THREE PATTERNS OF CYTOCHROME P450 GENE EXPRESSION DURING LIVER MATURATION IN MICE

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Running Title: Developmental gene expression patterns of mouse Cyps

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Discussion: 1042 words

Abbreviations: Cyp, Cytochrome P450
Abstract

The neonatal period of liver development is an often overlooked phase of development. For instance, ontogeny of xenobiotic metabolizing enzymes can markedly affect biotransformation as the liver matures. To systematically examine the ontogenic gene expression patterns of cytochrome P450 genes (Cyps) in mice, the gene expression profiles of 19 xenobiotic metabolizing Cyps in Cyp1-4 families were determined. The mRNA levels in C57BL/6 mouse livers were quantified using branched DNA technology at the following ages: gestational day 17 (2 days before birth), and postnatal days 0, 1, 3, 5, 10, 15, 20, 30, and 45. Among the 13 Cyp genes expressed in mouse livers, three distinct ontogenic expression patterns were identified by cluster analysis. Genes in Group 1 (Cyp3a16 as well as 3a41b in male) were expressed in the perinatal period, but were essentially non-detectable by 30 days of age. Genes in Group 2 (Cyp2e1, 3a11, and 4a10 as well as 3a41b in female) quickly increased after birth and reached maximum expression levels by day 5. Genes in Group 3 (Cyp1a2, 2a4, 2b10, 2c29, 2d22, 2f2, 3a13, and 3a25) were expressed at low levels until day 10-15, but markedly increased at day 20 to a high and stable level. In conclusion, the developmental expression of Cyps in mouse liver can be divided into three patterns, suggesting different mechanisms responsible for the expression of Cyps during liver maturation.
Introduction

About three-quarters of drugs primarily cleared via metabolism are biotransformed by members of the cytochrome P450 (CYP) superfamily. Humans have approximately 57 functional CYP enzymes, of which about 20 members in CYP1, 2, 3, and 4 families are likely involved in drug metabolism. It has been known since the 1950’s that CYP enzymes are not mature at birth (Fouts and Adamson, 1959), however at that time it was thought that there was only one, or perhaps two CYP enzymes. Since the cloning of the CYP genes, ontogenic expression of some CYP genes in human livers has been reported (de Wildt et al, 1999; Koukouritaki et al, 2004; Blake et al, 2005; Leeder et al, 2005; Gaedigk et al, 2006; Hines, 2007). Based on hepatic ontogenic gene expression patterns, human drug metabolizing CYP genes have been categorized into three developmental patterns (Hines, 2007). Considerable differences in the ontogenic gene expression contribute to variation in drug metabolism between children and adults. These differences result in children being less responsive to drug therapy and higher risks for adverse reactions for some drugs metabolized by CYP enzymes.

Variation in CYP gene expression among different individuals at different ages are due not only to ontogeny, but also to genetic polymorphisms, environmental factors (e.g. diet, toxicants), and therapeutic agents (e.g. drugs). Moral, ethical, and technical limitations for studying human fetal and neonatal samples have precluded an in-depth understanding of the mechanisms controlling ontogenic expression of human CYP genes (Rowell and Zlotkin, 1997). Therefore, to understand the factors and mechanisms governing the developmentally programmed CYP gene expression patterns, it would be advantageous to have a laboratory animal model that can minimize the influences of genetics, environment, and scarcity of tissues.
Mice have been used to study ontogenic expression of Cyp genes (Choudhary et al, 2003, 2005). These studies examined the expression of 40 Cyp genes in embryonic developmental stages from E7- E17 and in eight adult tissues. Some xenobiotic metabolizing Cyp genes are not expressed in embryonic developmental stages, but expressed in adult livers. However, there is a lack of expression data for the xenobiotic metabolizing Cyp genes during the postnatal period (i.e. after birth but before adult).

Mouse liver originates from the gut endoderm on embryonic day 8.5 (Douarin, 1975; Houssaint, 1980). One day later, the liver assumes the morphological appearance of hepatoblasts, capable of differentiating into either hepatocytes or bile duct epithelial cells (Rogler, 1997; Shiojiri and Mizuno, 1993). Hematopoietic stem cells migrate to the fetal liver at embryonic day 10.5 (E10.5) to generate numerous hematopoietic cells (Medvinsky and Dzierzak, 1996; Mukouyama et al, 1998; Sanchez et al, 1996). Thus, from E10.5 to E15, fetal liver is predominately a hematopoietic organ. By E14.5, immature hepatoblasts begin differentiate into hepatocytes and bile-duct epithelial cells (Douarin, 1975; Houssaint, 1980). At gestation day 17, only 30% of liver is devoted to hematopoiesis (Marie and Cresteil, 1989; Sasaki and Sonoda, 2000), although hematopoietic stem cells are still observed in liver until postnatal day 5 (Apte et al, 2007). From the day of birth (19 days post coitus) to postnatal day 9, the liver mass increases >3 folds (Sasaki and Sonoda, 2000). Rapid cellular proliferation between postnatal day 5 and day 20 has also been reported, and by postnatal day 30, mouse livers are histologically identical to livers of mice at 3 months (Apte et al, 2007). However, much is not known about how and when the xenobiotic metabolizing Cyp genes become activated or suppressed after hematopoiesis but before adulthood. Understanding the regulation of ontogenic expression is important since...
ontogeny affects both the expression and inducibility of xenobiotic metabolizing genes during postnatal liver development (Fouts and Adamson, 1959; Rich and Boobis, 1997).

Mice have over 102 CYP enzymes (Nelson et al, 2004), however the exact number involved in drug metabolism is not entirely clear. In the present study, 19 mouse Cyp genes from families 1-4 were selected based on their sequence homology to the 19 human drug metabolizing CYP genes (Table 1). Gene expression levels were quantified from gestational day 17 (day -2) to day 45 of ages using a branched DNA signal amplification assay. Because of lack of specific antibodies against most of the mouse Cyp proteins, gene expression at the protein level was not determined in this study. The data indicate that, in mice, there are also three developmental patterns of Cyp expression. Some mouse Cyp genes are similar, but others are not similar to their human homologous CYP genes.

Methods

Animals. Eight-week old C57BL/6 breeding pairs of mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed according to the American Animal Association Laboratory Animal Care guidelines, and were bred under standard conditions in the Lab Animal Resources Facility at the University of Kansas Medical Center. The use of these mice was approved by the Institutional Animal Care and Use Committee. Breeding pairs were set up at 4:00 pm, and separated at 8:00 am the following day. The body weights of the females were recorded each day to determine pregnancy status. Livers from male and female offspring were collected at the following ten ages: day -2 (gestational day 17), 0, 1, 3, 5, 10, 15, 20, 30, and 45 days of age (n=5), which represents periods of prenatal (day -2), neonatal (day 0 to 10), juvenile (day 15 to 30), and young adult (day 45). Due to the difficulty in distinguishing gender
from day -2 to day 5 mice, livers from male and female offspring of the same litter were pooled
at each age to achieve the desired amount of tissue. From days 10 to 45, male and female liver
samples were separated (5 males and 5 females per age). Livers were frozen immediately after
removal in liquid nitrogen, and stored at -80°C.

**Total RNA extraction.** Total RNA was isolated using RNAzol B reagent (Tel-Test Inc.,
Friendswood, TX) according to the manufacturer’s protocol. RNA concentrations were
quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at
a wavelength of 260 nm. The integrity of total RNA was evaluated by formaldehyde-agarose gel
electrophoresis, and confirmed by visualization of 18S and 28S rRNA bands.

**Cyp mRNA levels quantified by the Multi-plex Suspension Array.** The mouse Cyp1a1,
1a2, 1b1, 2a4, 2b10, 2c29, 2c39, 2c66, 2d22, 2e1, 2f2, 2j6, 3a11, 3a13, 3a16, 3a25, 3a41, 4a10,
and 4f18 mRNA levels were quantified by the Multi-plex Suspension Array according to the
manufacturer’s protocol (Panomics, Fremont, CA). Briefly, individual bead-based
oligonucleotide probe sets, specific for each gene examined, were developed by Panomics (panel
ID: 20044). The genes and accession numbers are listed in Table 1, and also available at
[www.panomics.com](http://www.panomics.com). Three types of oligonucleotide probes were utilized: capture extenders,
ligation extenders, and blockers. The capture extenders discriminate the target RNA via
cooperative hybridization among the different capture beads within the bead array. On the first
day, 6 μg of total RNA was loaded in each well, and incubated over-night with bead-based probe
sets. On the second day, signal amplification was mediated by amplification molecules that
hybridize to the tail of the ligation extenders. Each amplification unit contains multiple
hybridization sites for biotinylated label probes that bind Streptavidin-conjugated R-
Phycoerythrin (SAPE). The resulting fluorescence signals were analyzed using a Bio-Plex 200
System Array reader with Luminex 100 X-MAP technology, and data were acquired using Bio-
Plex Data Manager Software Version 5.0 (Bio-Rad, Hercules, CA). All data were standardized
to the internal control, Gapdh, and reported as mean fluorescence intensity of specific mRNA
normalized to Gapdh mRNA. The cutoff value for whether or not a gene is expressed at a
detectable level was conservatively set at 1% of *Gapdh* expression level. Information on method
validation is available at www.panomics.com.

**Statistical analysis.** Statistical differences of mRNA levels between male and female mice
were determined at ages after day 5 by the Student’s *t*-test (JMP v. 7.0, SAS Institute, Cary, NC).
Gene expression data from either male or female mice were analyzed using a two-way
hierarchical clustering method (JMP v. 7.0) using Ward’s minimum variance and displayed as a
dendrogram.

### Results

The NCBI HomoloGene Database
(http://www.ncbi.nlm.nih.gov/sites/entrez/query.fcgi?db=homologene) was interrogated to find
homologous genes in mice that are conserved for human CYP genes involved in drug
metabolism. Table 1 lists the human CYP genes queried and their murine homologous Cyp
genes, GeneID, mRNA accession, protein accessions, and identity of DNA and protein
sequences. Homologous sequences for human CYP2A6 and CYP2C9 were not annotated in
mice. Most human CYP genes have only one homologous mouse gene, but some human CYP
genes have multiple mouse homologues. For examples, *CYP2A13* is homologous to Cyp2a4 and
2a5, *CYP2C8* to Cyp2c66 and 2c65, *CYP3A4* to Cyp3a11, 3a41a, 3a41b, and 3a44, *CYP3A43* to
Cyp3a25 and 3a57, while *CYP4A11* is homologous to Cyp4a10 and 4a32. Only one mouse
homologue was selected for each human CYP gene, except two Cyp3a, Cyp3a11 and Cyp3a41b, for CYP3A4, which encodes the most important CYP enzyme for xenobiotic metabolism.

**Ontogenic gene expression patterns.**

**Cyp1 family.** The mRNAs of Cyp1a1, 1a2, and 1b1 in mouse liver samples were examined but only Cyp1a2 was detected. The developmental profile for Cyp1a2 mRNA is shown in Figure 1. Cyp1a2 expression gradually increased between 1 and 20 days of ages. Between 20 and 45 days of ages, Cyp1a2 mRNA remained relatively constant.

**Cyp2 family.** mRNA levels were detectable in perinatal mouse livers for Cyp2a4, 2b10, 2c29, 2d22, 2e1, and 2f2, but not for 2c29, 2c66, and 2j6. As shown in Figure 2, Cyp2a4, 2c29, and 2f2 had similar expression profiles in that they were not expressed or expressed at low levels until day 10 or 15, and then dramatically increased to reach their respective plateaus. In contrast, Cyp2b10 was low at all ages, but increased steadily over the first 20-30 days of life, reaching only ~10% of Gapdh expression at days 30 and 45. Cyp2d22 displayed a similar expression profile as Cyp1a2, with expression gradually increasing from day -2 to 20 days of age, followed by a relatively constant plateau from day 20 to day 45. A distinct developmental expression was observed for Cyp2e1 in which the mRNA increased sharply from day -2 to day 0, and remained relatively constant thereafter. Sexually dimorphic expression was observed after day 30 for Cyp2a4, with higher expression in female than in male mice. Cyp2c66, and 2j6 were non-detectable in the mouse livers at all ages.

**Cyp3 family.** Three general profiles of ontogenic development were also observed for the Cyp3a isoforms in mouse liver (Fig. 3). Cyp3a11 increases markedly between day -2 days and day 0, then remains constant until day 15, when it increases again to another steady level. Cyp3a13 and 3a25 show a similar increase in expression shortly after birth, decrease from days 3
to 5, and then increase to steady-state levels by day 20. Cyp3a16 is expressed around birth, increases at day 0, remains relatively constant until day 20, and then decreases to a nearly undetectable level by day 45. This expression profile was also quantified by Real-Time PCR with a similar ontogenic gene expression pattern (data not shown). Cyp3a41 in male mice have a similar expression profile as Cyp3a16, in that it is expressed high before birth and increased markedly during the first three days of life. However, the decrease in Cyp3a41b in female mice was not maintained, instead it increased at 30 days of age. The gender-difference data agree with the previous funding that Cyp3a41b is a female specific enzyme in adult mouse liver (Sakuma et al., 2002).

Cyp4 family. Cyp4a10 was expressed at very low levels at day -2 and increased at day 1. This surge at day 1 gradually declined throughout the next several days until day 30, when another surge in expression was noted. Although Cyp4a10 was detected in perinatal livers at high levels (Fig. 4), Cyp4f18 was very low at all ages (data not shown).

Hierarchical Clustering. The expression data from male and female genes were analyzed to determine the correlation of gene expression patterns among the Cyps and to estimate the age that reflects a dramatic change in Cyp gene expression in liver. Of the 13 Cyp genes found to be expressed in liver and separated by gender, two-way clustering of the gene expression patterns with respect to age revealed three distinct clades of genes, which we simply define as Groups 1, 2, and 3.

In males, Group 1 consists of Cyp3a16 and 3a41b; Group 2 includes 2e1, 3a11, and 4a10; and Group 3 has 1a2, 2f2, 2a4, 2c29, 2d22, 3a25, 2b10, and 3a13. (Fig. 5A). According to these data, the largest correlation distance with respect to age (as shown by the clade separation on the dendrogram scale) is observed between days 15 and 20. The mean relative expression levels for
members of these groups are plotted on the expression/time curve in Figure 5B. On average, Group 1 genes are expressed higher than Groups 2 and 3 genes before birth (day -2), and increase substantially until day 3, but then decrease and remain relatively constant until 15 days old, and then decreasing to non-detectable levels after 30 days. Group 2 genes are also expressed at very low levels on day -2, but their gene expression increases more rapidly after birth, essentially reaching maximal expression levels by day 5. Group 3 genes are expressed at low levels at day -2, remain at low levels through day 10, but dramatically increase to a plateau at day 20.

Female mice also fall into three distinct groups. Group 1 only has Cyp3a16; Group 2 includes 2e1, 3a11, 3a41b, 4a10; Group 3 contains Cyp1a2, 2a4, 2f2, 2b10, 2c29, 2d22, 3a13, and 3a25 (Fig. 6A). Similarly as with males, the largest correlation distance of the Cyp gene expression levels on the dendrogram scale with respect to age is between 15 and 20 days of age. Most of the genes in female mouse liver clustered into the same groups as males, thus, it is not surprising that their average expression profiles are also similar (Fig. 6B). Only Cyp3a41b exhibits significant gender differences, and it belongs to Group 2 in females, but Group 1 in males.

Discussion

The ontogenic expression patterns of 19 xenobiotic metabolizing Cyp genes in C57BL/6 mouse liver were characterized throughout postnatal liver maturation using a sensitive and quantitative technique, without potentially biasing amplification steps. Thirteen of the 19 Cyp genes were classified as being expressed during postnatal liver maturation. Three expression patterns were identified in both male and female mice by two-way clustering analysis (Figs. 5B
and 6B). Group 1 genes are restricted to the perinatal period, and essentially disappear in the adult. Group 2 genes are expressed at low levels before birth, but increase soon after birth, and reach their maximum expression by postnatal day 5. Group 3 genes are expressed at low levels until day 10-15, but markedly increase at day 20 to a stable high level thereafter. Only Cyp3a41b shows different mRNA expression patterns between male and female mice, and is a female-specific enzyme in adult livers.

Based on hepatic ontogenic gene expression patterns, human CYP genes have also been categorized into three groups (Hines, 2007), but they are not completely same as the mouse patterns reported in this study. CYP genes in the first group, such as CYP3A7, are expressed at high levels during prenatal and neonatal stages, and then are silenced or expressed at low levels at 1-2 years after birth (de Wildt et al., 1999; Lacroix et al., 1997; Stevens et al., 2003). Mouse Cyp genes in Group 1 identified in this study have a similar expression pattern. Although not as highly expressed as CYP3A7 in human fetal liver, mouse Cyp3a16 (and Cyp3a41b in males) appear to be best correspondence to the human CYP3A7 profile, but on a dramatically condensed time-scale. Human CYP genes in Hines’ second group, including CYP3A5 and CYP2C19, are expressed at highly variable levels throughout development, but generally independent of age (Wrighton et al., 1990; de Wildt et al., 1999; Stevens et al., 2003). No mouse Cyp genes in the current study were expressed similarly to human CYP3A5 or CYP2C19. Human CYP genes in the third group, such as CYP2C9, CYP2D6, CYP2E1, and CYP3A4, are not expressed or are expressed at low levels in the prenatal stage, but substantially increase their gene expression within the first 1-2 years of life (de Wildt et al., 1999; Hines, 2007; Koukouritaki et al., 2004; Stevens et al., 2008). Mouse Cyp genes in Group 2, including Cyp2e1, 3a11, and 4a10 (3a41b in female), are somewhat similar to the human Group 3 albeit in a dramatically condensed time-
For the majority of mouse Cyps examined (Group 3), the data show an alternative pattern not observed in human CYPs, where expression is relatively low until days 10-15, but then dramatically increases to adult levels by day 20.

Perhaps the most intriguing results are the marked differences in the expression profiles of the three groups of Cyps between days 15 and 20. Although purely speculative, one can envision that these gene expression changes observed between 15 and 20 days are in response to dietary changes, because mice transition from milk to chow during this period. If this hypothesis holds true, then dietary factors may serve as signaling molecules to initiate a cascade whereby the liver becomes capable of responding to its environment; highlighting the importance of diet on gene expression patterning. Further studies including measuring levels of individual Cyp protein isoforms and catalytic activities will be required when such technologies become available for mice.

The current study generates complementary knowledge on gene expression profiles during liver maturation to a previous study on the expression profiling of 40 mouse Cyp genes in embryonic and adult tissues (Choudhary et al., 2003). The previous study examined the expression patterns of 40 Cyp genes in four embryonic developmental stages (E7, E11, E15, and E17) and 8 adult tissues (8-12 weeks). The two sets of data together provide a complete picture of gene expression patterns of 12 Cyp genes through embryonic development (E7 to E17 before birth) and postnatal maturation (day 0 to day 45), to adult (older than 8 weeks). The two sets of data have good concordance in the most examined Cyp genes with one exception. Choudary et al. indicated Cyp1a1 as being expressed in adult mouse liver, whereas, this set of data indicates it is not expressed in liver of adult mice. Further interrogation of the SymAtlas Gene expression database [http://symatlas.gnf.org/SymAtlas/ (Su et al., 2004)], a web-application for publishing
experimental gene expression datasets, support our findings for absence of gene expression of Cyp1a1 in adult mouse liver. Furthermore, it is well recognized that CYPIA1 is expressed at very low levels in human livers (Westerink et al., 2008), but remains highly inducible (Drahushuk et al., 1998; Olinga et al., 2008; Pushparajah et al., 2008). Because Choudary et al. used pooled samples from BALB/c mice, the possibility exists that the conflicting data is the result of different mouse strains used. The current study also provides ontogeny of gene expression during liver maturation in the Cyp2c66, 2c39, 2e1, 2j6, 3a16, 3a41b, and 4f18 genes, which were not included in the Choudary studies.

The similarity in expression between mouse and human Cyp homologues has previously been reported for CYP1A1, CYP1A2, and CYP2E1 (Choudhary et al., 2005); however these comparisons were only made during fetal and adult stages, rather than determining patterns of expression occurring during the neonatal period. The ontogenic profiles of Cyp gene expression in this study yield insight into the functional transition period of the liver in a critical developmental stage: after hematopoiesis and before adult.

Taken together, these data indicate at least three different patterns of gene expression exist in xenobiotic metabolizing Cyp enzyme genes in mice. However, the mechanisms controlling the developmental patterning of Cyp expression has not yet been determined. It is also not clear whether human CYP expression is regulated through similar mechanisms as mouse Cyps. Nevertheless, it is interesting to ponder why some Cyp genes are expressed in the perinatal period (e.g. Cyp3a16) whereas others are not expressed until much later (e.g. Cyp1a2, 2a4, 2f2). No doubt, the epigenetic environment, such as DNA methylation and histone modifications, may shed clues into the observed ontogenic expression patterns. The mechanisms responsible for the
regulation of these expression patterns may be of considerable importance in understanding kinetics of xenobiotic metabolism during the neonatal period.

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References


Footnotes

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Figure Legends

Figure 1. Gene expression profile for Cyp1a2. Each bar represent the mean from n=5 animals per age. Mice aged between days -2 to 5 were not separated by gender (see text) and are denoted as a mixed population. Error bars indicate standard errors. Units are expressed relative to Gapdh.

Figure 2. Gene expression profiles for Cyp2a4, 2b10, 2c29, 2d22, 2e1, and 2f2. Bars represent the mean from n=5 animals per age. Mice aged between days -2 to 5 were not separated by gender (see text) and are denoted as a mixed population. Error bars indicate standard errors. Differences (p<0.05) in expression levels between males and females are indicated by an asterisk.

Figure 3. Gene expression profiles for Cyp3a11, 3a13, 3a16, 3a25, and 3a41b. Bars represent the mean from n=5 animals per age. Mice aged between days -2 to 5 were not separated by gender (see text) and are denoted as a mixed population. Error bars indicate standard errors. *p<0.05.

Figure 4. Gene expression profile for Cyp4a10. Bars represent the mean from n=5 animals per age. Mice aged between days -2 to 5 were not separated by gender (see text) and are denoted as a mixed population. Error bars indicate standard errors.

Figure 5. A) Hierarchical clustering of expression profiles for 13 Cyp genes expressed in livers of male C57/BL6 mice. The two trees describe the relationship between different gene expression profiles (left tree) and different ages (bottom tree). The dendrogram scale represents the correlation distances. Average expression from five animals per age is given by shaded
squares. Dashed lines categorize the grouping of expression patterns into either Group 1, 2, or 3.

B) Ontogenic profiles of average gene expression levels among the different groups.

Figure 6. (A) Hierarchical clustering of gene expression profiles for 13 Cyp isoforms expressed in livers of female C57/BL6 mice. The two trees describe the relationship between different gene expression profiles (left tree) and different ages (bottom tree). The dendrogram scale represents the correlation distances. Average expression from five animals per age is given by shaded squares. Dashed lines categorize the grouping of expression patterns into either Group 1, 2, or 3.

(B) Ontogenic profiles of the average gene expression levels among the different groups.
### Table 1. Human CYP genes responsible for xenobiotic metabolism, and their homologous mouse genes.

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<tr>
<td>CYP4A11</td>
<td>Cyp4a10</td>
<td>13117</td>
<td>NM 010011.2</td>
<td>NP 034141.2</td>
<td>79.3</td>
<td>77.6</td>
</tr>
<tr>
<td>CYP4F3</td>
<td>Cyp4f18</td>
<td>72054</td>
<td>NM 024444.1</td>
<td>NP 077764.1</td>
<td>82.2</td>
<td>81.5</td>
</tr>
</tbody>
</table>

-- indicates no data in HomoloGene
Figure 1

Relative expression of Cyp1a2 over age (days):

-2 0 1 3 5 10 15 20 30 45

M+F M F
Figure 4

Cyp4a10

Relative expression

Age (days)

-2  0  1  3  5  10  15  20  30  45

M+F  M  F
**Figure 6**

**A**

[Heatmap diagram showing gene expression levels across different age groups (Group 1, Group 2, Group 3).](#)

**B**

**Female patterns**

- **Maximum expression (%)**
  - Birth to Age (days)
  - Group 1
  - Group 2
  - Group 3

![Graph showing expression patterns over age](#)