Circadian Expression of Adiponectin and Its Receptors in Human Adipose Tissue

P. Gómez-Abellán, C. Gómez-Santos, J. A. Madrid, F. I. Milagro, J. Campion, J. A. Martínez, J. M. Ordovás, and M. Garaulet

Department of Physiology (P.G.-A., C.G.-S., J.A.Mad., M.G.), Faculty of Biology, University of Murcia, 30100 Murcia, Spain; Department of Nutrition and Food Sciences, Physiology, and Toxicology (F.I.M., J.C., J.A.Mar.), University of Navarra, 31080 Pamplona, Spain; Nutrition and Genomics Laboratory (J.M.O.), Jean Mayer United States. Department of Agriculture Human Nutrition Research Center on Aging, Tufts University, Boston, Massachusetts 02111; and Department of Epidemiology (J.M.O.), Centro Nacional Investigaciones Cardiovasculares, 28029 Madrid, Spain

Adiponectin is one of the most clinically relevant cytokines associated with obesity. However, circadian rhythmicity of adiponectin in human adipose tissue (AT) has not been analyzed. To assess whether the mRNA levels of adiponectin and its receptors (ADIPOR1 and ADIPOR2) might show daily circadian rhythms in visceral and sc fat explants obtained from morbid obese women, visceral and sc abdominal AT biopsies (n = 6) were obtained from morbidly obese women (body mass index ≥40 kg/m²). Anthropometric variables were measured and fasting plasma glucose, lipid, and lipoprotein concentrations were analyzed. To investigate rhythmic expression pattern, AT explants were cultured during 24 h, and gene expression was analyzed at the following times: 0800, 1400, 2000, and 0200 h, using quantitative real-time PCR. All genes investigated showed a circadian rhythmicity and oscillated accurately and independently of the suprachiasmatic nucleus in both AT explants (P < 0.05). Adiponectin gene expression fluctuated in the same phase as its receptors. Correlation analyses between the genetic circadian oscillation and components of the metabolic syndrome revealed that adiposity and abdominal obesity correlated with a decrease in adiponectin and adiponectin receptors ADIPOR1 and ADIPOR2 amplitude (P < 0.05). Visceral fat showed a trend toward a phase delay and dampening of the mRNA amplitude of adiponectin as compared with sc fat. The mRNA expression of adiponectin and its receptors showed 24-h rhythmicity in human AT from morbidly obese patients. (Endocrinology 151: 115–122, 2010)
confirmed (5, 7). The persistency of gene expression oscillations in vitro strongly suggests the existence of an intracellular circadian clock system directly regulating local cell functions.

In this context, adiponectin is one adipokine that has recently attracted much attention because it functions as a key modulator of insulin sensitivity (8, 9) and a protective factor against MetS alterations (10, 11). The beneficial effects of this hormone are predominantly mediated by binding to two cell membrane receptors, adiponectin receptor (ADIPOR)-1 and ADIPOR2 (12). ADIPOR1, a high-affinity receptor for globular adiponectin but a low-affinity receptor for full-length adiponectin, is most abundantly expressed in muscle, whereas ADIPOR2, an intermediate-affinity receptor for both full-length and globular adiponectin, is predominantly expressed in liver (12). Both receptors are also present in adipose tissue, suggesting that adiponectin may have biological effects in AT in an autocrine/paracrine manner (13).

The diurnal and ultradian rhythms of circulating adiponectin concentrations have been demonstrated in humans (14) and also in AT from experimental animals (15). However, it remains to be characterized whether this cytokine exerts a circadian oscillation in human adipose tissue and whether this oscillation differs between visceral and subcutaneous fat depots.

In view of the above mentioned, the objective of the present research was to analyze in human adipose tissue the putative circadian rhythmicity of the expression of adiponectin and its two receptors, ADIPOR1 and ADIPOR2, as well as to assess the potential differences between two adipose depots, sc and visceral AT, in morbid obese female subjects.

Materials and Methods

Subjects

Visceral and sc abdominal AT biopsies were obtained from morbidly obese women (n = 6), aged 51 ± 9 yr with body mass index (BMI) 44.1 ± 5.5 kg/m², undergoing laparoscopic gastric bypass surgery due to obesity at the General Surgery Service of Virgen de la Arrixaca Hospital (Murcia, Spain). The women studied were postmenopausal and were not under hormone replacement therapy. The day before surgery, all patients were synchronized having lunch at 1430 and dinner at 2100 h. The AT biopsies were taken as paired samples from the two AT depots (visceral and sc) at the beginning of the surgical procedure (estimated time of biopsies sampling at 1300 h).

The protocols were approved by the Ethics Committee of the Virgen de la Arrixaca University Hospital, and the subjects signed a written informed consent before the biopsies were obtained.

Clinical characteristics

Arterial pressure, BMI, and waist and hip circumference were assessed by standard procedures, whereas skinfolds (biceps, triceps, suprailiac, and subcapsular) were measured with a Harpenden caliper (Holtain Ltd., Crymych, Pembrokeshire, UK). Total body fat (percent) was evaluated by bioimpedance with a TBF-300 (Tanita Corp. of America, Arlington Heights, IL). Sagittal diameter and coronal diameter were measured at the level of the iliac crest (L4,5) using a Holttain Kahn abdominal caliper. Women were classified in visceral and sc obesity calculating the index visceral area (VA)/sc area (SA) after applying the following equation (16): \( \text{VA/SA}_{\text{predicted}} = 0.868 + 0.064 \times \text{sagittal diameter} - 0.036 \times \text{coronal diameter} - 0.022 \times \text{triceps skinfold} \). Those patients with VA/SA greater than 0.42 were classified as having visceral obesity. Fast- ing plasma concentrations of glucose, triacylglycerols, total cholesterol, and high-density lipoprotein cholesterol were determined with commercial kits (Roche Diagnostics GmbH, Mannheim, Germany).

Adipose tissue culture

Immediately after the surgery, a part of AT biopsies were immediately frozen at −80 °C and used for analyzing the basal gene expression. The rest of AT was used for culture; thus, 800-1000 mg AT explants (minimal pieces of 1–2 mm² to allow the maximal contact of adipose tissue with the medium) were transferred to cell culture bottles with membrane filter screw cap to safeguard the viability of the culture and placed in 5 ml of DMEM supplemented with 10% fetal bovine serum and kept at 37 °C for 24 h in a humidified atmosphere containing 7% CO₂.

On the next day, the adipose explants were collected to perform gene expression analysis at the following times (T): 0, 6, 12, and 18, T0 being arbitrarily defined as 0800 h because this was the usual waking time for patients, T6 as 1400 h, T12 as 2000 h, and T18 as 0200 h. Gene expression was measured for only one circadian cycle (24 h) but in the second day of cultured. All cultures were performed in duplicates.

Analysis of gene expression

Total RNA was extracted from AT explants using RNeasy kit (QIAGEN, Courtabeouf, France). Reverse transcription was performed using random hexamers as primers and Thermoscript reverse transcriptase (Invitrogen, Cergy Pontoise, France) with 1 μg total RNA for each sample. Quantitative real-time PCR was performed using an ABI PRISM 7000 HT sequence detection system (Applied Biosystems, Foster City, CA), using TaqMan universal PCR master mix and specific TaqMan probes (Applied Biosystems). The primers used in the study are the following references from Applied Biosystems: human AdipoQ (Hs00605917_m1), ADIPOR1 (Hs01114951_m1) and ADIPOR2 (Hs00226105_m1). We used 18S rRNA (reference Hs99999901_s1; Applied Biosystems) as internal control. We selected 18S because this gene showed a lower variance along time compared with the glyceraldehyde-3-phosphate dehydrogenase gene. In addition, we carried out a one-way (Zeitgeber time) ANOVA for 18S and observed no significant difference in any of the adipose depots studied \( P > 0.05 \). In addition, there was no significant difference in 18S cycle threshold (Ct) between sc (mean Ct = 23.3 ± 3.3) and visceral depots (mean Ct = 23.2 ± 3.3). In conclusion, we selected 18S as the better housekeeping gene for this experiment. Gene mRNA levels were normalized to 18S using the 2⁻ΔΔCt method (17).
Rhythm calculation and statistical analysis

Clinical and anthropometric data are presented as means ± sd. The results for gene expression, expressed in arbitrary units, are presented as means ± sem. Wilcoxon nonparametric paired test was used for comparing data from the samples derived from the two adipose depots in each individual subject. Pearson’s correlations coefficients were used for analyzing associations between the relative expression of adiponectin and ADIPOR1 and ADIPOR2 in both adipose depots.

The single cosinor method was used to analyze for circadian rhythm individually (18). This inferential method involves fitting a curve of a predefined period by the least squares method. The rhythmic characteristics and their 95% confidence intervals estimated by this method include the mesor (middle value of the fitted cosine representing a rhythm adjusted mean), the amplitude (half the difference between the minimum and maximum of the fitted cosine function), and the temporal location of maximum value or acrophase (the time at which the peak of a rhythm occurs, expressed in hours) and the percent rhythm (PR; percentage of variability accounted for by cosine curve). Relative amplitude was expressed as a percentage of mesor values [relative amplitude = (amplitude/mesor) × 100]. The significance of the rhythms was determined by rejection of the zero amplitude hypotheses with a threshold of 60%.

Mean circadian gene expression of the total population into each fat depot was analyzed by using repeated-measures ANOVA test, with a post hoc test of least significant difference (LSD) correction. Differences in acrophase between sc and visceral depots were analyzed by Mann-Whitney nonparametric test for the three genes studied. Pearson’s correlation analyses were used for finding associations between the genetic circadian oscillation (amplitude) and components of the MetS. All statistical analyses were carried out using SPSS for Windows (release 15.0; SPSS Inc., Chicago, IL). The level of significance for all statistical tests and hypotheses was set at P < 0.05.

Results

Characteristics of the population

Table 1 contains basal characteristics of the women studied. BMI was higher than 40 kg/m² in these patients and VA/SA predicted 0.54 ± 0.28.

| TABLE 1. Anthropometric characteristics of the population studied
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>51 ± 9</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>107.9 ± 11.2</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>156 ± 5.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>44.1 ± 5.5</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>43 ± 6</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>126 ± 8</td>
</tr>
<tr>
<td>HC (cm)</td>
<td>139 ± 9</td>
</tr>
<tr>
<td>WHR</td>
<td>0.91 ± 0.03</td>
</tr>
<tr>
<td>Sagittal diameter (cm)</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>Coronal diameter (cm)</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>Bicipital sinkfold (mm)</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>Tricipital sinkfold (mm)</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>Subscapular sinkfold (mm)</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>Suprailiac sinkfold (mm)</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>VA/SApredicted</td>
<td>0.54 ± 0.28</td>
</tr>
</tbody>
</table>

Data are presented as means ± sd. WC, Waist circumference; HC, hip circumference.

Circadian gene expression

Once we examined the basal expression of these genes, we focused on their circadian expression pattern in cultured adipose tissue explants. Parameters imputed from each subject obtained by cosinor analysis, defining the circadian rhythms as mesor, amplitude, relative amplitude, acrophase, and percent rhythm are reported in Table 3. According to these results, circadian expression patterns were found for all the genes investigated (represented in bold).

Figure 2 shows mean circadian rhythms for the examined group concerning the three genes studied (ADIPOQ, ADIPOR1, and ADIPOR2) in sc (Fig. 2A) and visceral
AT (Fig. 2B). When relative gene expression among different times was analyzed by using repeated-measures ANOVA test, statistical differences were found for ADIPOQ in sc (P = 0.010) and visceral (P = 0.045) ATs, evidencing the circadian rhythmicity of adiponectin expression.

When comparing both AT depots, different circadian patterns were observed. Thus, whereas the sc tissue displayed a strong oscillatory trend, adiponectin circadian expression in visceral fat was dampened in all patients, with only one exception, as observed when comparing relative amplitude from both AT locations (Table 3). Moreover, the three genes cycled in synchrony in both depots, but while in sc adipose tissue, they reached their zenith (highest levels) around Zeitgeber time 0 (0800 h) and their nadir (lowest levels) around time 12 (2000 h); in omental AT both zenith and nadir were significantly delayed 6 h with respect to sc AT for the three genes studied (P < 0.05).

Correlation analyses between the genetic circadian oscillation (amplitude) and components of the MetS revealed that higher adiposity and abdominal obesity (BMI, body fat percent, waist, sagittal diameter, and conicity index) correlated with a decrease in ADIPOQ, ADIPOR1, and ADIPOR2 amplitude (Table 4).

A simple statistical analysis was performed correlating the phase difference between both AT depots (differences in achrophase from sc and visceral fat) in adiponectin expression and obesity parameters such as BMI, waist circumference, waist to hip ratio (WHR), and sagittal diameter, and no significant associations were found for those parameters (BMI: r = 0.235, P = 0.70; waist circumference: r = 0.62; P = 0.26; WHR: r = 0.09; P = 0.89; sagittal diameter r = 0.08, P = 0.89).

![FIG. 1. Basal relative expression of genes studied (ADIPOQ, ADIPOR1, and ADIPOR2) in sc and visceral adipose tissue. mRNA levels were measured with the real-time PCR and normalized to 18S using the \( \Delta\Delta CT \) method of relative quantitation. Data are reported as means ± SEM, and results are presented as percent of ADIPOR2 relative expression (ADIPOQ = 1). Asterisk represents significant differences (Wilcoxon nonparametric paired test). NS, Not significant.](image)

### Table 3. Parameters imputed from cosinor analysis

<table>
<thead>
<tr>
<th>Patients</th>
<th>SC AT</th>
<th>Visceral AT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mesor (AU)</strong></td>
<td><strong>Amplitude (AU)</strong></td>
<td><strong>Relative amplitude (%)</strong></td>
</tr>
<tr>
<td>Adiponectin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>1.785</td>
<td>0.736</td>
</tr>
<tr>
<td>P3</td>
<td>2.856</td>
<td>2.59</td>
</tr>
<tr>
<td>P4</td>
<td>33.976</td>
<td>57.466</td>
</tr>
<tr>
<td>P5</td>
<td>14.463</td>
<td>20.047</td>
</tr>
<tr>
<td>P6</td>
<td>7.098</td>
<td>10.754</td>
</tr>
<tr>
<td><strong>Adiponectin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>1.703</td>
<td>0.587</td>
</tr>
<tr>
<td>P2</td>
<td>2.856</td>
<td>2.59</td>
</tr>
<tr>
<td>P3</td>
<td>33.976</td>
<td>57.466</td>
</tr>
<tr>
<td>P4</td>
<td>14.463</td>
<td>20.047</td>
</tr>
<tr>
<td>P5</td>
<td>7.098</td>
<td>10.754</td>
</tr>
<tr>
<td>P6</td>
<td>33.976</td>
<td>57.466</td>
</tr>
<tr>
<td><strong>PR (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>1.76</td>
<td>1.122</td>
</tr>
<tr>
<td>P2</td>
<td>2.16</td>
<td>1.487</td>
</tr>
<tr>
<td>P3</td>
<td>1.38</td>
<td>0.53</td>
</tr>
<tr>
<td>P4</td>
<td>53.61</td>
<td>2.279</td>
</tr>
<tr>
<td>P5</td>
<td>3.242</td>
<td>3.225</td>
</tr>
<tr>
<td>P6</td>
<td>3.509</td>
<td>1.495</td>
</tr>
</tbody>
</table>

Parameters imputed from each subject obtained by cosinor analysis defining the circadian rhythms as mesor, amplitude, relative amplitude, acrophase, and PR are represented in this table. Subjects in which expression of the gene was considered to have circadian rhythmicity are shown in bold. AU, Arbitrary units.
Discussion

Adiponectin is one of the most intensively studied cytokines. The interest in this novel adipocyte hormone is mainly derived from its protective role in the different alterations associated with obesity, such as insulin resistance and inflammation (20). Interestingly, in humans, circulating adiponectin levels exhibit diurnal variations (14). However, as far as we know, specific information concerning circadian rhythmic expression of this adipokine in human AT has not been reported. In the present study, we show in AT from obese women that adiponectin mRNA levels fluctuate during the day in the same phase as its receptors and that this circadian rhythmicity is attenuated with MetS features.

Women from the present work displayed significant differences in basal adiponectin expression between AT depots, being higher in the sc expression. These findings are in agreement with a previous study performed in a
similar population (21) and could be associated with the cardioprotective feature of this adipose area, termed by some authors as healthy adipose tissue (22). With respect to adiponectin receptors, no significant differences were found between both AT location, as previously shown in experimental animals (23).

The main objective of the present work was to assess whether the mRNA levels of adiponectin and its receptors showed daily circadian rhythms in visceral and sc fat explants obtained from morbidly obese women. Results indicate that the three genes examined (ADIPOQ, ADIPOR1, and ADIPOR2) exhibited 24-h rhythmicity, suggesting that the expression of adiponectin is regulated in a time-dependent manner, at least at the mRNA level.

Thus, our data suggest that these genes can oscillate accurately and independently of the suprachiasmatic nucleus in human AT during 24 h. This situation has been previously demonstrated for other genes in human AT cultures, such as clock genes (PER2, CRY1, and BMAL1) (7), cortisol-related genes, such as glucocorticoid receptor (GR) and 11β-hydroxysteroid dehydrogenase 1 (11βHSD1), and PPARG (24). However, this is the first study to show the circadian expression of adiponectin and its receptors in human AT. From a physiological point of view, these new data are not surprising if we consider that, besides glucose and lipid homeostasis, there are many hormones (insulin, glucagon, GH, and cortisol) and adipose-specific molecules implicated in energy metabolism and MetS (leptin, adipin, resistin, and visfatin), which display circadian oscillation with different daily pattern (2).

Only a limited number of studies previously examined ADIPOR expression in AT (13, 16, 25). Here we first explore their circadian rhythmicity, finding that they fluctuate in the same phase as adiponectin. This synergy could magnify the physiological effect of adiponectin and facilitate its autocrine/paracrine action. Indeed, the simultaneous circadian expression of adiponectin and its receptors may lead to an increase in adiponectin binding and thus result in increased adiponectin effects. These results emphasize the importance of circadian rhythmicity in the physiological role of adiponectin.

A previous study performed in experimental animals (26) have shown that adiponectin gene expression follows a circadian rhythm in adipose tissue depots with a nadir (minimum expression) during the morning (1000–1100 h). In the present study, performed in humans, the expression of adiponectin achieved its zenith (maximum) at the same hours. As expected, circadian expression of human adiponectin occurs in antiphase as compared with mice, a rodent characterized by its nocturnal behavior.

In humans, there are no studies showing circadian adiponectin gene expression in other tissues and none performed in women or obese women. When comparing AT adiponectin circadian expression in the present work with serum diurnal variability from a previous work (14), it can be observed that the nadir in AT in the present paper anticipated to that found in plasma, thus implying a 7-h phase delay between the transcription and the secretion of the protein to blood. These data must be discussed with caution because the study by Gavrila et al. (14) was performed in healthy males, whereas the present work is followed up in morbidly obese women. However, these results could be explained by the kinetics of expression, synthesis, and secretion of adiponectin.

Recent findings suggest that the circadian system regulates the metabolism in all tissues and organs implicated in the pathological changes leading the MetS. Moreover, glucose and lipid homeostasis are also known to exhibit circadian variation (27–30). In this sense, adiponectin is highly implicated in glucose metabolism (31). An additional relevant finding in our results was that adiponectin expression was decreased in the late evening (2000 h), which coincides with the hours of maximal insulin resistance in humans (32). Additionally, adiponectin has been called the fat-burning molecule because it is able to redirect fatty acids to the muscle for their oxidation (31). This special capability is of great interest because the influx of fatty acids to the liver decreases and so does total triglyceride content, leading to a higher insulin-sensitivity state (31). The fact that adiponectin displays its higher expression during the morning hours (1000 h) could be implicated in the maximal withdrawal of fatty acids at these times and the improvement in glucose tolerance, which characterized the subjects in these particular daily hours (32).

Circadian disruption or chronodisruption is characterized by an attenuation of the circadian rhythms (33). Recently some studies suggested that chronodisruption may lead to obesity (1). Disrupted biological rhythms lead to attenuated circadian feeding rhythms, hyperphagia, obesity, cancer proneness, and reduced life expectancy (1). In this regard, our correlation analyses performed between the genetic circadian oscillation (amplitude) and components of the MetS revealed that the rhythmicity of adiponectin expression in the women studied was attenuated with obesity. Indeed, our results show that an increase of adiposity markers and mainly abdominal obesity (BMI, body fat percent, weight, waist, sagittal diameter, and nicity index) significantly correlated with a decrease in adiponectin and ADIPOR1 and ADIPOR2 amplitude, which characterized a dampened rhythm. It is important to highlight that this situation was present only in the visceral fat. Another interesting result was that when comparing AT locations, visceral fat showed a trend toward a dampening of the mRNA amplitude of components of
adiponectin function, compared with sc fat. This consideration perhaps may help to explain why MetS features are highly related to visceral fat. Previously Ando et al. (15) showed that the rhythmic expression of clock genes (Bmal1, Per1, Per2, Cry1, Cry2, and Dbp) and adipocytokines (adiponectin, resistin, and visfatin) was attenuated in visceral adipose tissue from obese KK mice. Other animal models revealed that mice with Clock gene disruption are prone to develop obesity and a phenotype resembling MetS (34).

Another interesting characteristic that provides information about rhythmicity in AT is the potential phase delay between the different AT locations. In this line, in visceral AT from the women studied, both nadir and zenith were significantly delayed 6 h with respect to sc. A similar situation was present for two of the clock genes studied (bPer2 and bCry1) but not for bBmal1 (data not shown). It is noteworthy that this phase delay between both AT location was not related to obesity. Indeed, no significant correlations were obtained between obesity parameters and the differences in acrophase from both AT locations. Interestingly, in humans, alterations in the dynamics of circulating adiponectin with obesity have also been reported (35). Together with previous findings, we could then speculate that circadian rhythm disruption is clearly important in determining the severity of the metabolic impairment and vice versa (36).

One of the shortcomings of the current design is that it includes only morbidly obese women, and more information is needed about gender differences. Although in this study adiposity was associated with chronodisruption, even in morbidly obese women, another aspect to consider is whether the rhythms may be different for less obese individuals. However, to obtain enough AT sample from normal-weight individuals may be challenging, and it could be also an ethical problem, considering the difficulties in obtaining enough fat from visceral and sc locations within the same patients to perform four different adipose tissue explants cultures.

In conclusion, we have found for the first time that expression of adiponectin and its receptors (ADIPOR1 and ADIPOR2) showed 24-h rhythmicity in human visceral and sc AT explants from morbid obese patients. Interestingly, this rhythmic adiponectin expression is in phase with its receptors, and there is a zenith delay of 6 h in omental AT with respect to sc fat.

Acknowledgments

We thank Ana Lorente and Juan José Hernández-Morante for their excellent technical assistance.

Address all correspondence and requests for reprints to: Marta Garaulet, Department of Physiology, Faculty of Biology, University of Murcia, Campus de Espinardo, s/n 30100 Murcia, Spain. E-mail: garaulet@um.es.

This work was supported in part by National Institutes of Health Grant DK075030 and Contracts 53-K06-5-10 and 58-1950-9-001 from the U.S. Department of Agriculture Research Service; the Government of Education, Science, and Research of Murcia (Project BIO/FFA 07/01-0004); the Spanish Government of Science and Innovation (Project AGL2008-01655/ALI); the Spanish Ministry of Education and Science (Project BFU2007-60638/BFI); Seneca Foundation (PI/05700/07); the Institute of Health Carlos III (RETICEF, RD06/0013/0019); and Línea Especial of University of Navarra (LE/97).

Disclosure Summary: The authors have nothing to disclose.

References


