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CHAPTER 6
A disease model for iron overload in patients affected by transfusion-dependent diseases

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Summary
The understanding of iron overload dynamics and its progression is essential to establish an adequate therapeutic intervention in patients affected by transfusion-dependent diseases. The main objective of this analysis is to develop a disease model for iron overload on the basis of available literature data. A thorough literature search was performed in Pubmed to retrieve all pertinent publications that would allow characterising the different aspects of the disease. At first, the turnover of serum ferritin in healthy individuals was described by an indirect response model. Subsequently, the effect of blood transfusions on serum ferritin levels was quantified according to an Emax model that depicts the non-linearity of the relationship. Finally, the relationship has been integrated as an additive conversion rate in the turnover model to account for disease progression. Internal model validation diagnostics were satisfactory and visual predictive checks reveal that the model provides an adequate and non biased description of the data. In conclusion, a disease model for iron overload was successfully developed. The relationship between blood transfusions and serum ferritin levels was quantified for the first time through a model-based approach. This model puts the basis for a more structured evaluation of therapeutic intervention in this patient population.
6.1 Introduction
Patients affected by transfusion-dependent diseases, such as beta-thalassaemia major or sickle cell disease, require regular red blood cell (RBC) transfusions to survive. Without the chronic transfusion regimen, patients would die before the third decade of life. Based on the Guidelines for the Clinical Management of Thalassaemia, transfusions should aim at maintaining a pre-transfusion haemoglobin (Hb) level between 9 and 10 g/dl and a post-transfusion level of 14 to 15 g/dl. The most common transfusion interval in these patients is once every two to four weeks (equal to two to three blood units per three weeks).

Iron overload in patients affected by transfusion-dependent diseases
As generally known, iron is recycled within the body and the body itself does not have the capacity to remove the excess of iron that is introduced from continuous blood transfusions. In normal conditions (Figure 1), iron entry into the cells is regulated by the uptake of iron-transport protein transferrin from the plasma. Chronic blood transfusions induce an increased iron exposure from macrophages, resulting into saturation of transferrin transport capacity. This leads to the release of Non-Transferrin Bound Iron (NTBI) in the plasma which can enter important cells (e.g., heart and liver cells) resulting over time into tissue accumulation. Iron is stored in tissues mainly into ferritin complexes. Once ferritin storage capacity is overwhelmed, small clusters of ferritin particles are formed and are degraded by the lysosomes leading to the formation of insoluble masses of hemosiderin. Over time this accumulation would cause severe organ damage.

Even though a significant improvement has been achieved in the management of the chronic transfusion regimen in the past decades, the therapy will eventually lead to a series of complications. Iron overload is the most common and relevant one and it is associated with several (lethal) co-morbidities such as cardiac dysfunction, liver fibrosis, hypogonadism, hypothyroidism, hypoparathyroidism and diabetes mellitus. Cardiac disease caused by myocardial siderosis is the most relevant complication, causing death in 71% of the patients affected by transfusion-dependent diseases.
In the absence of an innate mechanism that allows removing iron excess from the body, treatment with iron chelators is therefore essential to prevent iron accumulation and related complications \(^{22-25}\). Iron chelators possess overall a similar mechanism of action. They act by 1) preventing the uptake of NTBI into organs such as liver and heart; 2) chelating intracellular iron and thus preventing its corporation into ferritin; or 3) intercepting iron released from degraded ferritin \(^{26}\).
Clinical assessment of iron overload

There are several clinical measures to evaluate the disease state of iron overload. The most common is the biomarker serum ferritin, due to its strong correlation with total body iron stores \(^{27}\). As a single clinical endpoint however, serum ferritin is not always reliable. It could also be influenced by other factors such as inflammatory disorders and liver disease \(^{28}\). On the other hand, serial measurements of serum ferritin are still the easiest and least invasive method to evaluate iron overload and efficacy of chelation therapy. Other assessment methods for iron status focus more on tissue specific accumulation. Liver iron concentration is considered as the gold standard for the evaluation of iron overload due to a high correlation with total body iron accumulation \(^{29}\). However, determination of liver iron concentration requires an invasive technique with complications and risks of false negative results \(^{30}\). Magnetic Bio-Susceptometry (SQUID) is another option for measurement of liver iron accumulation \(^{31}\). However, it is only available in a limited numbers of centres worldwide. Furthermore, cardiac complications due to iron accumulation in the heart have been associated with 50-70% of deaths in thalassaemia major patients, mainly at young age \(^{32}\). Methods that were developed for cardiac monitoring were based on keeping serum ferritin and LIC level below a certain threshold (<2500 μg/L and <7 mg/kg dwt respectively) that was associated with decreased cardiac risks. However, this method proved not to be sufficient for effective intervention, since any dysfunctions were often identified at relative late stage. In recent years, Magnetic Resonance Imaging (MRI) techniques for assessing iron loading in the liver and heart have been introduced and validated for the evaluation of tissue specific accumulation \(^{33}\).

Iron overload is thus a rather complex process, and the understanding of the dynamics of the disease and its progression is essential for an adequate improvement of the therapeutic intervention. Several clinical questions are still not fully understood, e.g. how much time is required in order to observe a true response in the patient, or in order to reach clinically acceptable serum ferritin levels (i.e. about 2500 μg/L). As generally recognised, ferritin reflects what happens at the organ level only up to a certain threshold. Above this threshold other mechanisms intervene (inflammatory disorders, liver status) \(^{27,28}\) that influence the relationship between serum ferritin and body iron accumulation and the iron interchange between organs and the circulatory system. This project puts its main focus on the use of model-based approach to gain more insights in key factors that play a role in iron overload. The specific objective is to develop a disease model on the basis of available literature data. In particular we aim at quantifying the impact of blood transfusions on the changes in serum ferritin levels.
6.2 Methods

Data
A thorough literature search was performed in Pubmed to retrieve all pertinent publications that would allow the development of the disease model. Stepwise mining and pooling of published data was subsequently performed to characterise the different aspects of iron overload. Data published by Dawkins et al. \textsuperscript{34} were used to quantify the turnover of serum ferritin in healthy individuals; whereas clinical data published by Worwood et al., George et al. and Letsky et al. \textsuperscript{35} were pooled to evaluate the impact of blood transfusions on serum ferritin levels in untreated patients (i.e., patients not receiving chelation therapy).

Modelling
The software R (v.2.14.0) was used for statistical summaries and literature data extraction (complemented with R Digitize Package \textsuperscript{36}), as well as data manipulation and preparation for modelling purposes. Nonlinear mixed effects modelling was performed in NONMEM version 7.2 (Icon Development Solutions, USA).

Model building criteria included: (i) successful minimisation, (ii) standard error of estimates, (iii) number of significant digits, (iv) termination of the covariance step, (v) correlation between model parameters and (vi) acceptable gradients at the last iteration. Comparison of hierarchical models was based on the likelihood ratio test. Goodness of fit was assessed by graphical methods, including population and individual predicted vs. observed concentrations, conditional weighted residual vs. observed concentrations and time, correlation matrix for fixed vs. random effects, correlation matrix between parameters and covariates and normalised predictive distribution error (NPDE) \textsuperscript{37}.

The validation of the final model was based on graphical and statistical methods, including visual predictive checks \textsuperscript{38}. Bootstrap was used to identify bias, stability and accuracy of the parameter estimates (standard errors and confidence intervals). The bootstrap procedures were performed in PsN v3.5.3 (University of Uppsala, Sweden) \textsuperscript{39}, which automatically generates a series of new data sets by sampling individuals with replacement from the original data pool, fitting the model to each new data set.

Iron homeostasis in healthy individuals (basal ferritin turnover)
To quantify serum ferritin changes in healthy individuals, data from 14 subjects were extracted from literature \textsuperscript{34} and combined into a single dataset. Data are presented in Table 1 and Figure 2. A turnover model was tested to describe serum ferritin profiles in this population:

\[
\frac{dFERRITIN}{dt} = Kin - Kout \times FERRITIN
\]
where Kin is the basal zero-order production rate of ferritin and Kout is the basal first-order degradation rate of ferritin.

**Table 1.** Summary of serum ferritin levels in healthy individuals: data is presented by study duration.

<table>
<thead>
<tr>
<th>Study duration</th>
<th>Mean ± s.d</th>
<th>Range (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 weeks (N=9)</td>
<td>49.67 ± 25.95</td>
<td>8.53 – 97.60</td>
</tr>
<tr>
<td>24 hours (N=5)</td>
<td>67.71 ± 31.62</td>
<td>19.7 – 119.4</td>
</tr>
</tbody>
</table>

**Figure 2.** Serum ferritin changes over time in healthy individuals. Individual profiles in 14 healthy individuals presented as mean (solid line) and 5th and 95th percentiles (dashed lines). Left panel: serum ferritin profiles during an observational period of 7 weeks (N=9). Right panel: serum ferritin profiles during an observational period of 24 hours (N=5).
Relationship between serum ferritin and cumulative blood units (effect of transfusions)

Data containing serum ferritin levels in untreated patients were extracted and pooled from literature \(^{35,40,41}\) into a single dataset (Figure 3, right panel). Relevant information regarding the study population mentioned in the published articles is summarized in Table 2.

**Table 2.** Summary of serum ferritin levels and transfusion history in patients affected by transfusion-dependent diseases not receiving iron chelation therapy.

<table>
<thead>
<tr>
<th>Study</th>
<th>N</th>
<th>Serum ferritin (µg/L)</th>
<th>Transfusion history (cum. blood units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± s.d</td>
<td>Range</td>
</tr>
<tr>
<td>Overall</td>
<td>188</td>
<td>4271.55 ± 3003.76</td>
<td>353-18780</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>153.8 ± 106.42</td>
</tr>
<tr>
<td>Worwood et al.</td>
<td>116</td>
<td>5023.4 ± 2512.38</td>
<td>445-14120</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>193.1 ± 107.4</td>
</tr>
<tr>
<td>Letsky et al.</td>
<td>24</td>
<td>4902.63 ± 4603.18</td>
<td>447.4-18780</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>116.5 ± 96.3</td>
</tr>
<tr>
<td>George et al.</td>
<td>48</td>
<td>2694.64 ± 2694.64</td>
<td>353-9046</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>77.6 ± 43.05</td>
</tr>
</tbody>
</table>

**Figure 3.** Comparison of the distributions of serum ferritin levels in healthy individuals (left panel) and patients affected by transfusion-dependent diseases not receiving iron chelation therapy (right panel).
Information regarding the volume of blood per unit was only available for the work carried out by Worwood et al. (500 ml per unit) and by George et al. (350 ml per unit). To ensure that equal volume of blood per transfusion was taken into account for the entire cohort, cumulative amount of blood units was normalized to a volume of 500 ml per blood unit.

Given the non-linear nature of the relationship between serum ferritin and cumulative blood units, an Emax model was tested to describe the relationship as described below:

\[
FERRITIN = E_0 + \frac{E_{max} \times BU}{(E_{bu50} + BU)}
\]

where, \(E_0\) represents baseline serum ferritin levels when no transfusion has yet occurred, \(E_{max}\) the maximum serum ferritin levels at saturation and \(E_{bu50}\) the cumulative amount of blood units (BU) when 50% of saturation is reached.

Ferritin data were log transformed for the analysis and the whole dataset was randomly divided into two subsets, resulting into 2/3 of the data used for the model building and 1/3 of the data preserved for external validation.

**Integration of the effect of blood transfusions in the turnover model: disease model for iron overload**

Once the relationship between cumulative blood units and serum ferritin was quantified, it was our goal to integrate this information within the turnover model. Our intent was to translate the relationship into a rate that would affect the basal ferritin production rate. Given that information on time was not provided in the data used to quantify the relationship, and given that we were mainly interested in translating the population profile, we assumed a constant interval of three weeks between subsequent units of blood transfused. This is on average the case in patients affected by transfusion-dependent diseases\(^2,4,5,7,8\).

Assuming this constant time interval we performed a simulation-estimation analysis to quantify the impact of blood transfusions on the production rate of serum ferritin. The simulations were performed using the Emax model in the range of 5 to 450 cumulative blood units and subsequently the simulated data were fitted with the turnover model where basal \(K_{in}\) and \(K_{out}\) were fixed and a new production rate (\(CRT =\) conversion rate) was estimated (Figure 4). This rate was non-linearly correlated to actual ferritin levels according to the following equation:

\[
CRT = SCL \times e^{-SHP \times FERRITIN}
\]
where SCL is a scaling factor and SHP is the shape factor of the correlation. The conversion rate was integrated in an additive manner in the turnover model as follows:

\[
\frac{dFERRITIN}{dt} = Kin + CRT - Kout \times FERRITIN
\]

CRT represents the effect of the disease (chronic transfusion therapy) on the biomarker ferritin.

Figure 4. Stepwise integration of the effect of blood transfusions on serum ferritin levels in the turnover model developed on healthy subjects. Left panel: open circles represent the observed data and solid line represents the fitting of the relationship between cumulative blood units and serum ferritin levels. Right panel: negative relationship between serum ferritin conversion rate in the presence of chronic transfusion regimen and serum ferritin levels. The relationship has been derived from the one presented on the left panel assuming a constant time interval between consecutive blood units transfused.

6.3 Results

Iron homeostasis in healthy individuals (basal ferritin turnover)

An indirect response model was developed to describe basal serum ferritin turnover in healthy individuals. The zero-order production rate constant (Kin) and the first-order degradation rate constant (Kout) were successfully estimated; inclusion of inter-individual variability on Kin allowed a better description of the data. Parameter estimates and
bootstrap results are shown in Table 3. Goodness-of-Fit (GoF) (Figure 7, see Appendix) plots as well as Normalized Prediction Distribution Errors (NPDE) (Figure 8, see Appendix) confirm the suitability of the model in describing adequately the data.

**Table 3.** Final model parameters for turnover model of serum ferritin in healthy individuals.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Estimates</th>
<th>Bootstrap (mean)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kin (μg/day)</td>
<td>0.00625</td>
<td>0.00416</td>
<td>34.2</td>
</tr>
<tr>
<td>Kout (day⁻¹)</td>
<td>0.000137</td>
<td>0.0000875</td>
<td>32.7</td>
</tr>
<tr>
<td>Eta on Kin</td>
<td>0.367</td>
<td>0.329</td>
<td>40.8</td>
</tr>
<tr>
<td>Residual error</td>
<td>0.000658</td>
<td>0.000641</td>
<td>31.2</td>
</tr>
</tbody>
</table>

**Relationship between serum ferritin and cumulative blood units (effect of transfusions)**
An Emax model was used to describe the relationship between serum ferritin levels and cumulative blood units. Inter-individual variability was estimated on the Ebu₅₀ parameters. The model allowed accurately quantifying the relationship; final parameter estimates are provided in Table 4 together with the estimates obtained with the external set of data. Goodness of Fit (Figures 9 and 11, see Appendix) plots as well as NPDE (Figures 10 and 12, see Appendix) reveal that the model provides a suitable description of the data.

**Table 4.** Summary of estimated relationship between cumulative blood units and serum ferritin levels in patients affected by transfusion-dependent diseases not receiving iron chelation therapy.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Estimates</th>
<th>External Validation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₀ (ug/L)</td>
<td>5.81</td>
<td>5.62</td>
</tr>
<tr>
<td>Emax (ug/L)</td>
<td>9.17</td>
<td>8.88</td>
</tr>
<tr>
<td>Ebu₅₀</td>
<td>26.5</td>
<td>16</td>
</tr>
<tr>
<td>Eta on Ebu₅₀</td>
<td>0.554</td>
<td>0.63</td>
</tr>
<tr>
<td>Residual error</td>
<td>0.0075</td>
<td>0.15</td>
</tr>
</tbody>
</table>

**Integration of the effect of blood transfusions in the turnover model: disease model for iron overload**
At first, ferritin levels corresponding to a range of cumulative blood units of 5 to 450 were simulated with the Emax model previously developed. Secondly, the data were fitted using an integrated model that consisted of the turnover model where basal Kin and Kout were fixed to the values estimated in the healthy population and an additive ferritin production rate (CRT) that was estimated during this process. The conversion rate (CRT) was non-linearly correlated to actual ferritin concentration. Final parameter estimates are provided in Table 5, whereas a schematic representation of the model is shown in Figure 5.
Table 5. Summary of final parameter estimates for the disease model of iron overload: integration of the effect of blood transfusions on serum ferritin levels in the turnover model developed on healthy subjects.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Estimates</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{\text{in}}$ ($\mu$g/h)</td>
<td>0.0000208 FIX</td>
</tr>
<tr>
<td>$K_{\text{out}}$ (h$^{-1}$)</td>
<td>0.00000458 FIX</td>
</tr>
<tr>
<td>SHP (h$^{-1}$)</td>
<td>0.00026</td>
</tr>
<tr>
<td>SCL ($\mu$g/h)</td>
<td>0.383</td>
</tr>
<tr>
<td>Residual error</td>
<td>0.0014</td>
</tr>
</tbody>
</table>

Figure 5. Disease model for iron overload. Kin and Kout represent respectively the basal zero order production rate and first order degradation rate of ferritin in healthy individuals. CRT represents the serum ferritin conversion rate in patients undergoing chronic transfusion therapy, which reflects the impact of the disease (blood transfusions) on serum ferritin levels. The dashed line represents the negative feedback that serum ferritin has on CRT.

Given the nature of the simulation, it was only possible to quantify the mean population profile for the integrated model. Inter-individual variability was added in a systematic manner to evaluate whether the model would capture the variation in the original data. Visual predictive checks (with the inclusion of 50% variability on both SCL and SHP) show that the model allows describing the data in an adequate and not biased manner (Figure 6)
left panel). In addition, Figure 6 (right panel) shows simulated profiles of serum ferritin over a period of 10 years in a virtual patient not receiving iron chelation therapy. The simulations provide insights on how the impact of the disease changes when patients start at different baseline levels, and allow quantifying the true underlying disease progression.

**Figure 6.** Visual predictive check of the disease model for iron overload and simulated mean serum ferritin profile in virtual patients not receiving iron chelation therapy. Left panel: VPC of the disease model for iron overload. Observed data are plotted using open circles; the black solid line represents the median of the simulated data; the dashed lines represent the 5th and 95th percentiles of the simulated data. Right panel: simulated ferritin profiles over a period of 10 years for a virtual patient not receiving iron chelation therapy. Each line represents a different ferritin baseline level: the solid, dashed (small), dotted, dashed-dotted, and dashed (big) lines represent 1000, 2500, 5000, 7500, and 10000 ug/L baseline ferritin levels.
6.4 Discussion and Conclusion

With this work we attempt for the first time to use a model-based approach in the field of transfusion-dependent diseases. An indirect response model was first developed in healthy subjects to account for the basal turnover of serum ferritin. Subsequently, the relationship between serum ferritin and cumulative blood units was quantified and integrated in the turnover model, and the non-linearity of the system was properly captured. Once the effect of the chronic transfusion regimen is introduced in the model, the contribution of the basal turnover of serum ferritin becomes negligible; the conversion rate (CRT) becomes the driving force of the changes in serum ferritin levels and gives a clear idea of the magnitude of iron overload in the absence of chelation therapy (Figure 6, right panel). As depicted in the same figure, the model allows exploring the natural course of the disease without treatment intervention; without such a model it would not be possible to appropriately quantify the true effect of iron chelation therapy. In addition, the nature of the model allows evaluating the drug effect of any available or future chelating agent.

Limitations

The lack of access to individual data did not allow a proper characterisation of the inter-individual variability and/or of a thorough covariate analysis. On the contrary, we could appropriately quantify the mean population changes in disease progression. When integrating the Emax model with the turnover model, we assumed a constant time interval between subsequent units of blood transfused; the interval chosen was based on available literature data \(^2,4,5,7,8\). Even though there is inter- and intra-patient variation in the transfusion regimen, we believe that the literature data support our assumption given that we could only evaluate the mean population profile of the integrated model.

Conclusions

In conclusion, despite some limitations due to incomplete availability of data a disease model was successfully developed in patients affected by severe iron overload that were not undergoing iron chelation therapy. The impact of blood transfusions on serum ferritin levels was quantified allowing a more mechanistic interpretation of the underlying disease progression. This model provides the basis for a more structured evaluation of therapeutic intervention in this patient population and gives the opportunity for further evaluation of the disease and its progression.
References


Appendix

**Figure 7.** Goodness-of-fit plots for the turnover model in healthy individuals. Upper panels show the observed data (Obs) vs. population predictions (Pred) (left) and the observed data vs. individual predictions (IPred) (right). Lower panels show the conditional weighted residuals (CWRES) vs. population predictions (left) and the CWRES vs time (right).
Figure 8. NPDE summaries for the turnover model in healthy individuals. Upper panels show the QQ-plot of the distribution of the NPDEs for a theoretical N (0, 1) distribution (left) and the histogram of the distribution of the NPDE together with the density of the standard normal distribution (right). Lower panels show the NPDEs vs. time (left) and NPDEs vs. individual predictions (right).
Figure 9. Goodness-of-Fit plots of estimated relationship between cumulative blood units and serum ferritin levels in patients affected by transfusion-dependent diseases not receiving iron chelation therapy: model building. Upper panels show the observed data (Obs) vs. population predictions (Pred) (left) and the observed data vs. individual predictions (IPred) (right). Lower panels show the conditional weighted residuals (CWRES) vs. population predictions (left) and the CWRES vs IPred (right).
**Figure 10.** NPDE summaries of estimated relationship between cumulative blood units and serum ferritin levels in patients affected by transfusion-dependent diseases not receiving iron chelation therapy: model building. Upper panels show the QQ-plot of the distribution of the NPDEs for a theoretical N(0, 1) distribution (left) and the histogram of the distribution of the NPDE together with the density of the standard normal distribution (right). Lower panels show the NPDEs vs. time (left) and NPDEs vs. individual predictions (right).
Figure 11. Goodness-of-Fit plots of estimated relationship between cumulative blood units and serum ferritin levels in patients affected by transfusion-dependent diseases not receiving iron chelation therapy: external validation. Upper panels show the observed data (Obs) vs. population predictions (Pred) (left) and the observed data vs. individual predictions (IPred) (right). Lower panels show the conditional weighted residuals (CWRES) vs. population predictions (left) and the CWRES vs IPred (right).
Figure 12. NPDE summaries of estimated relationship between cumulative blood units and serum ferritin levels in patients affected by transfusion-dependent diseases not receiving iron chelation therapy: external validation. Upper panels show the QQ-plot of the distribution of the NPDEs for a theoretical $N(0,1)$ distribution (left) and the histogram of the distribution of the NPDE together with the density of the standard normal distribution (right). Lower panels show the NPDEs vs. time (left) and NPDEs vs. individual predictions (right).