki et al., 1998) and promotes glycogen synthesis through inhibition of glycogen synthase kinase (GSK) (Ueki et al., 1998).

Given these findings, the present study was designed to evaluate the acute effect of carvedilol on glucose homeostasis and hepatic lipid signaling in standard chow diet (SCD)- and high-fructose, high-fat diet (HFrHFD)-fed mice. Furthermore, the current study investigated the chronic effects of carvedilol on insulin resistance and dyslipidemia in HFrHFD-fed mice focusing on the changes in the β -arrestin2 pathway in the classical insulin target tissues.

Materials and Methods

93 **Animals**

94 Adult male Swiss albino mice (20 ± 5 g, 8 weeks old) were purchased from the Faculty
95 of Veterinary Medicine, Zagazig University, Egypt, and housed in plastic cages with
96 wood shavings in the animal care unit of the Faculty of Pharmacy, Zagazig University.
97 The animals were kept under controlled temperature ($23 \pm 2^{\circ}\text{C}$) and humidity ($60 \pm 10\%$)
98 and a 12-h light/dark cycle. The mice were acclimatized for at least two weeks prior to
99 the experiments and had access to standard pellet chow diet and tap water.

100 **Ethical statement**

101 All procedures were conducted in accordance with the Canadian Council on Animal
102 Care guidelines and were approved by the Animal Ethics Committee of the Faculty of
103 Pharmacy, Zagazig University, Egypt (approval number: P2/12/2016).

104 **Drugs and Chemicals**

105 Carvedilol was obtained from Bio Pharma (Cairo, Egypt). Dimethyl sulfoxide (DMSO)
106 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

107 **Experimental design**

108 **Part I**

109 This part was designed to investigate the acute effect of carvedilol on glucose
110 homeostasis and hepatic lipid signaling in both SCD- and HFrHFD-fed mice. Mice were
111 fed SCD or HFrHFD (Panchal et al., 2011) for 16 weeks then were randomly distributed
112 into two sets, control and CARV ($n=6$ each). Control group received vehicle composed of
113 1-part DMSO: 3-parts distilled water at a dose of $100 \mu\text{L}/40$ g body weight, i.p. while
114 CARV group received (10 mg/kg, i.p.) dissolved in the same volume of vehicle. HFrHFD
115 was composed of chow diet (155 g), beef tallow (200 g), fructose (170 g), sweetened

condensed milk (320 g), corn glutin (60% protein, 100 g), salt mixture (25 g), and water (30 g) per kilogram of diet and received fructose (20% w/v) in drinking water for 16 weeks (Mohamed et al., 2019; Ibrahim et al., 2020). All nutritional parameters of this diet met or exceeded the guidelines of the National Research Council, Canada, for rats and mice (Table 1).

The mice were fasted overnight before receiving the drug or vehicle and were then given a glucose load (1 g/kg body weight, i.p.) as a 10% solution 30 minutes later for a glucose tolerance test. Carvedilol was given as a single dose 30 minutes before injection of glucose load. After glucose tolerance test, mice were fed SCD or HFrHFD again for 3 days. Then, mice were fasted overnight and carvedilol or vehicle was injected again as done in the glucose tolerance test. Thirty minutes later, mice were euthanized by decapitation and liver samples were collected to measure the hepatic levels of PIP2 and DAG. Fig. S1 shows the timeline of the experiment.

Part II

This part was designed to investigate the long-term effects of carvedilol on insulin resistance and dyslipidemia in HFrHFD-fed mice focusing on the changes in the β -arrestin2 pathway in the classical insulin target tissues.

After the acclimatization period, mice were randomly distributed into three experimental groups (n=12 each). In group 1, the mice were fed a standard chow diet (SCD) for 16 weeks. In groups 2 and 3, the mice were fed an HFrHFD for 16 weeks. In group 2 (the HFrHFD group), the mice received only the vehicle. In group 3 (the HFrHFD + CARV group), the mice received carvedilol (10 mg/kg/day, i.p., Von Möllendorff et al., 1987; Morsy et al., 2014). Carvedilol was dissolved in a mixture of

DMSO, Tween 80 and water (1:1:8) (100 μ L/40 g body weight). Carvedilol was administered for 4 weeks starting from week 13 of feeding [24]. Last dose of carvedilol was given one day before euthanasia and fasting was performed over-night. The dose of carvedilol was chosen following the methods in previous studies (Von Möllendorff et al., 1987; Morsy et al., 2014). Fig. S1 shows the timeline of the experiment.

Body weight gain

The body weight gain was calculated per mice per day in each group then the average was taken to obtain the average weight gain/mice/group (Abdulla et al., 2010).

Food intake

The diets were offered regularly at 3 PM daily and the daily food intake per group was calculated by subtracting the weight of the remained food from the weight of the offered food and then divided by the number of mice in each group to find out the average daily food intake per mice.

Intraperitoneal glucose tolerance test (IPGTT)

Blood drops obtained from mouse tails were used to measure the initial glucose levels (at 0 minutes; 30 minutes after carvedilol or vehicle injection) as well as the levels 30, 60 and 120 minutes after administration of a 10% glucose load (1 g/kg body weight, i.p.) (Jauncey and Ross, 1982) using an automated glucometer (GM100, Bionime GmbH, Berneck, Switzerland).

Blood and tissue sampling

At the end of the experiments, the mice were euthanized by decapitation, and then trunk blood was collected from the site of decapitation and centrifuged (4000 rpm, 4°C, 15 minutes). The serum was stored at -80°C for subsequent analyses. Liver, abdominal

skeletal muscle, and visceral adipose (epididymal) tissues were harvested and divided into three aliquots that were snap-frozen in liquid nitrogen and stored at -80°C for subsequent biochemical analyses.

Measurement of serum lipid profiles

Serum triglycerides (TGs), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) were measured using commercially available, quantitative, colorimetric assay kits obtained from Biodiagnostic (Giza, Egypt). All procedures were performed according to the manufacturer's instructions. Serum low-density lipoprotein cholesterol (LDL-C) was calculated according to the formula described by (Deisl et al., 2016) as follows:

$\text{LDL-C} = \text{TC} - \text{HDL-C} - (\text{TGs}/5)$. The units for all parameters were mg/dL.

Determination of serum insulin and calculation of the insulin resistance index

Serum insulin was measured by enzyme-linked immunosorbent assay (ELISA) using kits supplied by CUSABIO, Houston, USA (Cat. No. CSB-E05071m). The insulin resistance index was calculated using the homeostatic model of assessment (HOMA) according to the following equation (Friedewald et al., 1972): Insulin resistance HOMA (HOMA-IR) = Fasting glucose level (mg/dl) x fasting insulin level (mIU/mL)/405.

Determination of protein level of β -arrestin2 and downstream signals in liver, skeletal muscle, and adipose tissue

All samples were prepared according to the method described by Ibrahim et al (Ibrahim et al., 2020). Briefly, 100 mg was weighed from each tissue sample and homogenized using Con-Torque Eberbach's Tissue Homogenizer (Michigan, USA) in 500 μL 1x phosphate-buffered saline (PBS). Each homogenate was centrifuged at 10000

rpm and 5°C for 10 min using a cooling centrifuge. The supernatant was collected into new microcentrifuge tube (1.5 mL) and then used to assess all biochemical tests. The protein levels of β -arrestin2, PIP2, DAG, and phosphoserine 473 of Akt were measured by ELISA using kits supplied by LifeSpan BioSciences (WA, USA, cat. no. LS-F18999), Nova Lifetech Limited (Mongkok, Hong Kong, cat. no. CELI-66111m), BlueGene Biotech (Shanghai, China, cat. no. E03D0010), and Abcam (Cambridge, UK, cat no. ab176635), respectively. All procedures were performed according to the manufacturers' instructions. The ELISA technique for measuring β -arrestin2, PIP2, DAG, and phosphoserine 473 of Akt has been previously described (**Mohamed et al., 2019; Ibrahim et al., 2020**).

Statistical analysis

The data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical significance was tested using unpaired student's t-test or one-way ANOVA followed by Bonferroni multiple comparison tests for selected pairs. Correlation analysis was performed using Pearson r correlation test. $P < 0.05$ was used to indicate significance.

Results

Effect of HFrHFD on body weight gain, and food- and energy-intake

As depicted in Fig. 1A-C, feeding mice HFrHFD vs. SCD for 16 weeks significantly increased body weight gain % (41%, **Fig. 1A**), energy intake, (15.8%, **Fig. 1C**), and decreased food intake (41.6%, **Fig. 1B**).

Carvedilol acutely reduces fasting blood glucose levels and modulates hepatic lipid signaling

Feeding mice HFrHFD for 16 weeks significantly increased fasting blood glucose (24.5%, **Fig. 1D**), and hepatic DAG (332%, **Fig. 1F**), and decreased hepatic PIP2 (39%, **Fig. 1E**) levels compared to SCD-control group. On the other hand, carvedilol significantly decreased fasting blood glucose levels by 13% and 13.6% compared to SCD-control and HFrHFD-control groups, respectively (**Fig. 1D**). In addition, carvedilol significantly increased hepatic PIP2 levels (44.7% and 51.7%, **Fig. 1E**) compared to SCD-control and HFrHFD-control groups, respectively. Moreover, carvedilol significantly decreased hepatic DAG levels (38%, **Fig. 1F**) compared to HFrHFD-control group.

Carvedilol acutely maintains glucose homeostasis in SCD- and HFrHFD-fed mice

Feeding mice HFrHFD for 16 weeks significantly increased blood glucose levels (24% and 15%, **Fig. 2A**) after 0 and 60 minutes of glucose load compared to SCD-control group. On the other hand, carvedilol significantly decreased blood glucose level (13% and 13.6%, **Fig. 2A**) at 0 minute compared to SCD-control and HFrHFD-control groups respectively. In addition, carvedilol significantly increased blood glucose level (19.6%, **Fig. 2A**) after 60 minutes of glucose load compared to SCD-control group. No significant changes were observed in the area under the curve of the IPGTT (AUC of IPGTT) among all groups.

Carvedilol chronically ameliorates insulin resistance in HFrHFD-fed mice

Feeding mice HFrHFD significantly increased body weights (13% and 22%, **Fig. 3A**) after 7 and 10 weeks respectively compared to SCD group. No significant changes were observed in the visceral fat weights among studied groups (**Fig. 3B** and **3C**). On the other hand, feeding mice HFrHFD for 16 weeks significantly increased fasting blood

glucose levels (17.9%, **Fig. 4A**), serum insulin levels (611%, **Fig. 4B**) and HOMA-IR index values (1000%, **Fig. 4C**) compared to SCD group. In the same context, compared to HFrHFD-fed mice given vehicle, HFrHFD-fed mice given carvedilol, significantly decreased serum insulin levels (75%, **Fig. 4B**) and HOMA-IR index values (79%, **Fig. 4C**).

Carvedilol improves lipid profile in HFrHFD-fed mice

Feeding mice HFrHFD for 16 weeks significantly increased serum levels of TGs (112%, n=5, **Fig. 5A**), TC (85%, **Fig. 5B**), HDL-C (79%, **Fig. 5C**), and LDL-C (87%, **Fig. 5D**) compared to SCD-fed mice. Also, carvedilol-treated HFrHFD-fed mice showed significant decreases in serum TGs (26.8% decrease, **Fig. 5A**), TC (32.4% decrease, **Fig. 5B**), and LDL-C (28.5% decrease, **Fig. 5D**) compared to vehicle-treated HFrHFD-fed mice.

Carvedilol upregulates β -arrestin2 and its down-stream signals in the classical insulin target tissues, particularly in the liver

Feeding mice HFrHFD for 16 weeks significantly decreased β -arrestin2 level in skeletal muscle (56%, **Fig. 6A**), liver (78%, **Fig. 6A**), and adipose tissue (82%, **Fig. 6A**) compared to SCD-fed mice. Furthermore, compared to SCD-fed mice, HFrHFD-fed mice exhibited significantly decreased AKT phosphorylation at serine 473 in skeletal muscle (68%, **Fig. 6B**), liver (53%, **Fig. 6B**), and adipose tissue (90%, **Fig. 6B**). In the same context, compared to the SCD, the HFrHFD significantly decreased PIP2 levels in liver (35%, **Fig. 7A**) and adipose tissue (49%, **Fig. 7A**). However, compared to the SCD group, the HFrHFD group exhibited significantly increased DAG levels in skeletal muscle (181%, **Fig. 7B**), liver (41%, **Fig. 7B**), and adipose tissue (227%, **Fig. 7B**).

Treatment of HFrHFD-fed mice with carvedilol for 4 weeks significantly increased β -arrestin2 level in skeletal muscle (by 266%, **Fig. 6A**), liver (by 824%, **Fig. 6A**) and adipose tissue (by 709%, **Fig. 6A**) compared to the vehicle-treated mice. In addition, the HFrHFD + CARV group exhibited significantly increased AKT phosphorylation at serine 473 in skeletal muscle (434%, **Fig. 6B**), liver (340%, **Fig. 6B**), and adipose tissue (1800%, **Fig. 6B**) compared to the HFrHFD group. On the other hand, the HFrHFD + CARV group exhibited significantly increased PIP2 levels in the liver (40%, **Fig. 7A**) but significantly decreased DAG levels in skeletal muscle (26.6% decrease, **Fig. 7B**), liver (65% decrease, **Fig. 7B**), and adipose tissue (33.6% decrease, **Fig. 7B**) compared to the HFrHFD group.

Changes in AKT phosphorylation were highly correlated with the changes in β -arrestin2 level in all examined tissues ($r=0.93$, $P<0.001$), in contrast to both PIP2 ($r=0.49$, $P<0.01$) and DAG ($r=0.52$, $P<0.01$) levels.

Discussion

Sedentary lifestyles and increased consumption of fast food have markedly increased the worldwide prevalence of insulin resistance and type 2 diabetes to critical levels that pose high economic burdens on governments (**Muoio and Newgard, 2006**). Several clinical studies have shown interesting beneficial effects of carvedilol on glucose homeostasis (**Sica, 2005; Basat et al., 2006**). In the present study, we investigated the acute effects of carvedilol on glucose homeostasis and hepatic lipid signaling in SCD- and HFrHFD-fed mice. Moreover, we investigated the chronic effects of carvedilol on insulin resistance and dyslipidemia in HFrHFD-fed mice focusing on the changes in the

276 β -arrestin2 pathway in the classical insulin target tissues such as skeletal muscle, liver,
277 and adipose tissue.

278 In accordance with the results of previous studies (**Matthews et al., 1985; Ho Helen et**
279 **al., 1987; Panchal et al., 2011**), our results showed that feeding mice an HFrHFD for 16
280 weeks did not change body weight or visceral adiposity. However, we reported
281 significant increases in the body weights in HFrHFD-fed mice after 7 and 10 weeks
282 compared to SCD-fed mice. Moreover, the body weight of HFrHFD-fed mice was
283 markedly higher after 12 weeks ($P = 0.05$) compared to SCD-fed mice. In addition,
284 feeding mice HFrHFD for 16 weeks significantly increased FBG levels, serum insulin
285 levels and HOMA-IR index values.

286 Notably, saturated fatty acids in the HFrHFD can activate free fatty acid (FFA)
287 receptors 1 and 4, respectively (**Itoh et al., 2003; Basciano et al., 2005; Hara et al.,**
288 **2011**). FFA receptor 1 and 4 are $G_{\alpha q}$ protein-coupled receptors that promote PLC
289 activation and subsequent PIP2 breakdown into DAG and IP3 upon their induction
290 (**Talukdar et al., 2011**). In addition, previous studies have shown that chronic
291 consumption of fructose or saturated fats is associated with increased TG, TC, and LDL-
292 C levels (**Ho Helen et al., 1987; Holland et al., 2007**), which are similar findings to the
293 results of the current study.

294 In the same context and in accordance with previous studies, the current work showed
295 that feeding mice an HFrHFD compared to an SCD for 16 weeks significantly decreased
296 β -arrestin2 levels in skeletal muscle, liver, and adipose tissue (**Luan et al., 2009**).
297 Consistent with the increased insulin resistance, changes in β -arrestin2 levels, and
298 overactivation of the FFAR/ $G_{\alpha q}$ protein/PLC pathway (**Itoh et al., 2003; Hirasawa et**

al., 2008), feeding mice an HFrHFD for 16 weeks significantly decreased PIP2 levels in liver and adipose tissues and AKT phosphorylation at serine 473 in all examined classical insulin target tissues, while significantly decreased DAG levels.

Carvedilol is a third generation nonselective β - and α 1-blocker with β -arrestin-biased agonistic activity (Wisler et al., 2007). The use of traditional β -blockers in type 2 diabetic patients is not recommended due to acute hyperglycemic effects secondary to inhibition of insulin secretion (Kjekshus et al., 1990), and masking signs of hypoglycemia in diabetic patients receiving insulin (Tremblay et al., 2001). Moreover, long-term use of β -blockers is sometimes associated with impaired glucose homeostasis (Groop et al., 1983). However, vasodilatory β -blockers, such as carvedilol, have been found to improve glucose homeostasis in type 2 diabetic patients (Sica, 2005; Basat et al., 2006). Notably, no previous study has examined the role of β -arrestin2 pathway in mediating the in-vivo insulin sensitizing effect of carvedilol. Moreover, no previous study has examined the in-vivo acute effects of carvedilol on glucose homeostasis. For safe use of carvedilol in type 2 diabetic patients, it should at least maintain glucose homeostasis acutely and chronically. Therefore, the current study investigated both the acute and chronic metabolic effects of carvedilol.

In the present study, carvedilol significantly decreased FBG levels in both SCD- and HFrHFD-fed mice after 30 minutes of intraperitoneal injection. This effect was associated with significant increase in hepatic PIP2 levels in both SCD- and HFrHFD-fed mice and significant decrease in hepatic DAG level in HFrHFD-fed mice only. These findings are consistent with the insulin sensitizing effects of carvedilol and its β -arrestin biased agonistic activity. β -Arrestins promote the production of PIP2 by mediating the

interactions between phosphoinositides and phosphatidylinositol 4-phosphate 5-kinase $I\alpha$ (PIP5KI α) (Nelson et al., 2008). In the same context, β -arrestins mediate degradation of DAG into phosphatidic acid by activation of DAG kinase (Jean-Charles et al., 2017). The non-significant effect of carvedilol on hepatic DAG level in SCD-fed mice may be attributed to the low basal level of DAG in SCD-fed mice compared to its level in HFrHFD-fed mice.

On the other hand, carvedilol transiently increased the blood glucose level after 60 minutes of glucose load compared to SCD-control group but not HFrHFD-control group. This is consistent with the inhibitory effect of β -blockers on insulin secretion (Kjekshus et al., 1990). However, no significant changes were observed in the AUC of the IPGTT in all studied groups. This confirms that carvedilol can acutely maintain glucose homeostasis in insulin resistant subjects.

In the same context, administration of carvedilol to HFrHFD-fed mice for 4 weeks significantly reduced serum insulin levels and HOMA-IR index values compared to vehicle administration. These findings are compatible with previous clinical reports describing insulin-sensitizing effects of carvedilol (Sica, 2005; Basat et al., 2006). In addition, our results are in harmony with Nguyen et al. (2019) (Nguyen et al. (2019), who reported that feeding mice HFD increased circulating catecholamines levels leading to adrenergic overdrive. On the other hand, the authors reported that treating mice with carvedilol could block this effect and improve glucose tolerance and insulin sensitivity.

Carvedilol administration to HFrHFD-fed mice also significantly improved all lipid profile parameters. This observation is consistent with previous findings (Sharp et al., 2008). In harmony with these changes, carvedilol administration to HFrHFD-fed mice

significantly elevated PIP2 levels in the liver as well as β -arrestin2 and AKT phosphorylation at serine 473 in all examined classical insulin target tissues. On the other hand, carvedilol significantly decreased DAG levels in skeletal muscles, liver and adipose tissue.

Noteworthy, changes in AKT phosphorylation were highly correlated with the changes in β -arrestin2 level in all examined tissues, in contrast to both PIP2 and DAG levels which showed some deviations. These findings support the direct regulatory effect for β -arrestin2 on AKT phosphorylation (Luan et al., 2009). Moreover, carvedilol significantly increased both β -arrestin2 level and AKT phosphorylation at serine 473 in all examined tissues compared to SCD-fed mice. These findings support, at least in part, a potential role for the β -arrestin2/AKT pathway in mediating the insulin sensitizing effect of carvedilol rather than an indirect effect secondary to improved insulin resistance. In the last case, β -arrestin2 level and AKT phosphorylation should not exceed the normal level in SCD-fed mice as observed in the current study. On the other hand, the higher effect observed in the liver compared to other tissues may be attributed to higher expression level of β 2-adrenergic receptor in the liver than other tissues (McNeel and Mersmann, 1999).

The role of β -adrenergic receptors in glucose homeostasis is highly controversial. Activation of β 2-adrenergic receptors in skeletal muscles enhances glucose uptake in contrast to those in the liver and adipose tissues which mediate glycogenolysis, gluconeogenesis and lipolysis (Arner, 1992; Hunt and Ivy 2002; Erraji-Benchekroun et al., 2005; Ghosh et al., 2012). However, administration of β -agonists acutely induces systemic insulin resistance, while chronically ameliorating it (Sato et al., 2014; Hoff and

Koh, 2018). These effects were attributed to a shift of receptor coupling with G α S-protein during acute stimulation into coupling with G α i-protein during chronic stimulation (**Lefkowitz, 1998**). On the other hand, systemic administration of β -blockers acutely induces hyperglycemia secondary to inhibition of insulin secretion and chronically impairs insulin action (**Groop et al., 1983; Kjekshus et al., 1990**). Distinct from β -agonists and traditional β -blockers, the current study showed unique acute and chronic systemic metabolic effects for the vasodilatory β -blockers that possess β -arrestin biased agonistic activity.

In conclusion, to the best of our knowledge, the present study reports several findings for the first time. First, carvedilol at a dose of 10 mg/kg acutely maintains glucose homeostasis in both normal and insulin resistant mice and chronically ameliorates insulin resistance and dyslipidemia in HFrHFD-fed mice. Second, the carvedilol-insulin-sensitizing effects are highly correlated with the upregulation of the β -arrestin2 pathway in the classical insulin target tissues which may reflect promising insulin sensitizing actions for β -arrestin biased agonists (**Fig. 8**). Third, the use of carvedilol for cardiovascular complications in type 2 diabetic patients is safe and beneficial on both acute and chronic levels.

Author contributions AM, MM, and II: designed the study; HA, SM, WI, and AR: performed the experiments and collected the data; AM, MM, and II: analyzed the data; AM, MM, and II wrote the paper.

All authors approved the final manuscript and submission.

Funding No funding sources

Compliance with ethical standards

Conflicts of interest

The authors declare no conflicts of interest.

References

- Abdulla, M.H., Sattar, M.A., Abdullah, N.A., Khan, M.A.H., Anand Swarup, K.R.L., Rathore, H.A., Ibraheem, Z.O. and Johns, E.J., 2010. Effect of carvedilol and losartan on renal responses to ang ii and adrenergic agonists in fructose-fed rat. *FASEB J.* 605-8.
- Arner P, Engfeldt P, Hellström L, Lönnqvist F, Wahrenberg H, Sonnenfeld T, et al. (1990). β -adrenoreceptor subtype expression in human liver. *J Clin Endocrinol Metab*, 71(5), 1119-1126.
- Basat, O., Ucak, S., Seber, S., Oztekin, E. and Altuntas, Y., 2006. After myocardial infarction carvedilol improves insulin resistance compared to metoprolol. *Clin Res Cardiol.* Feb;95(2):99-104.
- Basciano H., Federico L., Adeli K., 2005. Fructose, insulin resistance, and metabolic dyslipidemia. *Nutr Metab.* 2(1):5.
- Deisl, C., Anderegg, M., Albano, G., Lüscher, B.P., Cerny, D., Soria, R., Bouillet, E., Rimoldi, S., Scherrer, U. and Fuster, D.G., 2016. Loss of sodium/hydrogen exchanger NHA2 exacerbates obesity-and aging-induced glucose intolerance in mice. *PloS one.* 11(9).
- DeWire, S.M., Ahn, S., Lefkowitz, R.J. and Shenoy, S.K., 2007. β -arrestins and cell signaling. *Annu Rev Physiol.* 69:483-510.
- Erraji-Benchekroun L, Couton D, Postic C, Borde I, Gaston J, Guillet J-G, et al. (2005). Overexpression of β 2-adrenergic receptors in mouse liver alters the expression of

- 414 gluconeogenic and glycolytic enzymes. *Am J Physiol Endocrinol Metab*, 288(4),
415 E715-E722.
- 416 Fox, C.S., Coady, S., Sorlie, P.D., Levy, D., Meigs, J.B., D'Agostino, R.B., Wilson, P.W.
417 and Savage, P.J., 2004. Trends in cardiovascular complications of diabetes. *JAMA*.
418 292(20):2495-9.
- 419 Friedewald W.T., Levy R.I., Fredrickson D.S., 1972. Estimation of the concentration of
420 low- density lipoprotein cholesterol in plasma, without use of the preparative
421 ultracentrifuge. *Clin Chem*. 18(6):499-502.
- 422 Frishman W.H., 1998. Carvedilol. *N Engl J Med*. 339(24):1759-65.
- 423 Gast, K.B., Tjeerdema, N., Stijnen, T., Smit, J.W. and Dekkers, O.M., 2012. Insulin
424 resistance and risk of incident cardiovascular events in adults without diabetes: meta-
425 analysis. *PLoS ONE*. 7(12).
- 426 Ghosh PM, Shu Z-J, Zhu B, Lu Z, Ikeno Y, Barnes JL, et al. 2012. Role of β -adrenergic
427 receptors in regulation of hepatic fat accumulation during aging. *J endocrinol*,
428 213(3), 251.
- 429 Groop L, Tötterman K, Harno K, & Gordin A., 1983. Influence of Beta-Blocking Drugs
430 on Glucose Metabolism in Hypertensive, Non-Diabetic Patients. *Acta med Scand*
431 213(1), 9-14.
- 432 Hara, T., Hirasawa, A., Ichimura, A., Kimura, I. and Tsujimoto, G., 2011. Free fatty acid
433 receptors FFAR1 and GPR120 as novel therapeutic targets for metabolic disorders. *J*
434 *Pharm Sci*. 100(9):3594-601.
- 435 Hirasawa, A., Hara, T., Katsuma, S., Adachi, T. and Tsujimoto, G., 2008. Free fatty acid
436 receptors and drug discovery. *Biol Pharm Bull*. 31(10):1847-51.

- 437 Ho HELEN, Hoffman B.B., Reaven G.M., 1987. Fructose-induced insulin resistance and
438 hypertension in rats. *Hypertension*. 10(5):512-6.
- 439 Hoff R, Koh C-K, 2018. Isoproterenol induced insulin resistance leading to diabetic
440 ketoacidosis in type 1 diabetes mellitus. *Case Rep Endocrinol*, 2018.
- 441 Holland, W.L., Knotts, T.A., Chavez, J.A., Wang, L.P., Hoehn, K.L. and Summers, S.A.,
442 2007. Lipid mediators of insulin resistance. *Nutr Rev*. 65:S39-46.
- 443 Hunt DG, Ivy JL., 2002. Epinephrine inhibits insulin-stimulated muscle glucose
444 transport. *J. Appl. Physiol*, 93(5), 1638-1643.
- 445 Ibrahim I.A.H., Nakaya M., Kurose H., 2013. Ezrin, Radixin, and Moesin
446 Phosphorylation in NIH3T3 Cells Revealed Angiotensin II Type 1 Receptor Cell-
447 Type-Dependent Biased Signaling. *J Pharmacol Sci*. 122(1):1-9.
- 448 Ibrahim, W.S., Ibrahim, I.A.H., Mahmoud, M.F. and Mahmoud, A.A., 2020. Carvedilol
449 Diminishes Cardiac Remodeling Induced by High-Fructose/High-Fat Diet in Mice
450 via Enhancing Cardiac β -Arrestin2 Signaling. *J Cardiovasc Pharmacol Ther*. 25(4),
451 pp.354-363.
- 452 Itoh, Y., Kawamata, Y., Harada, M., Kobayashi, M., Fujii, R., Fukusumi, S., Ogi, K.,
453 Hosoya, M., Tanaka, Y., Uejima, H. and Tanaka, H., 2003. Free fatty acids regulate
454 insulin secretion from pancreatic β cells through GPR40. *Nature*. 422(6928):173-6.
- 455 Jauncey, K. and Ross, B., 1982. A guide to tilapia feeds and feeding. Institute of
456 Aquaculture, University of Stirling, Stirling.
- 457 Jean-Charles, P.Y., Kaur, S. and Shenoy, S.K., 2017. GPCR signaling via β -arrestin-
458 dependent mechanisms. *J Cardiovasc Pharmacol*, 70(3), p.142.

- 459 Kjekshus, J., Gilpin, E., Cali, G., Blackey, A.R., Henningj, H. and Ross Jr, J., 1990.
460 Diabetic patients and beta-blockers after acute myocardial infarction. *Eur Heart J*.
461 11(1):43-50.
- 462 Lee S.B., Rhee S.G., 1995. Significance of PIP2 hydrolysis and regulation of
463 phospholipase C isozymes. *Curr Opin Cell Biol*. 7(2):183-9.
- 464 Lefkowitz R.J., 1998. G protein-coupled receptors III. New roles for receptor kinases and
465 β -arrestins in receptor signaling and desensitization. *J Biol Chem*. 273(30):18677-80.
- 466 Li, T., Ke, W., Sun, P., Chen, X., Belgaumkar, A., Huang, Y., Xian, W., Li, J. and Zheng,
467 Q., 2016. Carvedilol for portal hypertension in cirrhosis: systematic review with
468 meta-analysis. *BMJ open*. 6(5):e010902.
- 469 Luan, B., Zhao, J., Wu, H., Duan, B., Shu, G., Wang, X., Li, D., Jia, W., Kang, J. and Pei,
470 G., 2009. Deficiency of a β -arrestin-2 signal complex contributes to insulin
471 resistance. *Nature*. 457(7233):1146-9.
- 472 Malig, T.C., Ashkin, M.R., Burman, A.L., Barday, M., Heyne, B.J. and Back, T.G., 2017.
473 Comparison of free-radical inhibiting antioxidant properties of carvedilol and its
474 phenolic metabolites. *Med Chem Comm*. 8(3):606-15.
- 475 Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Treacher, D.F. and Turner,
476 R.C., 1985. Homeostasis model assessment: insulin resistance and β -cell function
477 from fasting plasma glucose and insulin concentrations in man. *Diabetologia*.
478 28(7):412-9.
- 479 McDonald, P.H., Chow, C.W., Miller, W.E., Laporte, S.A., Field, M.E., Lin, F.T., Davis,
480 R.J. and Lefkowitz, R.J., 2000. β -Arrestin 2: a receptor-regulated MAPK scaffold for
481 the activation of JNK3. *Science*. 290(5496):1574-7.

- 482 McNeel R.L., Mersmann H.J., 1999. Distribution and quantification of beta1-, beta2-, and
483 beta3-adrenergic receptor subtype transcripts in porcine tissues. *J Anim Sci.*
484 7(3):611-21.
- 485 Mohammed, S.G., Ibrahim, I.A.H., Mahmoud, M.F. and Mahmoud, A.A., 2019.
486 Carvedilol protects against hepatic ischemia/reperfusion injury in high-fructose/high-
487 fat diet-fed mice: Role of G protein-coupled receptor kinase 2 and 5. *Toxicol Appl*
488 *Pharm.* 382:114750.
- 489 Morsy, M.A., Ibrahim, S.A., Amin, E.F., Kamel, M.Y., Abdelwahab, S.A. and Hassan,
490 M.K., 2014. Carvedilol ameliorates early diabetic nephropathy in streptozotocin-
491 induced diabetic rats. *Biomed Res. Int.* 2014.
- 492 Muoio, D.M., Newgard, C.B., 2006. Obesity-related derangements in metabolic
493 regulation. *Annu Rev Biochem.* 75:367-401.
- 494 Nelson, C.D., Kovacs, J.J., Nobles, K.N., Whalen, E.J. and Lefkowitz, R.J., 2008. β -
495 arrestin scaffolding of phosphatidylinositol 4-phosphate 5-kinase $I\alpha$ promotes
496 agonist-stimulated sequestration of the β 2-adrenergic receptor. *J Biol Chem.*
497 283(30):21093-101.
- 498 Nguyen, L.V., Ta, Q.V., Dang, T.B., Nguyen, P.H., Nguyen, T., Pham, T.V.H., Nguyen,
499 T.H., Baker, S., Le Tran, T., Yang, D.J. and Kim, K.W., 2019. Carvedilol improves
500 glucose tolerance and insulin sensitivity in treatment of adrenergic overdrive in high
501 fat diet-induced obesity in mice. *PloS one.* 14(11):e0224674.
- 502 Panchal, S.K., Poudyal, H., Iyer, A., Nazer, R., Alam, A., Diwan, V., Kauter, K., Sernia,
503 C., Campbell, F., Ward, L. and Gobe, G., 2011. High-carbohydrate high-fat diet–

- 504 induced metabolic syndrome and cardiovascular remodeling in rats. *J Cardiovasc*
505 *Pharmacol.* 57(5):611-24.
- 506 Sato M, Dehvari N, Öberg AI, Dallner OS, Sandström AL, Olsen JM, et al., 2014.
507 Improving type 2 diabetes through a distinct adrenergic signaling pathway
508 involving mTORC2 that mediates glucose uptake in skeletal muscle. *Diabetes*,
509 63(12), 4115-4129.
- 510 Sawicki P, Siebenhofer A. Betablocker treatment in diabetes mellitus. *J intern med.*
511 2001;250(1):11-7.
- 512 Sharp R.P., Sirajuddin R., Sharief I.M., 2008. Impact of carvedilol on the serum lipid
513 profile. *Ann Pharmacother.* 42(4):564-71.
- 514 Sica D.A., 2005. Carvedilol: New Considerations for Its Use in the Diabetic Patient With
515 Hypertension. *J Clin Hypertens.* 7(1):59-64.
- 516 Talukdar S., Olefsky J.M., Osborn O., 2011. Targeting GPR120 and other fatty acid-
517 sensing GPCRs ameliorates insulin resistance and inflammatory diseases. *TRENDS*
518 *Pharmacol SCI.* 32(9):543-50.
- 519 Tremblay, F., Lavigne, C., Jacques, H. and Marette, A., 2001. Defective insulin-induced
520 GLUT4 translocation in skeletal muscle of high fat-fed rats is associated with
521 alterations in both Akt/protein kinase B and atypical protein kinase C (ζ/λ) activities.
522 *Diabetes.* 50(8):1901-10.
- 523 Ueki, K., Yamamoto-Honda, R., Kaburagi, Y., Yamauchi, T., Tobe, K., Boudewijn,
524 M.T., Coffer, P.J., Komuro, I., Akanuma, Y., Yazaki, Y. and Kadowaki, T., 1998.
525 Potential role of protein kinase B in insulin-induced glucose transport, glycogen
526 synthesis, and protein synthesis. *J Biol Chem.* 273(9):5315-22.

Wisler, J.W., DeWire, S.M., Whalen, E.J., Violin, J.D., Drake, M.T., Ahn, S., Shenoy, S.K. and Lefkowitz, R.J., 2007. A unique mechanism of β -blocker action: carvedilol stimulates β -arrestin signaling. *Proc Natl Acad Sci U S A*. 104(42):16657-62.

Wu Dianqing., Katz A., Simon M.I., 1993. Activation of phospholipase C beta 2 by the alpha and beta gamma subunits of trimeric GTP-binding protein. *Proc Natl Acad Sci U S A*. 90(11):5297-301.

Table 1: Components of standard chow diet (SCD) and high-fructose, high-fat diet (HFrHFD)

Figure legends

Fig. 1. Effect of high-fructose, high-fat diet (HFrHFD) on body weight gain, food intake and energy intake and acute effects of carvedilol on fasting blood glucose (FBG) level and hepatic lipid signaling. Graphical presentation of body weight gain (**A**), food intake (**B**), energy intake (**C**), FBG (**D**), phosphatidylinositol 4,5 bisphosphate (PIP2) (**E**) and diacylglycerol (DAG) (**F**). SCD: mice fed a standard chow diet for 16 weeks. HFrHFD: mice fed a high-fructose, high-fat diet for 16 weeks. CARV: mice treated with carvedilol (10 mg/kg, i.p.) for 30 minutes. Statistical analysis was performed using unpaired student's t-test and one-way ANOVA followed by Bonferroni post-test for selected pairs. The values are presented as the mean $\pm$ SEM. n= 3 cages (6 mice each) for body weight gain, food- and energy-intake; n=6 for FBG; n=3 for PIP2 and DAG. *, **, *** $P < 0.05$, 0.01, 0.001 respectively.

Fig. 2. Acute effect of carvedilol on glucose tolerance. Graphical presentation of intraperitoneal glucose tolerance test (IPGTT) (**A**), area under the curve of the IPGTT (AUC of IPGTT) (**B**). SCD: mice fed a standard chow diet for 16 weeks. HFrHFD: mice

550 fed a high-fructose, high-fat diet for 16 weeks. CARV: mice treated with carvedilol (10
551 mg/kg, i.p.) for 30 minutes. Statistical analysis was performed using one-way ANOVA
552 followed by Bonferroni post-test for selected pairs. The values are presented as the
553 mean $\pm$ SEM. n= 6. % $P<0.05$ vs. SCD group; @ $P<0.05$ vs. HFrHFD group; \$ $P<0.05$ vs.
554 SCD + CARV group.

555 **Fig. 3.** Chronic effect of carvedilol on body weight and visceral fat weight. Graphical
556 presentation of weekly changes in the body weights (**A**), visceral fat weight (**B**) and visceral
557 fat weight normalized to tibial length (**C**). SCD: mice fed a standard chow diet for 16
558 weeks. HFrHFD: mice fed a high-fructose, high-fat diet for 16 weeks. CARV: HFrHFD-
559 fed mice treated with carvedilol (10 mg/kg/day, i.p.) for 4 weeks starting at week 13.
560 Statistical analysis was performed using one-way ANOVA followed by Bonferroni post-
561 test for selected pairs. The values are presented as the mean $\pm$ SEM. n= 10 for weekly
562 changes in body weights, n=7-8 for visceral fat weight, and visceral fat weight normalized
563 to tibial length. * $P<0.05$.

564 **Fig. 4.** Chronic effect of carvedilol on insulin resistance. Graphical presentation of fasting
565 blood glucose (FBG) (**A**), serum insulin (**B**) and homeostasis model of assessment of
566 insulin resistance (HOMA-IR) (**C**). SCD: mice fed a standard chow diet for 16 weeks.
567 HFrHFD: mice fed a high-fructose, high-fat diet for 16 weeks. CARV: HFrHFD-fed mice
568 treated with carvedilol (10 mg/kg/day, i.p.) for 4 weeks starting at week 13. Statistical
569 analysis was performed using one-way ANOVA followed by Bonferroni post-test for
570 selected pairs. The values are presented as the mean $\pm$ SEM. n= 8 for FBG, n= 5 for serum
571 insulin and HOMA-IR. *, *** $P<0.05$, 0.001 respectively.

572 **Fig. 5.** Changes in lipid profiles. Quantitative analyses of serum triglycerides (**A**), serum

total cholesterol (**B**), serum high-density lipoprotein cholesterol (HDL-C) (**C**), and serum low-density lipoprotein cholesterol (**D**) were performed. SCD: mice fed a standard chow diet for 16 weeks. HFrHFD: mice fed a high-fructose, high-fat diet for 16 weeks. CARV: HFrHFD-fed mice treated with carvedilol (10 mg/kg/day, i.p.) for 4 weeks starting at week 13. Statistical analysis was performed using one-way ANOVA followed by Bonferroni post-test for selected pairs. The values are presented as the mean $\pm$ SEM. $n=5$. **, *** $P<0.01$, 0.001 respectively.

Fig. 6. Changes in β -arrestin2 and phosphoserine 473 of protein kinase B (AKT). Quantitative analysis of β -arrestin2 (**A**), and phosphoserine 473 of AKT (**B**). SCD: mice fed a standard chow diet for 16 weeks. HFrHFD: mice fed a high-fructose, high-fat diet for 16 weeks. CARV: HFrHFD-fed mice treated with carvedilol (10 mg/kg/day, i.p.) for 4 weeks starting at week 13. Statistical analysis was performed among groups within each tissue using one-way ANOVA followed by Bonferroni post-test for selected pairs. The values are presented as the mean $\pm$ SEM. $n=3$. *** $P<0.001$.

Fig. 7. Changes in phosphatidyl inositol 4,5 biphosphate (PIP2) and diacylglycerol (DAG). Quantitative analysis of PIP2 (**A**), and DAG (**B**). SCD: mice fed a standard chow diet for 16 weeks. HFrHFD: mice fed a high-fructose, high-fat diet for 16 weeks. CARV: HFrHFD-fed mice treated with carvedilol (10 mg/kg/day, i.p.) for 4 weeks starting at week 13. Statistical analysis was performed among groups within each tissue using one-way ANOVA followed by Bonferroni post-test for selected pairs. The values are presented as the mean $\pm$ SEM. $n=4$. *, **, *** $P<0.05$, 0.01 , 0.001 respectively.

Fig. 8. Carvedilol ameliorates insulin resistance and upregulates β -arrestin2 pathway in the classical insulin target tissues

596 **Fig. S1.** Timeline of the experiment. IPGTT: Intraperitoneal glucose tolerance test; SCD:
597 Standard chow diet; HFrHFD: High-fructose high-fat diet.

Draft

Table 1:

Component	SCD	HFrHFD
Carbohydrate	60%	46%
Protein	22%	18%
Fat	2.5%	24%
Others (fibers, minerals, etc)	15.5%	12%

Draft

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8