PHENOTYPING CYP3A4/5 USING AN ENDOGENOUS BIOMARKER IN CHILDREN WITH DOWN SYNDROME

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\textbf{Background:} Down syndrome (DS); a common chromosomal abnormality in humans can affect multiple organ systems. DS individuals usually use a wide range of medications. There is a gap in knowledge about the extent of contribution of CYP3A4/5 in altered clinical response to medications in DS children.

\textbf{Objectives:} Phenotyping of CYP3A4/5 in DS children using the ratio of 4β-hydroxycholesterol / Cholesterol (4β-OHC/C) as an endogenous biomarker for CYP3A4/5 activity.

\textbf{Method:} The study was an observational case control study, conducted in the DS clinic, King Abdul-Aziz University Hospital. Blood samples were taken for thyroid and liver function test by automated immunoassay procedure and analysis of cholesterol and 4β-hydroxycholesterol by gas chromatography.

\textbf{Results:} 16 DS and 29 non-DS children were enrolled (1-12 Y). Children with DS showed a lower median 4β-OHC/C molar ratio of 0.19×10\textsuperscript{-4} compared to 0.45×10\textsuperscript{-4} in the control group and with interquartile range (IQR) 0.17, 0.36 respectively (p< 0.001 Mann Whitney U test). DS children also showed an abnormality in liver enzymes and hypercholesteremia.

\textbf{Conclusion:} Children with DS had about two-fold lower CYP3A4/5 activity compared to children without DS. More studies to confirm these observations are required, however, drugs should be used cautiously in DS children.

\section*{INTRODUCTION}

Down syndrome (DS) is one of the most common genetic disorders, and an epidemiological study of children in Saudi Arabia showed an incidence of 18 per 10,000\textsuperscript{1}. It is known that DS patients suffer many health problems that require the use of drugs\textsuperscript{2}, and many studies have shown that these patients have an altered clinical response compared to non-DS patients\textsuperscript{3,7}.

A major source of variability in drug clinical response can be attributed to the difference in drug-metabolizing enzymes (DMEs), especially cytochrome P450 3A4 and 3A5 (CYP3A4/5) which metabolizes more than 50\% of all marketed drugs\textsuperscript{8,10}. The clinical relevance of genetic and non-genetic factors that influence CYP-mediated metabolism can be appreciated by estimating in-vivo enzyme activity (i.e., the phenotyping) through the use of “probe drugs” or by using an endogenous biomarker\textsuperscript{11}.

Cytochrome P450 phenotyping provides information about their activity; It can be assessed by administration of a sub-therapeutic dose of the probe drug(s) which are the substrate for the intended CYP450. Blood or
urine samples are collected and sometimes saliva for drug/metabolites analysis by chromatography. Due to the limitations of this approach increasing interest was given for utilizing endogenous biomarkers in the phenotyping of drug-metabolizing enzymes. In this context, the metabolite of cholesterol e.g. 4β-OHC reflects the activity of CYP3A4/5 so it was utilized as an endogenous biomarker for phenotyping of activity of these enzymes. Studies deals with phenotyping Cytochrome P450 in DS are very limited. Therefore, we conducted the present study to demonstrate the difference in CYP3A4/5 activity between children with and non-DS children.

MATERIALS AND METHODS

Settings

The study was implemented at the DS clinic, King Abdul-Aziz University Hospital (KAUH), the study protocol was approved by the Research Ethics Committee of the Faculty of Medicine, (KAU). The study was approved by the Unit of biomedical ethics Faculty of Medicine at King Abdul-Aziz University. Consent was taken from children's parents to be voluntarily involved in the study.

Sample size determination

The sample size was estimated using the following parameters: SD= 25 ng/ml and \( p=0.05 \) two-sided The sample size in each group \( \approx 16 \)

\[
n = \frac{2(Z_{\alpha/2} + Z_{p})^2 \times SD^2}{d^2}
\]

Taking into account down syndrome is a relatively rare disease, this number is satisfactory to provide a reliable conclusion.

Regarding sample size, 16 DS children enrolled in the study was considered sufficient to provide a reliable conclusion regarding children with the relatively rare congenital disorder as the prevalence of DS in KSA (18/10.000) as suggested by a Consultant biostatistician.

Inclusion criteria

Sixteen healthy children with DS and 26 healthy controls in the age range of 1-12 years were enrolled from KAUH. All children with DS underwent cytogenetic analysis and had trisomy 21. (All children are Arab residents in Makkah region of SA).

Exclusion criteria

History of hepatic dysfunction; two siblings; congestive heart failure and using prescribed drugs that are known to induce or inhibit CYP3A4/5.

Samples collection and storage

Single venous blood sample (1-2 ml) ordered as part of routine clinical care) was withdrawn in EDTA-tubes from each subject. Samples were centrifuged and serum was separated for routine biochemical analysis. The remaining serum was kept at -80°C until GCMS analysis for determination of total cholesterol and 4β-OHC was done.

1. Biochemical analysis

Serum thyroid-stimulating hormone (TSH) and free triiodothyronine (FT3) were analyzed at KAUH, biochemistry lab via the cobas™ clinical chemistry system (Roche Diagnostics, Germany). The method is an immunoassay for the in-vitro quantitative determination of TSH and FT3 in the serum. It is done by two incubations assays of the sample using the sandwich principle for TSH and the competition principle for FT3. The first incubation is to form an immunocomplex and the second is to bind the complex to a solid phase. After unbound substances are removed the reaction mixture is aspirated magnetically onto an electrode, a photomultiplier is used to measure a voltage induced emission. Results are determined via a calibration curve. Albumin (Alb), and liver function tests Alanine transaminase (ALT); Aspartate transaminase (AST), were done by Dimension Clinical Chemistry System (Stream lab.). Calibration and analysis were done as specified by the reagent manufacturer. The lab joined an external quality control program to ensure high quality of analytical results.

2. Phenotyping CYP3A4/5 using 4β-OHC

The ratio of 4β-OHC/Cholesterol in serum was used as an endogenous probe for CYP3A4/5 activity.
3. Chromatography analysis

The concentrations of cholesterol and 4β-OHC were determined in each serum sample according to reported methods with slight modification20. The serum samples were thawed at room temperature, 250 µL was transferred to an autosampler 1.8 mL vial by auto-pipette, mixed with 250 µL 2M KOH (prepared in water), then was capped and vortexed for 2 sec., and allowed to stand at room temperature for 20 min (to hydrolyze combined cholesterol or its hydroxyl forms). The whole amount of serum was transferred by Pasteur pipette to the C8 extraction column, a vacuum was started and half a milliliter of distilled water was added for washing. The vacuum was then applied for 10 min to evaporate the water. The elution solution consisted of 1 mL solvent composed of 80% ethyl acetate and 20% n-hexane and 1 mL CH2Cl2 was added. This extraction solution was then dried under dry Nitrogen gas at 40°C. Twenty-five microliter MSTFA was added (using silanized glass syringe) to the remaining residue and was heated at 80°C for 35 min (half inserted at thermostated block heater). One microliter was injected for GC-MS (Clarus 5000 (Perkin Elmer), analysis using the programmed split-split less mode, after leaving the vial to cool to room temperature. 4β-OHC/C was calculated as a molar ratio by dividing the molar concentration of 4β-OHC by the molar concentration of cholesterol (Molecular weight; 402.65 and 386.65 g/mol of 4β-OHC and cholesterol respectively).

Statistical analysis

All data were statistically processed with the SPSS program, version 16.0 (SPSS Inc., Chicago, IL, USA). Data when show normal distribution are presented as mean ± S.E (standard error of the mean) to evaluate confidence interval or ± SD (Standard deviation) to express the extent of variability. Median (IQR) is used in case of absence of normal distribution.

The normal distribution of variables was checked with the Shapiro-Wilk test. Mann–Whitney U-test was used to compare 4β-OHC/C between groups because the groups have deviated from the normal distribution. For all tests, values of $p < 0.05$ (two-tailed) were considered statistically significant. To assess if there is any cofounding factor, the study of the correlation between both age, gender, and thyroid function (TSH and FT3) and the molar ratio was done using the Spearman correlation test. Ethnicity was not considered confounding factors as all children are Arab most of them are Saudi.

RESULTS

Chromatographic analysis

Figure 1 represents the (EI) GCMS chromatogram. The analytical procedure was validated; the limit of quantification (LOQ) ($n=5$) of 0.4 ng/ml and 0.8 for 4β-OHC and cholesterol in serum was obtained, respectively. At the LOQ, the RSD ($n=3$) of the measured concentration was 5.2% and 7.1% respectively, and the accuracy of measurement was 7.2%.

Summary of demographic and relevant data

Sixteen DS individuals (50% males, 50% females) and 29 non-DS (41% males, 59% females) were included in the study; All are Arab population, most are Saudi.

None of the non-DS subjects was on medications that were known substrates, inhibitors, or inducers of CYP3A. Six DS children were on thyroxin therapy, non of them use other medications that may induce or inhibit CYP3A4/5.

Thyroid function

The results of thyroid function are shown in figure 2 and the results of liver function are shown in figure 3 for both DS and non-DS children. DS individuals showed a significantly lower mean value of FT3 ± SD; 5.8±0.24 (pmol/L) compared to non-DS children 6.5±0.15 (pmol/L), ($p=0.025$); while they showed a higher mean TSH value of 4.1±0.7 (µIU/L) compared to 2.9±0.3 (µIU/L) in non-DS children. The latter result is statistically insignificant ($p=0.112$). Correlations between both FT3 and TSH versus 4β-OHC/C ratio were studied using Spearman correlation coefficient, no significant correlation was observed. DS individuals showed significantly higher mean values of (ALT, AST,) and lower mean Alb compared to non-DS (Fig. 3). Using Spearman’s correlation test no significant correlation between the 4β-OHC/C ratio with either liver enzymes or age was observed in tables 1&2.
Fig. 1: GC-EI-MS chromatogram of: (A) and (B) two different non-DS samples, (C) cholesterol, 4β-OHC, and internal standards. All are MSTFA derivatized.

Fig. 2: Mean values of serum TSH and FT3 in DS and non DS children.

TSH: Thyroid-stimulating hormone, FT3: Free triiodothyronine,
*Significantly different from control (p< 0.05, Mann Whitney U test).
Fig. 3: Mean values(±S.E) of liver function in Down syndrome (DS) and non-DS children (control).

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase, Alb: Fig albumin, TP: Total protein.
*Significantly different from control (p< 0.05, Mann Whitney U test).

Table 1: Correlations of some biochemical parameters with 4β-OHC/C ratio in DS using Spearman’s coefficient.

<table>
<thead>
<tr>
<th>RATIO</th>
<th>Correlation coefficient</th>
<th>Age</th>
<th>FT3</th>
<th>TSH</th>
<th>ALT</th>
<th>AST</th>
<th>Alb</th>
<th>TP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.144</td>
<td>-0.113</td>
<td>-0.012</td>
<td>0.209</td>
<td>-0.052</td>
<td>-0.072</td>
</tr>
<tr>
<td>Sig.</td>
<td>0.594</td>
<td></td>
<td>0.545</td>
<td>-0.198</td>
<td>-0.325</td>
<td>-0.122</td>
<td>-0.271</td>
<td>0.360</td>
</tr>
<tr>
<td></td>
<td>(2-tailed)</td>
<td></td>
<td>0.029</td>
<td>0.086</td>
<td>0.529</td>
<td>0.155</td>
<td>0.055</td>
<td>0.068</td>
</tr>
<tr>
<td>N</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>13</td>
<td>13</td>
<td>15</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

*Correlation is significant at the 0.05 level (2-tailed).

Table 2: Correlations of some biochemical parameters with 4β-OHC/C ratio in non-DS using Spearman’s correlation coefficient.

<table>
<thead>
<tr>
<th>RATIO</th>
<th>Correlation coefficient</th>
<th>Age</th>
<th>FT3</th>
<th>TSH</th>
<th>ALT</th>
<th>AST</th>
<th>Alb</th>
<th>TP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.020</td>
<td>-0.198</td>
<td>-0.325</td>
<td>-0.122</td>
<td>-0.271</td>
<td>0.360</td>
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<tr>
<td>Sig.</td>
<td>0.921</td>
<td></td>
<td>0.303</td>
<td>0.086</td>
<td>0.529</td>
<td>0.155</td>
<td>0.055</td>
<td>0.068</td>
</tr>
<tr>
<td></td>
<td>(2-tailed)</td>
<td></td>
<td>0.303</td>
<td>0.086</td>
<td>0.529</td>
<td>0.155</td>
<td>0.055</td>
<td>0.068</td>
</tr>
<tr>
<td>N</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>29</td>
</tr>
</tbody>
</table>

*Correlation is significant at the 0.05 level (2-tailed).
Serum total cholesterol levels
The median (IQR) serum concentration of total cholesterol showed a significantly higher value in DS4.06 (3.09) compared to Non-DS 2.18 (1.4) (ng/ml) with p< 0.0001. There is no significant difference observed between males and females. Hypercholesteremia was observed in about 40% of DS (total serum Cholesterol >5 mM).

Serum 4β-OHC levels
The median (IQR) (ng/ml) of 4β-OHC in non-DS and DS is 30.7 and 40.8, respectively. A trend for lower median 4β-OHC is noticed in DS (p= 0.07) in comparison with non-DS.

Serum 4β-OHC/C ratio (Phenotyping biomarker)
Table 3 shows the serum 4β-OHC/C represented as median (IQR) in both groups. The DS children have a less varied and significantly lower value of 4β-OHC/C (0.19×10⁻⁴) compared to the non DS control children with corresponding value of (0.45 × 10⁻⁴), (p< 0.0001). Figure 4 shows the frequency of 4β-OHC/C ratio in the serum of DS and non-DS children. DS children showed a more variable 4β-OHC/C ratio, the range [0.15-0.85] × 10⁻⁴. In contrast, children with DS showed lower variability in 4β-OHC/C, the range [0.05-0.35] × 10⁻⁴. Neither gender nor thyroxine therapy showed a statistically significant effect on 4β-OHC/C ratio in DS-Children.

Table 3: Serum 4β-OHC/C ratio × 10⁻⁴ in DS and non-DS.

<table>
<thead>
<tr>
<th>Comparing*</th>
<th>Median (IQR)</th>
<th>CV%</th>
<th>95% CI</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS vs. Non-DS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS n= 16</td>
<td>0.19 (0.17)</td>
<td>66.67</td>
<td>0.14-0.28</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td>Non-DS n= 29</td>
<td>0.45 (0.36)</td>
<td>66.07</td>
<td>0.41-0.70</td>
<td></td>
</tr>
<tr>
<td>DS (Gender)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male n= 8</td>
<td>0.21 (0.13)</td>
<td>45.00</td>
<td>0.13-0.28</td>
<td>0.833</td>
</tr>
<tr>
<td>Female n= 8</td>
<td>0.16 (0.19)</td>
<td>81.81</td>
<td>0.07-0.37</td>
<td></td>
</tr>
<tr>
<td>Non-DS (Gender)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male n= 12</td>
<td>0.48 (0.45)</td>
<td>65.57</td>
<td>0.36-0.86</td>
<td>0.425</td>
</tr>
<tr>
<td>Female n= 17</td>
<td>0.41 (0.36)</td>
<td>71.15</td>
<td>0.33-0.71</td>
<td></td>
</tr>
</tbody>
</table>

DS; Down syndrome. Non-DS; Children. CI; Confidence interval. 4β-OHC/C; 4β-hydroxycholesterol/cholesterol; coefficient of variation. IQR; Interquartile range. *Significant differences (Mann Whitney U test).
DISCUSSION

Altered drug response is frequently observed in DS individuals which may be due to pharmacokinetics, pharmacodynamics; genetics, or other variables. There is a gap in knowledge regarding the contribution of liver metabolizing enzyme in these variabilities. This study tried to use an endogenous biomarker to predicate the activity of one of the most important Phase I drug metabolizing enzymes, namely CYP3A4/5. Other variables including thyroid function will be also addressed.

In this study, no correlation between liver function parameters (ALT, AST) and the molar ratio of the 4β-OHC/C ratio was observed in both DS and non-DS children. Mild elevation in ALT, AST, and lower Alb was observed in DS children compared to non-DS children. Elevated liver enzymes, in DS children, may be explained by multiple factors, including obesity, low activity, sleep apnea, thyroid abnormality low vitamin D; glutathione deficiency, and increase in oxidative stress.

Dyslipidemia in DS children was common; Unachak et al. pointed out that sub-clinical hypothyroidism was the most common thyroid abnormality in children with DS the thyroid hormone, FT3 has a positive effect on the binding of LDL to its receptor. Hypothyroidism was suggested as a contributing factor in hypercholesterolemia. Given this background and increase in TSH (Fig. 2) in our study, we suggested a potential link between subclinical hypothyroidism and hypercholesteremica.

Interest is increasing regarding use of endogenous biomarkers for phenotyping of CYP450 activity, in this context, 4βOHC/C was frequently used. In our study, we found a significant difference in FT3 between DS and non-DS $p<0.05$, but there was no correlation between FT3 with either 4β-OHC or 4β-OHC/C (using Spearman correlation test), this indicates that FT3 is not a confounding factor in our phenotyping CYP3A4/5.
Some studies have shown that body weight is negatively correlated with $4\beta$OHC/C ratio in healthy volunteers and that obese patients have lower $4\beta$OHC levels than healthy controls. Studies on the clearance of several other CYP3A substrates suggest that CYP3A activity is reduced by 10-35% in obese patients. Further-more, Ulvestad et al. reported a strong negative correlation between body mass index (BMI) and CYP3A protein expression in the liver and intestines. Altogether, this indicates that CYP3A activity decreases with increasing body weight. In our study, DS children expected to have higher BMI compared to non DS which may contribute to the observed lower CYP450 activity.

In the case of high interpatient variability in the measured serum cholesterol levels, the ratio of $4\beta$-OHC/C should be used as a marker for CYP3A4/5 activity.

Concerning $4\beta$-OHC/C molar ratio in both DS and non-DS, the median (IQR) in DS was 0.19 (0.17) ($\times 10^{-4}$), while in non-DS the corresponding value was (0.45-$10^{-4}$) (Table 3). The DS group exhibited a significantly lower median molar ratio ($p<0.001$) compared to non-DS. No significant difference between females and males in CYP3A4/5 activity in DS or non-DS was observed.

Based on the above findings, we suggested that DS children exhibited lower CYP3A4/5 activity compared to non-DS children. Our finding supports and explains the reduced clearance of certain drugs whose metabolism is mediated by CYP3A4. Palle et al. reported lower median etoposide clearance in DS children (13.6 ml/ min/ m$^2$) compared to that of non DS children (17.6 ml/ min/ m$^2$).

A study done by Zwaan et al. confirmed that cells of DS children with Acute myeloid leukemia (AML), AML possess greater sensitivity to several chemotherapeutic agents such as cytarabine, etosopide, busulfan, and prednisolone. These studies did not explain the higher incidence of adverse effects in DS children. The disposition of all the previously mentioned drugs involves CYP 450 metabolism. Given our findings of low CYP3A4/5 activity in DS children, we strongly suggest that reduced clearance of chemotherapeutic drugs in DS has a major contribution to both a better prognosis and a higher incidence of adverse effects to chemotherapeutic agents which are the substrate for CYP3A4/5 enzymes.

Infants with DS showed prolonged theophylline clearance. Theophylline metabolism in the liver is known to be mainly mediated by CYP1A2 while at higher plasma concentrations CYP2E1 is also involved. We suggested that CYP1A2 and CYP2E possibly have reduced activity, in DS patients that require further investigations.

Limitations
The study has limitations of not determining the body weight at the time of sampling; and lack of genotyping analysis of CYP3A4.

Conclusion
CYP3A4/5 activity was determined by using $4\beta$-OHC/C in DS and non-DS children, non-DS children show 2.4 fold higher CYP3A4/5 activity comparing to DS. We recommend that special precaution with medications that depend on CYP3A4/5 should be followed in DS children. Further studies are needed to phenotype other CYPs in DS, as well as genotyping of these enzymes.

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تحديد النشاط الظاهري لإنزيمات الأيض في أطفال متلازمة داون باستخدام مؤشر حيوي داخلي

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الخلفية: متلازمة داون (م د)؛ من الأمراض الوراثية الأكثر شيوعًا في البشر وغالبًا ما يؤثر على أجهزة وأعضاء الجسم المختلفة. يستخدم أفراد (م د) العديد من الأدوية. هناك فجوة معقدة حول مدى مساهمة بعض إنزيمات الأيض في CYP3A4/5 في احتفاظ الاستجابة السريرية للأدوية لدى أطفال م د.

الأهداف: دراسة النمط الظاهري (نشاط) إنزيمات الأيض في CYP3A4/5 (السيتوكروم 3 أ ف /) في أطفال (م د) باستخدام نسبة (4-بيتا-هيديروكسي كيتوستيرويل/كوليستيرويل (4β-OHC/C) كمؤشر حيوي داخلي لنشاط إنزيمات الأيض السابق ذكرا.

الطريقة: دراسة وصفية مستقبلية جُرت في عيادة (م د) بمستشفى جامعة الملك عبدالعزيز، السعودية. تم تأخذ عينات الدم لفحوصات وظائف الغدة الدرقية والكبد باستخدام التحليل المناعي الآلي وتحليل الكوليستيرويل و-4-هيديروكسي كوليستيرويل باستخدام كروماتوغرافيا الغاز.

النتائج: أشتملت الدراسة على 16 (م د) و29 طفلًا طبيعيًا (1-16 سنة). جميعهم من العرب وأكثرهم سعوديون. أظهر الأطفال الذين يعانون من (م د) انخفاضًا ملحوظًا في أيض الكوليستيرويل مقارنة بالأطفال الطبيعيين، كما لوحظ أن لديهم زيادة (غير خطيرة) في مستوى بعض إنزيمات الكبد وفرط كوليستيرويل الدم.

الخلاصة: اثبَت البحث أن نشاط إنزيمات الأيض (السيتوكروم 3 أ ف /) منخفضًا بمقدار ضعفي لدى الأطفال المصابين بـ (م د) مقارنة بالأطفال الطبيعيين، مؤكداً أن هناك حاجة إلى مزيد من الدراسات، ومع ذلك، يجب استخدام الأدوية بحذر مع أطفال (م د).