Overweight in mice, induced by perinatal programming, exacerbates doxorubicin and trastuzumab cardiotoxicity

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ABSTRACT

Purpose: Trastuzumab (TRZ) is believed to potentiate doxorubicin (DOX) cardiotoxicity, resulting in left ventricular dysfunction. There is some evidence that overweight could influence anticancer drug-induced cardiotoxicity, though no study has evaluated the impact of moderate overweight, induced by postnatal nutritional programming, on the cardiotoxic effects of DOX alone or in combination with TRZ.

Methods: Immediately after birth, litters of C57BL/6 mice were either maintained at 9 pups (normal litter, NL), or reduced to 3 (small litter, SL) in order to induce programming of ~15% overweight through postnatal overfeeding. At 4 months, NL and SL mice received a single intraperitoneal injection of either saline, DOX (6 mg/kg), or TRZ (10 mg/kg) or both (DOX-TRZ). Transthoracic echocardiography was performed 24 hours before as well as 10 and 20 days after treatments.

Results: Twenty days after DOX administration, systolic dysfunction was observed only in the overweight SL group, while NL mice group had a normal left ventricular ejection fraction (LVEF). However, in the NL group, functional impairment appeared when TRZ was co-administered. Forty-eight hours after drug administration, gene expression of natriuretic peptides (ANP, BNP) appeared to be potentiated in DOX-TRZ mice of both the NL and SL group, whereas the expression of β-MHC increased significantly in overweight-SL mice only.

Conclusions: In an acute model of DOX cardiotoxicity, moderately overweight adult mice were more sensitive to cardiac systolic impairment. Moreover, our results confirm the potentiating action of TRZ on DOX-induced cardiotoxicity in lean mice.

Keywords: Doxorubicin, trastuzumab, cardiotoxicity, overweight, postnatal programming

1. Introduction

Doxorubicin (DOX, Adriamycin®) is an anthracycline with a broad spectrum of activity in oncological practice [1]; however, its use is limited by its dose-dependent cardiotoxicity, which may result in both acute and chronic cardiotoxic effects [2,3]. Following the administration of anthracyclines in the adjuvant settings of breast cancer, trastuzumab (TRZ), a humanized monoclonal antibody against the human epidermal growth factor receptor 2 (HER 2) protein [4], is effective in patients that overexpress this receptor. The association of TRZ and chemotherapy provides a clinical benefit in terms of recurrence-free survival and overall survival compared with chemotherapy alone [5-8]. Nonetheless, while preclinical studies did not reveal cardiotoxicity [9], later clinical studies showed that treatment with TRZ led to an unexpected incidence of cardiac side effects [10]. The most frequent effect was reduced systolic ventricular function, which was asymptomatic or associated with heart failure [11].

In fact, clinical trials have shown a greater incidence of cardiac dysfunction when anthracycline chemotherapy was associated with TRZ than with chemotherapy alone [10] and TRZ is now believed to potentiate the cardiotoxic effects of anthracyclines [6,7,12]. In *in vivo* murine models that combined DOX with TRZ, a synergetic interaction leading to deterioration in cardiac function [13] and to an increase in cardiomyocyte apoptosis was reported [14,15]. In addition, *in vitro* in cardiomyocytes, the blockage of HER2 was accompanied by apoptotic processes and by an increase in the level of oxidative stress possibly through mitochondrial and reactive oxygen species-dependent pathways [16,17].

Several factors may increase the incidence of cancer in overweight and obese individuals [18]. Although it has been reported for many years that patients with metabolic syndrome have a higher risk of cardiovascular disease [19], their susceptibility to chemotherapy-induced cardiac disease has only recently been documented. In fact, few studies have shown that obese patients [20] or rats fed with a high-lipid diet [21] are more sensitive to the cardiotoxic effects of anthracyclines. Moreover,

obesity has recently been associated with a poor outcome in breast cancer patients treated with DOX-based chemotherapy [22,23], a finding that could in part be related to greater sensitivity to the side-effects of anticancer agents. Therefore, it is important to explore more deeply the influence of overweight and obesity as aggravating factors in the cardiotoxic effects of anticancer drugs, particularly when several types of chemotherapy are combined.

We recently demonstrated that overfeeding during the immediate postnatal period in mice induced perinatal programming of cardio-metabolic risk. Indeed, postnatal overfeeding not only induced permanent moderate overweight, but also, during adulthood, increased blood pressure, glucose intolerance and insulin-resistance [24]. This rodent model, which presents several features of mild metabolic syndrome, is also characterized by greater susceptibility to myocardial ischemia-reperfusion injury [25,26]. Therefore, this model is potentially relevant to study the impact of overweight on heart sensitization to the cardiotoxic effects of anticancer drugs, and particularly the association of anthracyclines with TRZ.

Thus, the aim of our study was to evaluate in mice the role of overweight in the cardiac impact of acute DOX treatment associated or not with TRZ.

2. Material and methods

2.1. Animals

Adult female C57BL/6 mice (Charles River, L'Arbresle, France) were mated with male mice, then housed in individual cages and given water and standard chow *ad libitum* during pregnancy and lactation. On day-2 after birth, male pups were randomly distributed among the mothers and the litter size was adjusted to 9 pups (normal litter, NL group), or reduced to 3 pups in order to induce postnatal-overfeeding (small litter, SL group) as previously described [25]. The investigation was approved by the local ethics committee (Comité d'Ethique de l'Expérimentation Animale, Université- Bourgogne-Franche-Comté, Dijon, France, protocol agreement number: 00412.03). Throughout the procedure, care was taken to avoid suffering and to ensure animal welfare.

2.2. Treatments

At 4 months of age, considered as adulthood for mice, control groups originating from NL and overweight groups from SL were subjected to different types of treatment.

A preliminary study was conducted to determine the acute dose of DOX that could impair cardiac contractile function in both groups of mice. For this purpose, DOX (Sigma-Aldrich, St Louis, MO) was tested at 0, 6 and 10 mg/kg (intra-peritoneal (IP) single injection) in 29 NL and 28 SL mice. These low-doses of DOX were chosen because of their mild impact on mortality, which is the case for doses up to 15 mg/kg [27]. All mice underwent transthoracic echocardiography and our results showed that in mice treated with 6 mg/kg DOX, the impairment of systolic function appeared only in overweight SL mice. At 10 mg/kg, DOX induced systolic dysfunction at D10 and D20 in all groups. Therefore, in order to determine the possible deleterious interaction between DOX and TRZ, the dose of 6 mg/kg of DOX was chosen for the following experiments.

In order to assess the impact of overweight on the development of cardiotoxicity by DOX, TRZ or their association, 74 NL mice and 79 SL mice aged 4 months received at D1 a single IP injection of

either saline solution (NaCl 0.9%), or DOX (6 mg/kg), or TRZ (10 mg/kg [14,27,28]) or DOX (6 mg/kg) + TRZ (10 mg/kg). Thus, 8 groups were constituted (Figure 1).

Body weight was measured at baseline, before each echocardiography and before sacrifice. Transthoracic echocardiography was performed 24 hours before (day 0, D0), ten (D10) and twenty (D20) days after the treatments. A subset of 6 mice per group was euthanized 48 hours after the injections (D3), in order to evaluate early circulating and cardiac modifications. At the end of the protocol (D20), all of the animals were euthanized after anaesthesia under pentobarbital (60 mg/kg, IP); blood and tissue were harvested, immediately frozen in liquid nitrogen and stored at -80 C until further analysis.

2.3. Transthoracic echocardiographic imaging

Transthoracic echocardiography was performed using the Vevo770™ imaging system (VisualSonics Inc., Toronto, Canada), equipped with a 30 MHz probe as described previously [29]. Briefly, mice were anesthetized with isoflurane and their body temperature was maintained at 37±0.5°C. An ECG signal was also monitored. The ultrasound probe (RMV-707B) was placed on the mouse's chest. Two-dimensional images were recorded in the parasternal long- and short-axis views. Pulsed Doppler studies of LV diastolic function were performed in the apical 4-chamber view with the Doppler cursor oriented parallel to the long-axis plane of the left ventricle.

2.4. Quantitative measurement of BNP and cTnI

Plasma brain natriuretic peptide (BNP) and cardiac troponin I (cTnI) levels were determined with sandwich enzyme immunoassays (Uscn, Life science Inc) on the VICTOR3-V multilabel reader (Wallac-PerkinElmer Life Sciences, Courtaboeuf, France) according to the instructions of the manufacturer (Cloud-Clone Corp., Houston, USA).

2.5. Myocardial gene expression

Total RNA was extracted from frozen heart samples and real-time quantitative polymerase chain reaction (RT-PCR) was performed as described before [25]. Cardiac mRNA levels of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and beta-myosin heavy chain (β-MHC) were normalized against the housekeeping hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene expression. The primers used for the amplification of mouse genes are provided in Table 1.

2.6. Statistical analysis

Continuous data were expressed as means ± standard deviations (SD) and dichotomous data as numbers (percentages). For continuous variables, the normality of distribution was tested by the Shapiro-Wilk test. For body weight and echocardiographic data, values were compared using 2-way ANOVA for repeated measures followed by a Tukey post-hoc analysis. Because of the presence of two factors in treatment groups (SL vs NL and the treatment administered), all other continuous data were compared using 2-way ANOVA. This allowed the analysis of the effects of TRZ and DOX, and the potential interaction of these two factors. Post-hoc analyses were done with Tukey test if appropriate.

3. Results

3.1. Mice body and heart weight

At D0, aged 4 months, SL mice were almost 14% heavier than NL mice (32.6 g *vs.* 28.8 g, $p<0.001$) and this difference persisted throughout the duration of the experiment (Figure 2).

The administration of DOX, alone or in combination with TRZ, induced a transient decrease in body weight at D10 in the SL groups but not in the NL groups (Figure 2B and 2D).

In control groups, there was a significant reduction of the ratio of left ventricular weight to body weight (Table 2) at any time point in SL groups as compared to NL, due to the fact that body weight was increased, but that heart weight was not significantly modified. No changes were induced by DOXO or TRZ alone between DO and D10 and/or D20. However, a significant increase in the ratio was observed at D 20 in both the NL and SL groups, revealing myocardial hypertrophy.

3.2. Cardiac function in vivo

The echocardiographic measurements are sumarized in Table 3.

At D0, the left ventricular ejection fraction (LVEF) in NL groups was no different from that in SL groups (Table 3). LVEF remained unchanged over time in CONT saline-treated animals (Figure 3A). DOX did not modify the LV systolic function in the NL group (NL DOX) at D10 or D20 (Figure 3B). However, in the SL DOX group, there was a significant decrease in LVEF at D10 (49%±7 *vs.* 61%±5), which persisted at D20 (47%±4). TRZ alone did not alter LVEF in either the NL or SL groups (Figure 3C). The administration of a combined treatment with DOX and TRZ modified LVEF in both groups: at D10, there was a significant decrease in LVEF only in the SL DOX-TRZ (49%±4 vs 58%±3) group but at D20 (48%±5), the systolic function was similarly affected in both NL and SL groups. At D20, LVEF values in the NL DOX-TRZ group were significantly lower than those in the NL DOX group (52%±6 vs 58%±3, $p<0.01$) whereas there was no significant difference between the SL DOX-TRZ and SL DOX groups.

In the DOX and DOX-TRZ groups, left ventricular diameter (LVEDD) in SL mice was higher than that in NL mice at D10 and D20 ($p<0.005$), suggesting more severe cardiac alteration.

In the present study, we did not observe the development of an abnormal diastolic function profile for mice treated with DOX and TRZ (E/A or E/E').

3.3. Quantitative measurement of plasma cTnI and BNP

Cardiac troponin I and BNP levels were measured in blood plasma collected by intra-cardiac puncture at D3 and D20. These results showed low or undetectable levels that were similar in all groups of mice (data not shown).

3.4. Myocardial expression of genes involved in remodelling and heart failure

The mRNA expression of BNP, ANP and β-MHC were measured in myocardial tissue harvested 48 hours after treatment (D3) or at the end of the experimental protocol (D20).

Our result showed early but not late expression of these genes. At D3, myocardial expression of ANP and BNP was significantly higher in NL and SL groups treated with the combination of DOX and TRZ than in the untreated groups (Figure 4A and 4B). The combined treatment induced significantly higher ANP gene expression than did DOX or TRZ alone (Figure 4B) in both SL and NL groups.

Conversely, an increase in β-MHC expression was only observed in overweight SL groups treated with the combination of DOX and TRZ, but not in lean NL groups nor in mice treated with DOX or TRZ alone (Figure 4C).

Discussion

The main objective of our study was to investigate the role of moderate overweight in mice in the cardiac consequences of DOX treatment, in combination or not with TRZ. The results clearly confirmed, through the evaluation of cardiac function with *in vivo* echocardiography, that overweight mice are more prone to DOX-induced cardiotoxicity than their lean counterparts, as they show earlier impairment of LVEF and increased ventricular diameters. TRZ potentiated the cardiac effects of DOX, as shown by higher heart-to-body weight, a marker of myocardial hypertrophy, a higher myocardial expression of genes involved in heart failure (ANP, BNP), and increased impairment of left ventricular function in lean NL mice. To our knowledge, this is the first study to evaluate, in a mouse model of postnatal programming of "cardio-metabolic risk", the consequences of anticancer drugs, and to show that overweight individuals are more sensitive to anthracycline cardiotoxicity.

One of the major findings of our study is that moderately overweight SL adult mice present a greater likelihood of developing cardiomyopathy with systolic dysfunction for very low doses of DOX (6 mg/kg), whereas normal weight NL mice did not. For the higher 10 mg/kg dose of DOX, impairment of left ventricular function was observed in all groups at D10 and D20. In our study, in order to acquire multiple echocardiographic images over time, we intentionally used low doses of DOX that did not cause death, but were still able to induce acute cardiac toxicity. As a comparison with the clinical situation, in the management of acute leukaemia, for which intensive combination chemotherapy schedules are employed, the recommended dose of doxorubicin is 2.4 mg/kg of body weight (approximately corresponding to 75-90 mg/m^2), to be administered divided over three consecutive days (one cycle).

The results presented here suggest that our murine model of moderate overweight, obtained by postnatal programming through over-nutrition during the suckling period, is highly sensitive to

DOX-induced cardiotoxicity. To the best of our knowledge, no previous study has shown that moderate overweight could aggravate DOX-mediated cardiotoxicity. Our results could be linked to those of Mitra *et al*., who reported evidence of increased DOX-cardiotoxicity in a rodent model of obesity [21]. In their work, male rats were obese (30% overweight) after 43-days of a 40% high-fat diet and cardiotoxicity was evaluated after a single IP dose of DOX (8 mg/kg). The results showed that while only 10% of the normal diet rats died after DOX administration, 80% of the obese rats died, thus exhibiting the increased sensitivity of obese rats to DOX-induced toxicity. Our study, using both functional and molecular cardiac assessments, supports their hypothesis and emphasizes the major role of overweight in DOX cardiotoxicity.

The mechanisms by which obesity is able to promote DOX-induced cardiotoxicity must be specified with further studies. In a study from our laboratory, we reported that overweight 6-month-old rodents had higher cardiac susceptibility to ischemia-reperfusion injury, increased cardiac oxidative stress and metabolic alterations such as increased leptin and decreased adiponectin levels [26]. From a clinical point of view, obesity is an established risk factor for several cancers [30]. Unfortunately, the occurrence of cardiac events during anthracycline-based chemotherapy has almost never been recorded in trials. Thus, clinical data are required to determine whether obese patients are more prone than non-obese patients to the cardiotoxicity of DOX or TRZ treatments.

Our results showed that TRZ could potentiate the cardiotoxicity induced by DOX, especially in the context of lean NL mice. Indeed, this finding was supported both by increased and earlier deterioration of systolic function and by early elevation of markers of heart failure and remodelling (ANP, BNP and β-MHC) in hearts of mice treated with a combination of TRZ and DOX. Walker *et al*. [27] evaluated over 10 days mice receiving a combination of DOX (20 mg/kg) and TRZ (10 mg/kg). Although the mortality rate for DOX-treated mice was high (50%) at day 5, the mortality rate was even worse for DOX+TRZ-treated mice (80%). Jassal *et al.* [14], using an acute murine model of chemotherapy-induced cardiotoxicity, observed that the mortality rate was extremely high

(80%) in DOX+TRZ treated mice compared with that in mice with DOX treatment only. Mice treated with DOX or DOX+TRZ demonstrated progressive LV dilatation and reduced LVEF, compared with the group control, but systolic dysfunction appeared earlier in the group treated with DOX+TRZ.

The pharmacological target of TRZ in mice is still a matter of great debate. The potentiation of DOX-induced cardiomyopathy by TRZ could be explained by the impossibility of HER-2 survival pathways to overreact to the anthracycline-related stress in cardiomyocytes. Indeed, in mice, TRZ-induced cardiotoxicity has been attributed to the blockade of HER-2 signalling in cardiomyocytes, which lead to the alteration of antiapoptotic signalling pathways [27] and to an increase in oxidative stress. Gordon *et al.* showed that blockade of HER-2 enables the accumulation of reactive oxygen species (ROS) within neonatal rats' cardiomyocytes through a mitochondrial pathway, leading to the development of cardiac dysfunction by stimulating cardiomyocyte apoptosis [16]. In addition, increased angiotensin II levels as a result of HER-2 inhibition lead to an increase in ROS production and the inhibition of neuregulin signalling [31].

4. Conclusion

Our model of postnatal programming suggests, for the first time, an association between overweight and DOX-induced cardiotoxicity, resulting in greater sensitivity to the cardiotoxic effects of DOX, with significant acute systolic function impairment assessed by echocardiography. Furthermore, our results confirmed that TRZ administration potentiates the acute cardiotoxicity induced by DOX, especially in lean mice treated with DOX.

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Tables

Table 1. Forward and reverse sequences of primers used for the amplification of mouse genes

	Forward (5' to 3')	**Reverse (5' to 3')**
HPRT	CTGGTGAAAAGGACCTCTCG	TGAAGTACTCATTATAGTCAAGGGCA
ANP	GAGAAGATGCCGGTAGAAGA	AAGCACTGCCGTCTCTCAGA
BNP	ATGGATCTCCTGAAGGTGCTG	GTGCTGCCTTGAGACCGAA
β-MHC	GCCAACACCAACCTGTCCAAGTTC	TGCAAAGGCTCCAGGTCTGAGGGC

Table 2. Ratio (x 10^3) of left ventricular (LV) weight, measured by echocardiography, and body weight

LV weight/ body weight (x 10^3)	NL			SL		
	D0	D10	D20	D0	D10	D20
Control	4.34 ± 0.13	4.26 ± 0.21	4.22 ± 0.23	4.00 ± 0.09*	3.96 ± 0.10*	3.99 ± 0.09*
DOX	3.99 ± 0.15	3.74 ± 0.15	3.78 ± 0.18	3.73 ± 0.13	3.99 ± 0.19	3.88 ± 0.14
TRZ	4.11 ± 0.22	4.10 ± 0.28	4.22 ± 0.25	3.85 ± 0.10	4.22 ± 0.13	3.95 ± 0.15
DOX + TRZ	4.02 ± 0.08	4.04 ± 0.12	4.30 ± 0.08†	4.17 ± 0.10	4.23 ± 0.09	4.38 ± 0.09†

*; significantly different from NL group, $p<0.05$

†; significantly different from DO and D10, $p<0.05$

Table 3. Left ventricular parameters

Variables	Baseline (Day 0)	Day 10	Day 20
HR (beats/min)			
NL CONT	392±51	398±40	409±38
NL DOX	390±47	388±51	407±56
NL TRZ	356±24	376±25	388±27
NL DOX-TRZ	354±56	370±42	373±40
SL CONT	388±14	391±27	432±116
SL DOX	402±49	395±56	398±57
SL TRZ	393±45	345±23	400±72
SL DOX-TRZ	356±39	362±40	375±22
LVEF (%)			
NL CONT	57±5	57±4	56±4
NL DOX	60±4	57±4	58±3
NL TRZ	58±5	54±7	52±2
NL DOX-TRZ	59±2	55±7	52±6†
SL CONT	56±3	56±3	55±3
SL DOX	61±5	49±7†*	47±4†*
SL TRZ	58±4	53±3	52±5
SL DOX-TRZ	58±3	49±4v*	48±5†
LVEDD (mm)			
NL CONT	4.2±0.3	4.2±0.2	4.3±0.3
NL DOX	4.0±0.2	4.0±0.2	4.0±0.3
NL TRZ	4.1±0.4	4.1±0.3	4.1±0.2
NL DOX-TRZ	4.0±0.2	4.0±0.2	4.0±0.1
SL CONT	4.3±0.2	4.2±0.2	4.2±0.2
SL DOX	4.1±0.3	4.4±0.3†*	4.3±0.2†*
SL TRZ	4.0±0.2	4.3±0.3	4.1±0.2
SL DOX-TRZ	4.2±0.3	4.4±0.3*	4.4±0.2*
LVMN (mg/g)			
NL CONT	4.3±0.5	4.3±0.8	4.2±0.9
NL DOX	3.9±0.7	3.8±0.7	3.8±0.8
NL TRZ	4.1±0.5	4.1±0.6	4.2±0.6
NL DOX-TRZ	4.0±0.3	4.0±0.4	4.3±0.3
SL CONT	4.0±0.3	3.9±0.3	4.0±0.3
SL DOX	3.7±0.7	4.0±0.9	3.9±0.7
SL TRZ	3.9±0.3	4.2±0.3	4.0±0.4
SL DOX-TRZ	4.2±0.4	4.5±0.8	4.4±0.3
E/A ratio			
NL CONT	1.4±0.1	1.4±0.2	1.4±0.2
NL DOX	1.4±0.1	1.5±0.2	1.3±0.2
NL TRZ	1.5±0.1	1.4±0.1	1.3±0.1
NL DOX-TRZ	1.6±0.2	1.6±0.6	1.5±0.5
SL CONT	1.4±0.3	1.4±0.2	1.5±0.6
SL DOX	1.5±0.2	1.4±0.2	1.4±0.2
SL TRZ	1.5±0.2	1.4±0.1	1.5±0.2
SL DOX-TRZ	1.5±0.2	1.4±0.1	1.3±0.1

*Data are means ± standard deviation. DOX indicates doxorubicin; HR: heart rate; LVEDD: left ventricular end-diastolic diameter; LVEF: left ventricular ejection fraction; LVMN: left ventricular mass normalized to body weight; n: number of mice; NL: normal litter; SL: small litter. TRZ: trastuzumab. † p<0.01 vs D0 in each group, * p<0.01 compared with NL group with the same treatment, using 2-way ANOVA for repeated measures with a Tukey test as the post-hoc analysis*

Figure Legends

Figure 1. Study protocol for the assessment of the cardiac effects of DOX, TRZ or their combination in 4-month-old control mice from a normal litter (NL) and overweight mice from a small litter (SL): Eight study groups were created, according to the single intra-peritoneal (IP) injection of DOX (DOX, 6mg/kg), TRZ (TRZ, 10mg/kg) or DOX+TRZ (DOX-TRZ) or saline at D1. At D0, D10 and D20 all mice underwent transthoracic echocardiography. A subset of mice was killed 48 hours after the injections (D3) and blood and tissue samples were harvested. At the end of the protocol, all mice were killed and blood and tissue samples were harvested.

Figure 2. Evolution of body weight in control mice from a normal litter (NL) and overweight mice from a small litter (SL) after a single intra-peritoneal (IP) injection at D1 of (A) saline, (B) DOX (DOX, 6mg/kg), (C) TRZ (TRZ, 10mg/kg) or (D) DOX+TRZ (DOX-TRZ).

* Different from NL group, $p<0.01$

† Different from D0, $p<0.05$

Figure 3. Evolution of left ventricular ejection fraction (LVEF) in control mice from a normal litter (NL) and overweight mice from a small litter (SL) after a single intra-peritoneal (IP) injection at D1 of (A) saline, (B) DOX (DOX, 6mg/kg), (C) TRZ (TRZ, 10mg/kg) or (D) DOX+TRZ (DOX-TRZ).

* Different from NL group, $p<0.01$

† Different from D0, $p<0.01$

Figure 4. Relative mRNA expression of (A) brain natriuretic peptide (BNP), (B) atrial natriuretic peptide (ANP) and (C) beta-myosin heavy chain (β-MHC) at D3 in control mice from a normal litter (NL) and overweight mice from a small litter (SL) after single intra-peritoneal (IP) injection of DOX (DOX, 6mg/kg), TRZ (TRZ, 10mg/kg) or DOX+TRZ (DOX-TRZ) or saline at D1.