Chapter 5:

A novel life span regulating locus at chr13q34 influencing serum triiodothyronine level

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1. Abstract

The genetic propensity for an attenuated thyroid function and lifespan extension co-occur in long-lived families and suggest the existence of a pleiotropic genetic mechanism underlying human longevity as well as attenuation of the thyroid function. Attenuation of the thyroid function is even more profound in long-lived sibships whose parents also exhibited an excess survival compared to their respective birth and sex cohorts, and thus suggests an enrichment of variants explaining the pleiotropic relation in sibships with this marked family history (FH(+)). Linkage analyses among all 415 nonagenarian sibships from the Leiden Longevity Study (LLS) identified suggestive linkage at chr13q34 (LOD=2.96) that was highly specific to the 239 FH(+) sibships (LOD=3.35). The FH(+) subset was characterized by significantly lowered levels of serum free triiodothyronine (fT3) and its interaction with prospective survival thereof. Subsequent fine mapping of the 1-LOD-drop 13q34 region by QTL analyses on serum fT3 levels using variants from whole genome sequencing data identified a fT3 lowering haplotype tagged by the rs9515460 minor C allele, that also associates with prospective survival. Hence, we hereby report chr13q34 as a novel locus harbouring variants underlying lifespan regulation as well as an attenuated thyroid function.

2. Introduction

Attenuation of the thyroid function has been implicated in life span extension in rodents[1-3], as well as in several large longitudinal human cohort studies[4-6]. Active thyroid hormone, triiodothyronine (T3), and its precursor, thyroxine (T4), are produced by the thyroid gland under influence of thyroid stimulating hormone (TSH), and are both key factors in the regulation of the basal metabolic rate and cardiac output. A mildly lowered thyroid function (subclinical hypothyroidism), marked by an increased serum level of TSH and a reduced level of free T4, has been associated with a lowered risk of cardiovascular disease from middle age onward[6] and a decreased cardiovascular mortality in the oldest old[4]. Interestingly, first-degree relatives of long-lived persons have, compared to the general population, an increased probability of becoming long-lived themselves[7-9], while exhibiting from middle age onward an attenuated thyroid function[10] and an improved cardiovascular health[7-9]. Moreover, attenuation of the thyroid function seems to be more profound in long-lived families with a family history of excess survival, as compared to long-lived families without such a marked family history[11]. These findings suggest a genetic mechanism for human longevity in which a life-long survival advantage, especially with respect to cardiovascular mortality, is promoted by attenuation of the thyroid function.

The relation of the active thyroid hormone itself with respect to cardiovascular mortality is less clear, possibly because serum T3 as a marker integrates cues from both the endocrine system, through conversion of T4, as non-thyroidal routes[12]. Parle *et al.* finds levels of free T4 (fT4) and free T3 (fT3) to inversely correlate with the level of TSH in hyperthyroid patients aged over 60 years, but observes a significant association with cardiovascular mortality only for TSH and not for fT4 nor for fT3[13]. In contrast, Gussekloo *et al.* finds significant associations with mortality for TSH, fT4 and fT3 in patients aged above 85, but only reports an inverse correlation between levels of TSH and fT4, and not for TSH and fT3[4]. More importantly, whereas lowered fT4 levels confer a protective effect, they find lowered levels of fT3 to convey an increased risk on mortality and argue that the association of fT3 with mortality can be explained by disability at baseline. Thus, whereas the findings of Parle *et al.* and Gussekloo *et al.* agree on the protective effect of high serum TSH levels, they disagree on the relation between thyroid parameters, thereby questioning the causality of fT3 in conveying the protective effect on life span regulation. Since the fT4 precursor also exhibits a very modest regulatory capacity, but is present in a much higher abundance, it raises the possibility that the life-prolonging effects of an attenuated thyroid function are either totally dependent on or directly transmitted trough serum fT4. Genetic research on the main parameters of the thyroid system e.g. TSH, fT4 and fT3, may shed light on the causal mechanism relating an attenuated thyroid function to life span regulation.

The genetics of thyroid homeostasis, human longevity and interactions thereof

have been studied with varying degrees of success. The genetic influences on the phenotypic variation of the main parameters involved in thyroid metabolism have been shown to be moderate to strong (30-60%)[11,14]. Furthermore, genome wide associations studies (GWASs) have identified over 60 genome-wide significant loci associated with serum levels of either TSH or fT4[15-19]. However, despite this strong evidence for genetic variation to influence the phenotypic variation in thyroid metabolism and the strong indications that attenuation of the thyroid system, at least with respect to TSH, leads to a prolonged life span, little evidence has been reported for genetic variations to affect both traits. An important exception is the work by Atzmon *et al*. reporting two common variants upstream of the TSH receptor gene (*TSHR*) to be enriched amongst the oldest old in the Askhenazi Jewish population, as compared to middle aged controls, while also marking increased TSH serum levels[20]. Neither of these two variants, however, was re-identified in any GWAS on either serum TSH levels[15-19] or human longevity[21-24], suggesting considerable heterogeneity in both traits. In summary, whereas genetic approaches have been able to relate common variants to phenotypic variation in each of TSH, fT4 and life span regulation separately, little evidence exists to date for genetic factors influencing both the thyroid metabolism and human longevity.

Here we study the genomes of members of long-lived families of the Leiden Longevity Study (LLS) for the presence of rare variants that explain the pleiotropic interactions between an attenuated thyroid function and human life span regulation. To this end, we focussed on nonagenarian sibships of the Leiden Longevity Study (LLS) with parents exhibiting the largest excess survival relative to their respective sex and birth cohort specific life expectancies[11]. Since long-lived sibships with this marked family history (FH(+)) exhibit an even more attenuated thyroid function, as compared to long-lived sibships without such a marked family history (FH(-))[11], we hypothesize the FH(+) subset to be enriched for genetic determinants underlying familial longevity by attenuating the thyroid function. To this end, we performed affected sibling pair analyses to identify genomic regions exhibiting linkage for familial longevity in the whole study, and show that one of the suggestive signals (chr13q34) is explained by significant linkage among the FH(+) sibships specifically. Since the FH(+) sibships are characterized by significantly lower fT3 levels, as compared to FH(-) sibships, which moreover seems to affect their prospect of survival, we hypothesize the 13q34 locus to harbour variants underlying human lifespan regulation by lowering serum fT3. Subsequent QTL analyses for loci associating with serum fT3 employing variants from whole genome sequencing data residing in chr13q34, identified a serum fT3 lowering haplotype tagged by the minor C allele of rs9515460_T<C. This serum fT3 lowering haplotype was also shown to associate with prospective survival in a 10 years follow up. Hence, we hereby report chr13q34 as a novel locus harbouring variants contributing to lifespan regulation as well as an attenuated thyroid function.

3. Results

3.1 Demographic and thyroid characteristics

Demographic and thyroid characteristics in all 415 nonagenarians sibships of the Leiden Longevity Study (LLS), the 239 sibships enriched for family history of extended survival (FH(+)), the 176 non-enriched sibships (FH(-)) and the 214 unrelated nonagenarians from enriched families selected for whole genome sequencing (SEQ_FH(+)) are given in Table 1 (Experimental Procedures 1). Previously, we showed that a family history of excess survival, as indicated by a lower Family History Score, significantly associated with higher levels of serum TSH and lower levels of serum fT4 and serum fT3[11]. Although all thyroid parameters show directions of correlation consistent with those reported earlier, the current dichotomization into two subsets of sibships yielded significant differences in baseline levels for serum TSH ($N_{FH(-)}$=344, $N_{FH(+)}$=482 β=1.06, 95% CI 1.00-1.12, p=0.039) and serum fT3 ($N_{FH(-)}$=348, $N_{FH(+)}$=492, β=-0.10, 95% CI -0.19--0.01, p=0.026), but not for serum fT4 (p=0.17, see Experimental Procedures 2 and 3.1). This suggests that in the current setting, we have most power to identify genetic determinants underlying lifespan regulation and an attenuated thyroid function through influencing either serum TSH or fT3 levels.

3.2 Prospective survival of study subsets and thyroid parameters

We compared the prospective survival above 90 years between the FH(-) and FH(+) subsets and between the FH(-) and the SEQ_FH(+) subsets, but found no significant differences, indicating that a family history of excess survival has a negligible effect on the survival beyond age 90 (Experimental Procedures 3.2). Except for a suggestive association in the FH(-) subset, serum TSH levels did not display any significant associations with prospective survival beyond 90 years, neither in the whole study nor for any of the analysed subsets of the LLS (Table 2). However, when investigating the influence of the thyroid parameters fT4 and fT3 on prospective survival, we observed significant associations for both these thyroid parameters in the whole LLS (ALL) and some interesting interactions with the FH strata (Table 2). The association of fT4 with prospective survival in the whole study was explained by the protective effect of low serum fT4 levels specifically observed in the FH(-) subset. In contrast, the association of fT3 with prospective survival in the whole study was explained by the deleterious effect of low serum fT3 levels most profoundly observed in the FH(+) subset, confirming the observation of Gussekloo *et al*. that low serum fT3 becomes detrimental in the oldest old (Figure 1). Joint modelling of fT4 and fT3 with prospective survival in the whole study and its subsets indicates that these associations are independent (data not shown). From these findings we conclude that the FH(-) subset seems to exhibit relations between the thyroid parameters and prospective survival that are very similar to those reported by Gussekloo *et al*. for the oldest old in the general population[1].

1 High TSH and low fT4 levels are beneficial, low fT3 levels are detrimental.

	ALL 415 sibships	FH(+) 239 sibships	FH(-) 176 sibships	SEQ_FH(+) 214 cases
Total # individuals	931 (37.4% male)	540 (36.7 % male)	391 (38.4% male)	214 (37.9% male)
Age at inclusion [years]	92.9 (91.5 – 94.8)	93.1 (91.5 – 95.1)	92.8 (91.4 – 94.7)	93.7 (91.8 – 95.5)
Follow-up time* [years]	3.4 (1.5 – 5.8)	3.4 (1.5 – 5.6)	3.4 (1.6 – 6.0)	4.0 (1.8 – 6.1)
Number of deaths [N, %]	863 (92.7%)	504 (93.3%)	359 (92.0%)	200 (93.5%)
Age at censoring [years]	97.1 (94.6 – 99.9)	97.2 (94.8 – 99.9)	97.0 (94.3 – 99.6)	97.9 (95.7 – 100.2)
TSH availability [N, %]	826 (88.7%)	482 (89.3%)	344 (88.0%)	206 (96.3%)
TSH [mU/L]	1.52 (0.98 – 2.42)	1.64 (1.03 – 2.61)	1.38 (0.93 – 2.15)	1.58 (1.04 – 2.43)
FT4 availability [N, %]	840 (90.2)	493 (91.3%)	347 (88.8%)	209 (97.7%)
FT4 [pmol/L]	15.9 (14.4 – 17.5)	15.8 (14.2 – 17.5)	16.1 (14.6 – 17.6)	15.9 (14.3 – 17.3)
FT3 availability [N, %]	840 (90.2%)	492 (91.0%)	384 (89.0%)	208 (97.2%)
FT3 [pmol/L]	4.0 (3.7 – 4.4)	4.0 (3.6 – 4.4)	4.1 (3.8 – 4.5)	4.0 (3.7 – 4.3)

* at February 2014

TABLE 1: BASELINE CHARACTERISTICS OF THE STUDIED STUDY SUBSETS. Baseline characteristics of the whole Leiden Longevity Study (ALL) and the currently defined subsets: FH(+): 239 nonagenarian sibships with a marked family history of an extended survival into old age; FH(-): 176 nonagenarian sibships without such a marked family history; SEQ_FH(+): 214 independent index cases selected from the 239 sibships exhibiting a marked family history (FH(+)) of whom the whole genome has been sequenced previously (**Chapter 4** of this thesis).

In contrast, the FH(+) subset, selected for their genetic propensity of excess survival, displays relationships between thyroid parameters and prospective survival that is governed solely by levels of serum fT3. Together these findings suggest that family history, as reflected by the FHS, is not so much a marker of prospective survival beyond age 90, but instead it may indicate a distinct mechanism through which the thyroid function may affect life span regulation.

	ALL 415 sibships	FH(+) 239 sibships	FH(-) 176 sibships	SEQ_FH(+) 214 cases
TSH				
Ndeath [%]	767 [92.9%]	451 [93.6%]	316 [91.9%]	194 [94.2%]
HR [95% CI]	0.86 [0.80-1.21]	1.22 [0.94-1.58]	0.75 [0.55-1.01]	1.20 [0.78-1.85]
p	0.85	0.14	0.059	0.40
FT4				
Ndeath [%]	780 [92.9%]	462 [93.7%]	318 [91.6%]	197 [94.3%]
HR [95% CI]	1.04 [1.01-1.07]	1.00 [0.96-1.04]	1.09 [1.04-1.14]	0.99 [0.92-1.07]
p	**0.013**	0.94	**1.4×10^{-4}**	0.827
FT3				
Ndeath [%]	781 [93.0%]	462 [93.9%]	319 [91.7%]	197 [94.7%]
HR [95% CI]	0.73 [0.64-0.83]	0.68 [0.56-0.82]	0.80 [0.65-0.97]	0.56 [0.41-0.77]
p	**4.25×10^{-6}**	**5.49×10^{-5}**	**0.023**	**2.55×10^{-4}**

Table 2: Prospective survival on thyroid parameters. Prospective survival on TSH, fT4 and fT3 in a 10 years follow up performed in the whole Leiden Longevity Study (ALL) and the currently defined subsets: FH(+): 239 nonagenarian sibships with a marked family history of an extended survival into old age; FH(-): 176 nonagenarian sibships without such a marked family history; SEQ_FH(+): 214 independent index cases selected from the 239 sibships exhibiting a marked family history (FH(+)) of whom the whole genome has been sequenced previously (**Chapter 4** of this thesis). HR indicates the hazard ratio for mortality.

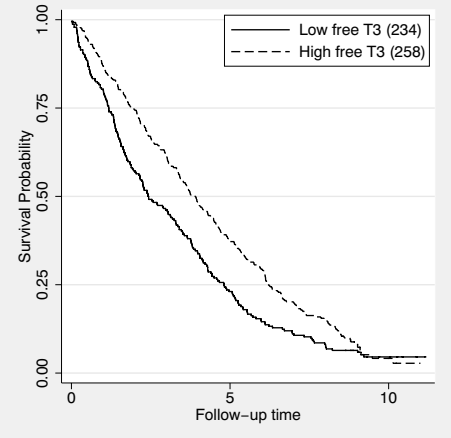

Figure 1: Prospective survival of fT3 in FH(+). Kaplan-Meier curves of fT3 in the 239 sibships with a marked family history of an extended survival into old age (FH(+)): A clear mortality risk is observed for subjects with low levels of fT3.

3.3 Linkage analysis identifies 13q34 as a longevity locus

To identify genomic regions harbouring variants predisposing to familial longevity and an attenuated thyroid function, affected sib pair analyses were performed on all nonagenarian sibships (ALL) and the FH(+) and FH(-) study subsets (Experimental Procedures 4). The locus displaying the largest disparity in linkage results between FH(+) and FH(-) in presence of suggestive linkage in the whole study was found at 13q34 (rs752342 at 120 cM; LOD_{ALL}=2.96; $LOD_{FH(+)}$=3.35; $LOD_{FH(-)}$=0.28, Figure 2 and Supplemental Figure 1). The 1-LOD-drop interval in the FH(+) subset is 8.36 cM at chr13:110,823,340-113,522,717 (GRCh37/hg19 coordinates) and harbours 16 RefSeq genes: (part of) *COL4A1*, *COL4A2*, *COL4A2-AS1*, *RAB20*, *CARKD*, *CARS2*, *ING1*, *LINC00346*,

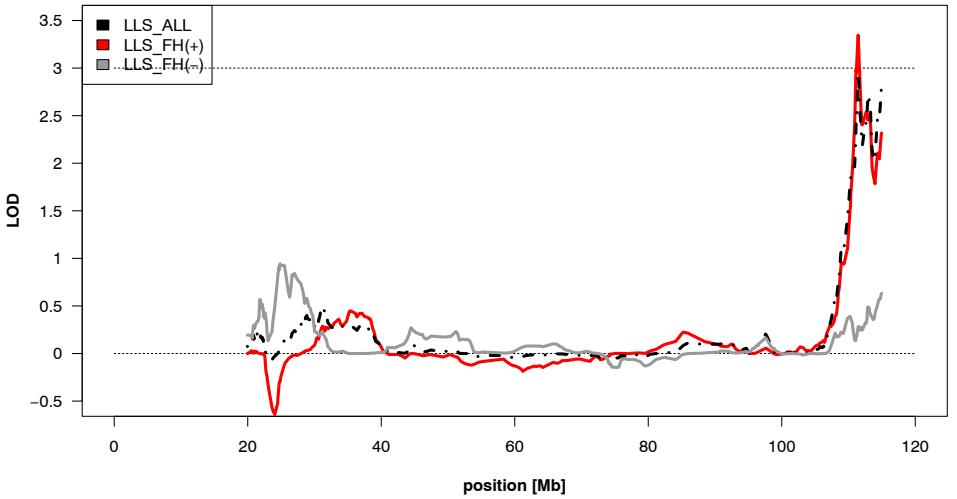

FIGURE 2: LINKAGE RESULTS AT CHR13Q34. Significant linkage for familiar longevity was established at locus 13q34 in the FH(+) subset. Linkage was computed using the 239 sibships with the most profound family history of longevity (FH(+): N=540, LOD_{max}=3.35 at rs752342, red), all 415 sibships (ALL: N=931, $LOD_{rs752342}$=2.96, black dotted), and the remaining 176 nonagenarian sibships without a marked family history (FH(-): N=391, $LOD_{rs752342}$=0.28, grey).

ANKRD10, *ARGHEF7*, *TEX29*, *SOX1*, *SPACA7*, *TUBGCP3*, *C13orf35* and (part of) *ATP11A* (Supplemental Figure 2 and Supplemental Table 1). Linkage at chr13q34 is highly specific to the FH(+) subset, which are more hypothyroidal as compared to the FH(-) subset, e.g. higher TSH and lower fT3, and exhibit a thyroid prospective survival profile solely depending on serum fT3. We therefore hypothesize that this locus contributes to a family history of excess survival by harbouring variation attenuating the thyroid function, through constitutively lowering levels of serum fT3.

3.4 Next Generation Sequencing identifies an intergenic variant under the linkage peak marking lowered serum levels of fT3

Thus far we have observed that nonagenarians sibships with a marked family history of excess survival (FH(+)) exhibit a deviating thyroid prospective survival profile, that seems to largely depend on only levels of serum fT3. To investigate whether any variants in the chr13q34 region could explain this deviating thyroid function observed in the FH(+) subset, we obtained the whole genome sequence of 214 unrelated index cases from the FH(+) subset, termed the SEQ_FH(+) subset (Table 1 and 2, Experimental Procedures 1.2). Depending on DNA availability, the sibling that showed the most extended life span was preferably included for sequencing to further enrich the SEQ_FH(+) study subset for longevity variants.

Using this data, we performed two types of association tests with serum fT3 levels. First, we employed a sliding window of 25kb, 50kb, 100kb, and 150kb to bin and jointly associate common and rare variants in 13q34 ($N_{variants}$=15,612)

to fT3 levels using the Sequence Kernel Association Test (SKAT-O[25], Experimental Procedures 3.3). Though none of these tests achieved significance ($\alpha \leq 0.05$) after Bonferroni correction, it is noteworthy that highest significance was obtained using the broadest window size (150kb) at a genomic position roughly coinciding with the maximal linkage score (Supplemental Figure 3).

Common Single Nucleotide Variants (SNVs) (MAF ≥ 5%, N=5,997) residing in the 13q34 candidate region were tested for association with serum fT3 levels using a standard regression model (Experimental Procedures 3.4). Using this approach, we identified one independent association with serum fT3 levels that passed the Bonferroni correction (rs9515460, T/C, β=-0.52, 95% CI -0.73--030, p=3.43×10^{-6}, Supplemental Figure 4 and 5 and Supplemental Table 2). The identified SNV is in full disequilibrium with two neighbouring SNVs rs80043005 and rs74128254 and is situated between *TEX29* (266 kb) and *SOX1* (459 kb). Thus, within the 214 individuals selected for sequencing (SEQ_FH(+)), taken from sibships with a marked family history of excess survival (FH(+)), carriers of the rs9515460-C allele exhibit lower fT3 (Figure 3), potentially explaining the lower fT3 levels in the FH(+) subset as compared to the FH(-) subset.

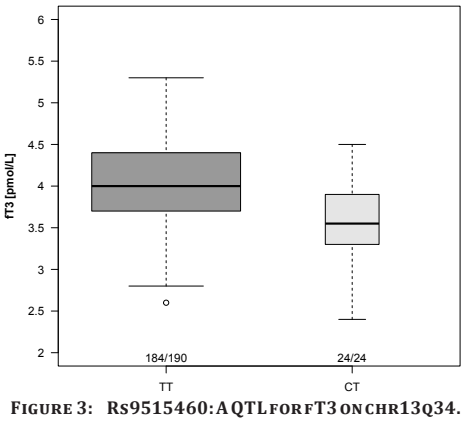

FIGURE 3: RS9515460: A QTL FOR FT3 ON CHR13Q34. Within the individuals selected for sequencing (SEQ_FH(+)), the 24 rs9515460-CT carriers have a 12% lower serum level of fT3 as compared to the 190 rs9515460-TT carriers.

3.5 Rs9515460 association with fT3 serum levels confined to siblings that contribute to linkage at chr13q34

Next we questioned whether the association of rs9515460 with serum level of fT3 is confined to sibling pairs contributing to the linkage at chr13q34 or is observed in the complete LLS nonagenarian cohort independent of IBD status or FH assignment. For this purpose, we performed Sequenom MassArray genotyping (Experimental Procedures 5) on rs9515460 in all 415 nonagenarian sibships in the LLS study. Sequenced genotypes of rs9515460 in the SEQ_FH(+) cohort were in perfect concordance with those obtained with Sequenom. Whereas evidence for association of fT3 with rs9515460 was validated in the whole FH(+) subset (β=-0.23, 95% CI -0.40--0.07, p=0.006), such association was not found in the FH(-) subset (p=0.32), nor in the whole LLS (p=0.21). This either implies that the observed association of fT3 serum levels with rs9515460 in the FH(+) subset is a spurious finding, or alternatively, that not rs9515460 itself, but rare variants specific to the FH(+) subset and linkage disequilibrium with rs9515460 are causal for the lowered serum fT3 level.

To investigate whether the association of rs9515460 with fT3 can be explained by rare variants in partial linkage with

rs9515460 we employed the Identical By Descent (IBD) status at chr13q34. Nonagenarian sibships that have inherited identical strands of DNA from their parents (IBD2), while carrying the rs9515460-CT genotype should be enriched for fT3 lowering variants, as compared to sibships that inherited different strands of DNA (IBD0), but by coincidence carry the rs9515460-CT genotype. Thus to investigate whether there is a significant interaction between the rs9515460 SNV and IBD status at chr13q34, we selected those sibling pairs being IBD2 at the marker with the highest linkage signal (rs752342), while carrying the rs9515460-CT genotype. In total, 12 sib pairs fulfilled these criteria, 9 of which were included with 1 sibling in the SEQ_FH(+) subset. When stratified by rs9515460 genotype (CT or TT) and IBD status (IBD0 or IBD2) a significant lower fT3 level was observed in the 12 sibling pairs (IBD2-CT) as compared to the remaining sibling pairs in the whole LLS carrying CT, but not linking (IBD0-CT) or not carrying CT (IBD0-TT and IBD2-TT) (β=-0.41, 95% CI -0.62--0.21, p=6.33×10^{-5}, Figure 4). Since this shows that the fT3 lowering effect of the rs9515460-CT genotype is conditional on IBD status, these results indicate that not the rs9515460 SNV itself, but rare variants in linkage with rs9515460 explain the association with serum levels of fT3.

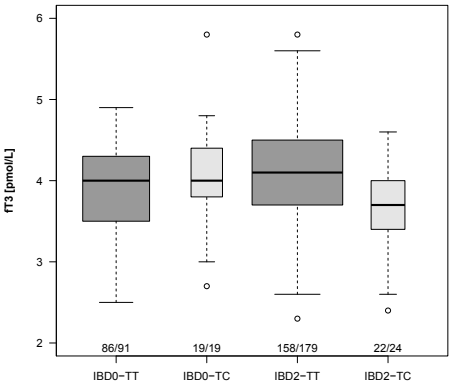

FIGURE 4: IBD × RS9515460 GENOTYPE INTERACTIONS FOR FT3. FT3 serum levels in nonagenarians of all 415 sibships stratified by the most contrasting IBD statuses (IBD0 or IBD2) and rs9515460 genotypes (CT and TT) were plotted for the nonagenarians for whom data on fT3 levels were available, indicated by the ratios at the bottom.

3.6 Variation at rs9515460 associates with prospective survival in nonagenarians

In population-based studies of elderly above 85 years[4,26], low serum fT3 associates with a poor prospect of survival, presumably not so much as a causal factor but because it marks frailty among the elderly. Since the rs9515460-CT genotype seems to tag a fT3 lowering phenotype, we investigated whether carriership of the rs9515460-CT allele associates with a poor prospective survival beyond age 90 and again observed interesting interactions with the FH strata (Table 3). Whereas the rs9515460-CT genotype significantly associates with a poor prospective survival in the FH(+) subset (HR=1.47, 95% CI 1.11-1.95, p=0.008, Figure 5), it does not for the FH(-) subset (p=0.98). This can be explained by the fact that the rs9515460-CT genotype only marks a fT3 lowering haplotype enriched in the FH(+), but not in the FH(-) subset. This would imply that the association of rs9515460-CT with a poor prospective survival is also explained by rare variants in linkage with the rs9515460-CT allele.

To confirm whether the association of rs9515460 with mortality is also dependent on IBD status at chr13q34, we repeated

	ALL 415 sibships	FH(+) 239 sibships	FH(-) 176 sibships	SEQ_FH(+) 214 cases
rs9515460				
Ndeath [%]	848 [92.7%]	493 [93.9%]	355 [92.0%]	199 [93.4%]
HR [95% CI]	1.19 [0.98-1.46]	1.47 [1.11-1.95]	1.00 [0.75-1.34]	1.85 [1.19-2.88]
p	0.084	**0.008**	0.983	**0.006**

TABLE 3: PROSPECTIVE SURVIVAL ON RS9515460. Prospective survival on a QTL for fT3, rs9515460, in a 10 years follow up performed in the whole Leiden Longevity Study (ALL) and the currently defined subsets: FH(+): 239 nonagenarian sibships with a marked family history of an extended survival into old age; FH(-): 176 nonagenarian sibships without such a marked family history; SEQ_FH(+): 214 independent index cases selected from the 239 sibships exhibiting a marked family history (FH(+)) of whom the whole genome has been sequenced previously (**Chapter 4** of this thesis). HR indicates the hazard ratio for mortality.

the survival analysis in the whole study, while stratifying for the most contrasting IBD statuses (IBD0 or IBD2, as indicated by rs752342). Indeed we observe that the association of rs9515460 and mortality is also confined to the sibling pairs that link on chr13q34 (N_{tot}=313, N_{death}= 274, HR=1.94, 95% CI 1.23-3.04, p=0.004, Figure 6). Hence, both the associations of a lowered serum fT3 level as a poor prospective survival of rs9515460-CT carriers are conditioned on sibling pairs linking on chr13q34. This indicates that the rs9515460-CT genotype partially tags a fT3 lowering haplotype that conveys a poor survival prospect in late life. As hypothesized, chr13q34 appears to contribute to the familial history of excess survival by harbouring variation attenuating the thyroid function thereby influencing lifespan regulation, however, apparently not in a protective sense in the oldest old.

4. Discussion

In this paper we report chr13q34 as a novel locus harbouring genetic variants contributing to lifespan regulation as well as an attenuated thyroid function. Within this locus, we identified the rs9515460_T<C polymorphism as a QTL for serum levels of fT3, the unbound active thyroid hormone. Nonagenarian minor C allele carriers of rs9515460 exhibit a significantly lowered fT3 level, which may have contributed to their survival to the age of 90, as a moderately lowered fT3 level at middle age is assumed to be beneficial for cardio-metabolic health. Since the minor allele rs9515460-C carriers display higher mortality risk above the age of 90, we conclude that, in concordance with literature[4,26], low fT3 levels in exceptional old age may negatively influence survival.

Like previous genome wide linkage studies for longevity, we do not observe any overlap with any of the previously reported loci 4q25[27], 3p24-22, 9q31-34, 12q24[28], 6p12.1, 7q11.21, 14q22.1[29] or 14q11.2, 17q12-q22, 19p13.3-p13.11, and

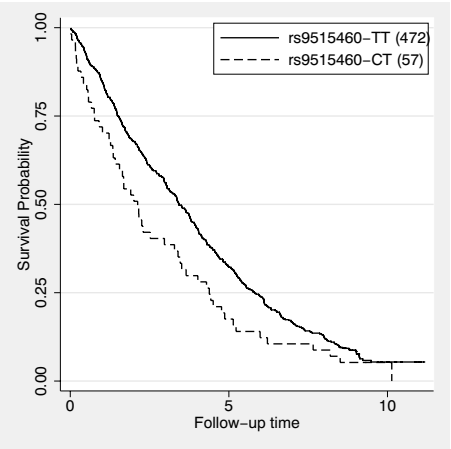

FIGURE 5: RS9515460 AND PROSPECTIVE SURVIVAL. Kaplan-Meier curves were drawn for rs9515460 genotypes in the 239 nonagenarian sibships with a marked family history of an extended survival into old age (FH(+)): Carriers of the rs9515460-CT genotype have an increased risk on mortality during a 10 years follow up, as compared to the TT carriers.

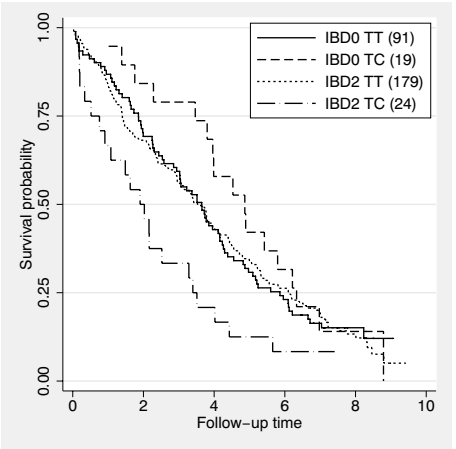

FIGURE 6: IBD × RS9515460 GENOTYPE INTERACTIONS FOR PROSPECTIVE SURVIVAL. Kaplan-Meier curves of rs9515460 genotypes in nonagenarians of all 415 sibships stratified by IBD status. IBD2-TC nonagenarians have an increased risk on mortality during a 10 years follow up, as compared to the TT genotype carriers or IBD0 sibling pairs.

19q13.11-q13.32[30], suggesting that each of the studied populations have their private mechanisms leading to the longevity phenotype. In contrast to previous linkage studies, we have strong indications of the nature of the potential private mechanisms underlying the longevity phenotype in our study cohort. Previously we have shown that long-lived families with the most profound family history of excess survival (FH(+)) are characterized by an attenuated thyroid function. Together, these findings are a strong indication that chr13q34 harbours variants that constitutively attenuate the thyroid function and thereby promote survival, especially in middle age. Fine-mapping of the chr13q34 region was performed by associating each of the common variants under the linkage peak separately with fT3 and indicated one independent significant signal (rs9515460) situated between *TEX29* (266 kb) and *SOX1* (459 kb). The fact that this association with fT3 was conditional on IBD status indicated that the rs9515460 was probably not causal itself, but tagged a haplotype carrying the causal variants. Two aspects about the proximal genomic region are noteworthy. First, the rs9515460 is situated in the first intron of a putative protein-coding transcript (RP11-65D24.2) predicted by the GENCODE consortium on basis of a single spliced testis expressed EST. Secondly, nearby GWAS-hits are at 42kb and 82kb and respectively associate with age at menopause[31] and age at menarche[32], which themselves have been attributed to a gene upstream of *TEX29*, namely *ARHGEF7* (305kb). *ARHGEF7* is known to be an activator of *FOXO3a*[33], which is one of the most well studied longevity genes and has been shown to extend lifespan

upon disruption in multiple organisms[34,35]. Overall, the rs9515460-C allele tags an fT3 lowering haplotype and its proximal genomic region seems to relate to hormone functioning.

As an alternative, we attempted to fine map the linkage region by associating groups of variants jointly with fT3 levels using the Sequence Kernel Association Test (SKAT) in conjunction with a sliding window for variant grouping. Benefits of SKAT lie in its ability to handle mixtures of rare and common variants with opposite signs of association, thus fitting our expectations with respect of the genetic heterogeneity in both human longevity and an attenuated thyroid signalling. The most significant signal (p=0.0032) was detected in a window neighbouring the one containing the highest observed linkage signal (~44.8kb) and coincided with the *ING1* gene and the first exons of the *CARS2* gene. However, none of the association tests performed with SKAT remained significant after correction for multiple testing, indicating that we might have insufficient power in the current study for performing joint association analyses of variants.

Since the FH(+) and FH(-) subsets show a very comparable prospective survival, we concluded that the genetic propensity to exhibit excess survival has no significant effect on the survival beyond age 90, which seems to be counterintuitive. Nonagenarian sibships with a genetic propensity to exhibit an excess survival were selected on basis of a so-called Family History Score (FHS), which expresses the mean survival advantage of the parents of a nonagenarian sibship relative to their respective sex and birth cohort specific life expectancies. Hence, the FHS relates to the probability of the parents of a nonagenarian to outlive their sex and age matched peers derived from the general population, who typically did not live up to age 90, nor their age-equivalent of the oldest old. Hence, the genetic propensity to exhibit an excess survival relates to survival into old age, and therefore not necessarily to survival of old into oldest old, as these two periods in life history are characterized by very distinct disease specific mortality rates.

Nonagenarian sibships with the most profound family history of excess survival are enriched for an fT3 lowering haplotype that is assumed to be beneficial for survival into old age, but is apparently detrimental for survival of old into oldest age. This antagonistic pleiotropic effect of the fT3 lowering haplotype on human lifespan regulation may be explained by considering the thyroid axis in relation to blood pressure and its subsequent effect on cardiovascular mortality. Increased thyroid levels promote an increased heart rate and cardiac output, leading to an increased pulse pressure. Whereas a low systolic blood pressure is beneficial from middle age onward (65 to 84), as indicated by a lower risk on cardiovascular death, it becomes detrimental in the oldest old (age > 84)[36]. Hence, a haplotype carrying variants constitutively lowering fT3 serum levels is expected to contribute to human longevity by transmitting its beneficial health effects prior age 90. The fact that many of the health parameters, that distinguish members of long-lived families from the general population, display such inverse health correlations, suggests that the hereby-proposed antagonistic pleiotropic

genetic mechanism for longevity might be common amongst long-lived families. Hence, the key to uncover the genetic basis for healthy ageing and human longevity lies in the knowledge on what conditions and life-timing health associated phenotypes actually confer their health benefits.

A limitation of the current study is that we thus far assumed that carriership of fT3 lowering haplotype confers a health benefit at middle age, especially with respect to the cardio-metabolic make up. To test whether this hypothesis holds, also in the general population, we first need to further characterize the fT3 lowering haplotype. Once a clearly defined haplotype or the causal variants on this haplotype have been established, we first will verify whether fT3 levels co-segregate with the presumed casual variants in the offspring of the studied nonagenarians. To verify that the causal variants indeed predispose to a beneficial cardio-metabolic health status at middle age, we will investigate whether carriership of the causal markers can explain the lowered incidence of cardio-vascular morbidity observed in the offspring of the studied nonagenarians[9]. A following step would be to generalize these observations to the general population in multiple cohorts of European decent, to validate that the chr13q34 region harbours genetic predispositions underlying a public mechanism for human longevity marked by low serum fT3 levels.

To conclude, we have performed in depth genetic analyses to disentangle the pleiotropic relation observed in long-lived families between the propensity to exhibit an excess survival and an attenuation of the thyroid function. Using linkage analyses followed by QTL analyses for fT3 on NGS variants within the 1-LOD-drop interval, we were able to identify an fT3 lowering haplotype that might causally explain the attenuated thyroid function. Unlike previously reported longevity loci, that confer their beneficial effect either from middle age onward, e.g. *APOE*-ε2 allele[37], or in late life only, e.g. *FOXO3A*[38-40], we hereby report the chr13q34 locus, that exhibits an antagonistic pleiotropic relation with life span regulation. Whereas lowered fT3 levels contribute to cardio-metabolic health from middle into old age, it associates with a poor prospective survival in the oldest old. These findings collectively warrant further investigations into the mechanism how fT3 levels in specific and other ageing markers displaying inverse health associations in general contribute to human longevity and lifespan regulation.

5. Experimental Procedures

5.1 Study population: Leiden Longevity Study

5.1.1 Study Design: Families participating in the Leiden Longevity Study[41] have at least two siblings meeting four inclusion criteria: (i) men are at least 89 years old and women are at least 91 years old, (ii) participants have at least one living brother or sister who fulfils the first criterion and is willing to participate, (iii) the nonagenarian sibship has an identical mother and father, and (iv) the parents of the nonagenarian sibship are Dutch and Caucasian. Using these criteria, a total of 421 nonagenarian sibships (N=944) have been recruited. For 415 sibships (N=931) genome wide SNP genotypes[22] for at least two nonagenarian siblings were

available for the genetic linkage analyses.

5.1.2 Subset definitions: A so-called Family History Score (FHS)[11] was computed per sibship, which expresses the mean survival advantage of the parents of a nonagenarian sibship relative to their respective sex and birth cohort specific life expectancies. A threshold on the FHS at -1.05 was used to assign sibships to either the FH(+)(FHS≤-1.05, 239 sibships, N=540) or FH(-) (FHS>-1.05, 176 sibships, N=391) subset (Table 1). From the thus created FH(+) subset, 214 independent cases were selected for sequencing using the following criteria: (i) the availability of at least 5 µg of genomic DNA from whole blood for whole genome sequencing, (ii) the participation of children of one of the siblings for future research and (iii) the most extended lifespan compared to his/her siblings. Thus selected participants of the LLS were whole genome sequenced according to procedures fully described in **Chapter 4** of this thesis.

5.2 Thyroid serum parameters

Details regarding the measurement protocols for the thyroid serum parameters TSH, fT4 and fT3 have been described in full detail elsewhere[10]. Serum levels of TSH were log10 transformed in order to obtain an approximately normal distribution. Outliers in thyroid serum parameters were defined as observations deviating more than three standard deviations of the mean on basis of measurements performed in the whole population of nonagenarian participants in the Leiden Longevity Study (N=859).

5.3 Statistical analyses

5.3.1 Differences in thyroid parameters between sub-populations: Differences in serum levels of the thyroid parameters TSH, fT4 and fT3 between sibships with a marked family history and without were tested using linear mixed models implemented in the *lme4*[42] and lmerTest[43] packages of the statistical language R[44]:

$$TP \sim \beta_1 \times age + \beta_2 \times sex + \beta_3 \times status + \mu_1 \times famID \quad (1)$$

Where *TP* indicates the level of a thyroid serum parameter, *age at inclusion* is provided in years, *sex* is provided as 1 (male) or 2 (female), *status* indicates the assignment to sibships with (status = 1) or without (status = 0) a family history of extended survival and *famID* indicates the family membership. Family membership was modelled using a random effect μ_1 to account for the phenotypic correlations observed between family members.

5.3.2 Associations with prospective survival: Analyses were performed with the *Survival* package[45] of R[44] using an age at inclusion and sex-adjusted, left-truncated Cox proportional hazards model to adjust for late entry into the dataset according to age. Mortality analyses between different subsets of the study were performed using:

$$\lambda(t) \sim \lambda 0(t) \times exp(\beta_1 \times age + \beta_2 \times sex + \beta_3 \times sel + \mu_1 \times famID) \quad (2)$$

Where the covariates *age* designates *age at inclusion* and is provided in years, *sex* as either 1 (male) or 2 (female), *sel* as either 1 or 2 to indicate study subset membership and *famID* indicates the family membership. Again, family membership was taken into account to correct for phenotypic correlations observed between family members and was done by supplying the term *cluster(famID)* in the Cox regression. Similarly, mortality analyses on the thyroid parameters were performed using:

$$\lambda(t) \sim \lambda 0(t) \times exp(\beta_1 \times age + \beta_2 \times sex + \beta_3 \times TP + \mu_1 \times famID) \quad (3)$$

Where *TP* indicates the serum levels of TSH, fT4 or fT3. Finally, mortality analyses on the rs9515460 SNV were performed using:

$$\lambda(t) \sim \lambda 0(t) \times exp(\beta_1 \times age + \beta_2 \times sex + \beta_3 \times SNV + \mu_1 \times famID) \quad (4)$$

Where *SNV* indicates the C allele dosage (0,1 or 2) of the rs9515460_T>C polymorphism.

5.3.3 Sequence Kernel Association Test (SKAT-O): To jointly associate groups of genotype markers with serum levels of fT3, we employed the package SKAT[46] of the statistical language R[44] at default settings and used the following formula to describe the null hypothesis (containing covariates only):

$$fT3 \sim \beta_1 \times age + \beta_2 \times sex \quad (5)$$

5.3.4 QTL associations: Associations with single genotypic genotypes was done for common variants within the 1-LOD-drop linkage area (MAF ≥ 5%, N = 5,480), using the *lm* function of the R package *stats*[44], using the following model:

$$fT3 \sim \beta_1 \times age + \beta_2 \times sex + \beta_3 \times SNV \quad (6)$$

Where the covariates *age* is provided in years and *sex* as 1 (male) or 2 (female). Genotypes were recoded to minor allele dosages: 0 (homozygous common allele), 1 (heterozygous) or 2 (homozygous rare allele). Genotype data was filtered on missingness (≤ 5%) and MAF (≥ 5%) with respect to the 208 out of 214 samples for which also data on fT3 levels was available. Obtained *p*-values were corrected for multiple testing (Bonferroni).

5.4 Linkage analysis

The Illumina 660Quad and Illumina OmniExpress arrays have been used for genotyping the participants of the Leiden Longevity Study. Details on data acquisition and pre-processing are have been described elsewhere[21]. For linkage analysis 12,000 equally spaced SNVs were selected that are genotyped on both arrays with a MAF>0.3 and a mutual R^2<0.4. MERLIN-0.10.2[47] was used to estimated the information content per genome and to estimate IBD probabilities in sibling pairs without parents. Nonparametric affected sibling pair analyses were performed using a score test statistic for affected sibling pairs[48].

5.5 Sequenome MassArray Genotyping

Genotyping of rs9515460 in the LLS study was performed using the Sequenom MassARRAY iPLEX Gold. Genotypes were successfully measured for 909 out of the 925 participants (98.3%). Sequenom genotypes in the 214 individuals with whole genome sequencing data were in perfect concordance with sequenced genotypes of rs9515460.

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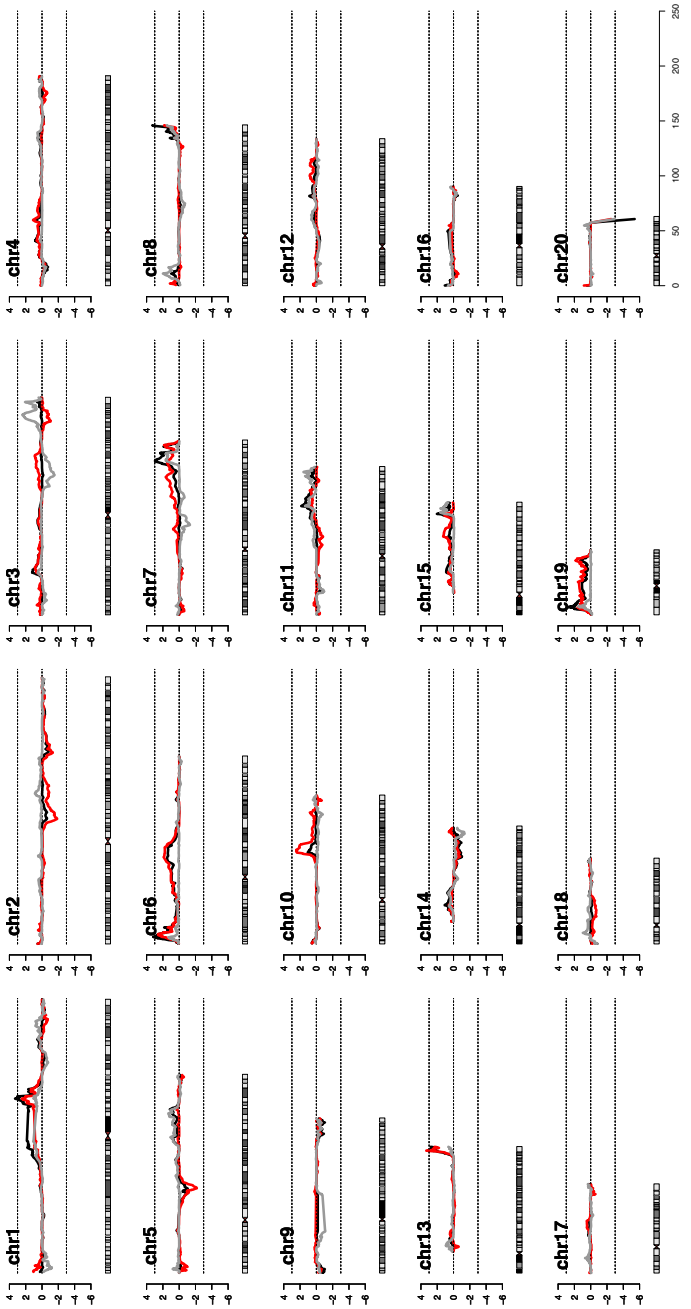
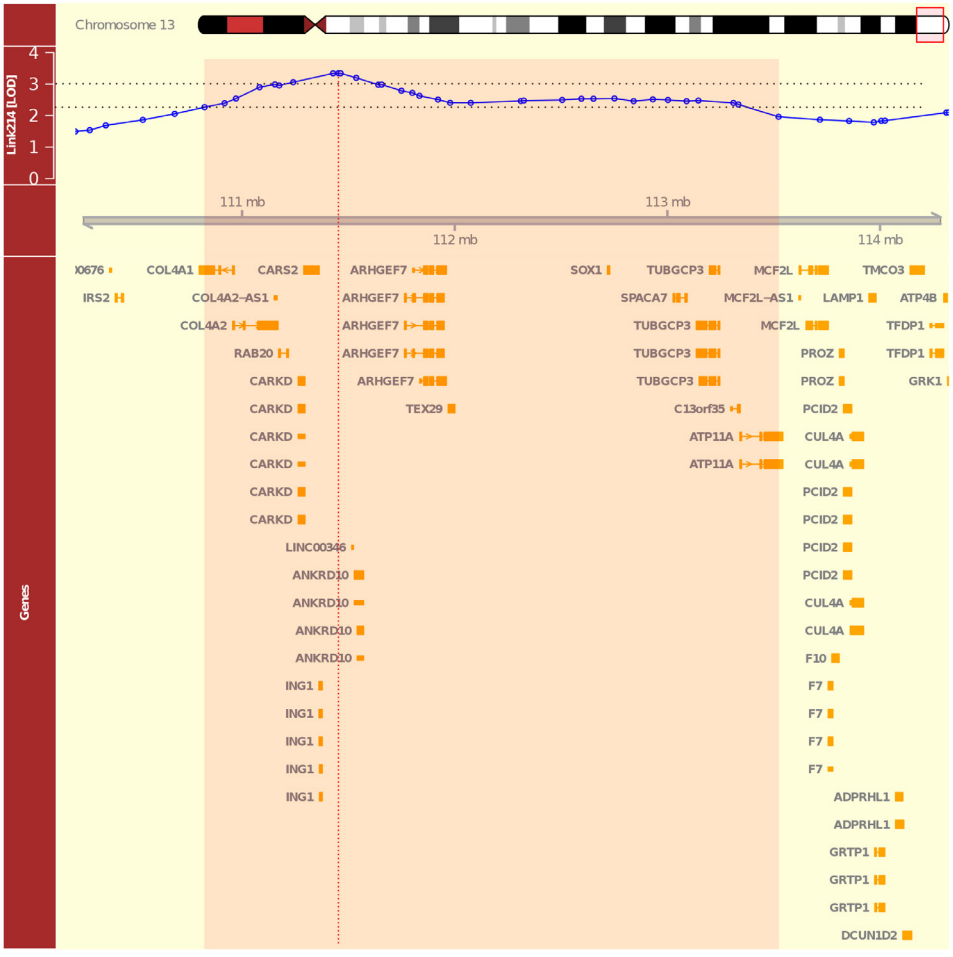
Supplemental Materials



SUPPLEMENTAL FIGURE 1: GENOME-WIDE LINKAGE RESULTS ON THE SUBSETS OF THE STUDY. Genome wide linkage results for familial longevity using all 415 nonagenarian sibships of the LLS for which genetic data was available (N=931, black), the subset of sibships 239 sibships with a marked family history of an extended survival into old age (FH(+), red) and the remaining 176 sibships without such a marked family history (FH(-), grey). On chrom13q34, linkage highly specific for sibships with a profound family history is observed (rs752342, LOD_{ALL}=2.96, $LOD_{FH(+)}$=3.35, $LOD_{FH(-)}$=0.28).

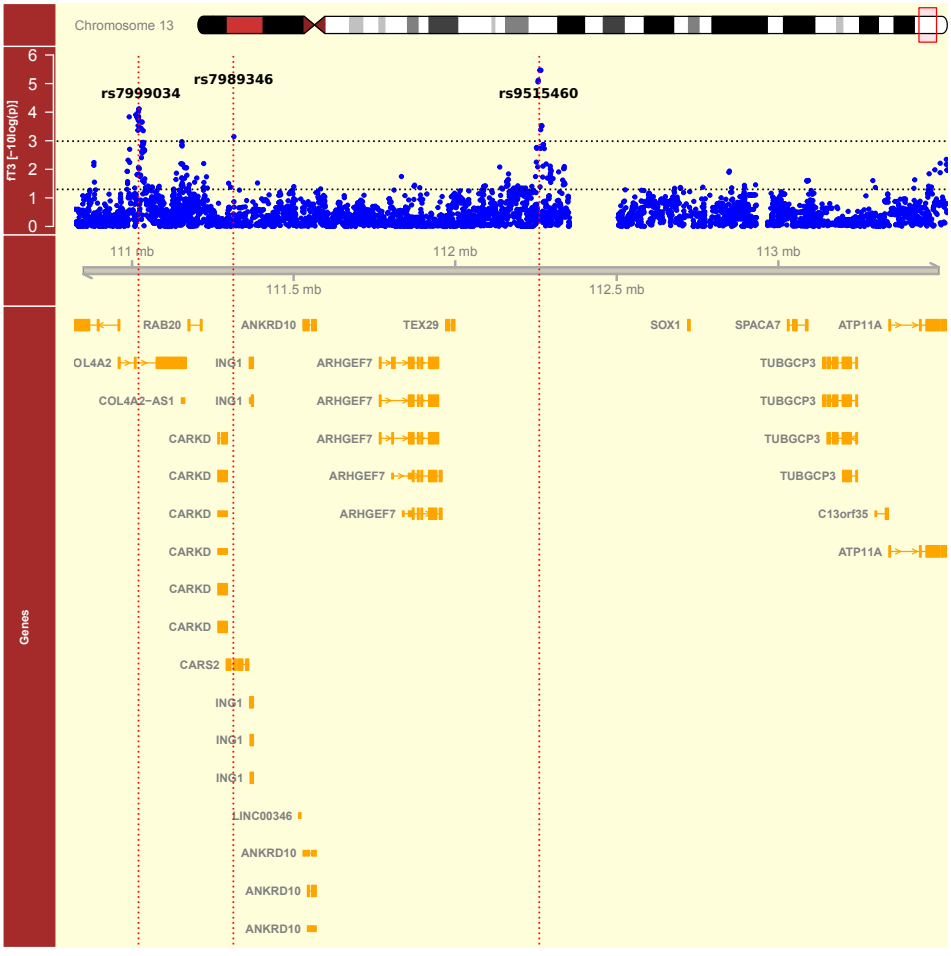


SUPPLEMENTAL FIGURE 2: AN OVERVIEW OF THE 1-LOD-DROP INTERVAL ON CHR13Q34. The red area indicates genes within the 1-LOD-drop interval around the top marker on chr13q34, rs752342, as determined in the FH(+) subset.

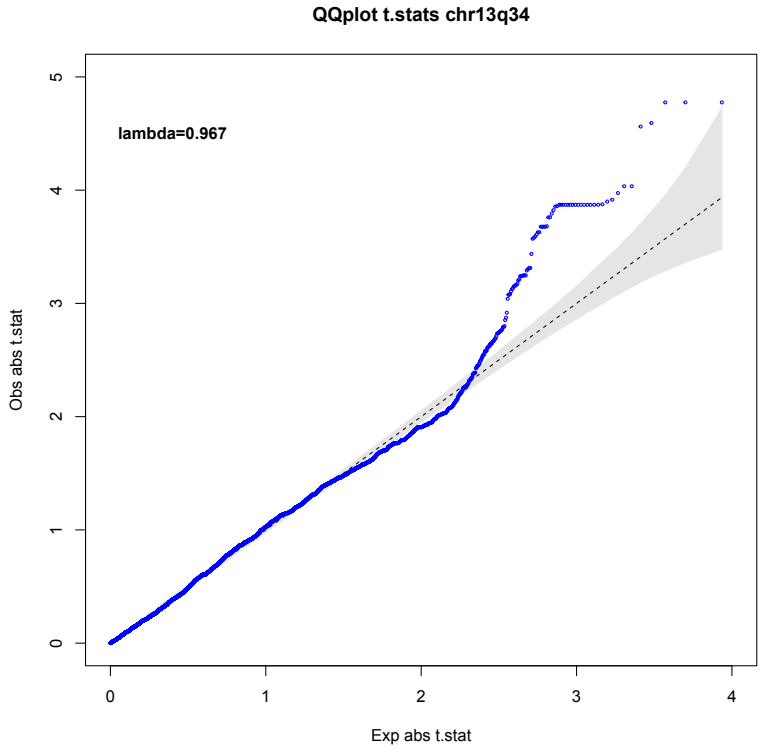
GeneSymbol	EntrezGeneID	TxAcc	chrom	strand	start	end
COL4A1	1282	NM_001845	chr13	-	110801310	110959496
COL4A2	1284	NM_001846	chr13	+	110959631	111165373
COL4A2-AS1	100874203	NR_046583	chr13	-	111154923	111160526
RAB20	55647	NM_017817	chr13	-	111175413	111214071
CARKD	55739	NM_001242882	chr13	+	111267807	111292342
CARKD	55739	NM_001242883	chr13	+	111267807	111292342
CARKD	55739	NR_040103	chr13	+	111267807	111292342
CARKD	55739	NR_040104	chr13	+	111267807	111292342
CARKD	55739	NM_001242881	chr13	+	111267931	111292342
CARKD	55739	NM_018210	chr13	+	111267931	111292342
CARS2	79587	NM_024537	chr13	-	111293757	111358480
ING1	3621	NM_198217	chr13	+	111364970	111373421
ING1	3621	NM_198218	chr13	+	111365610	111373421
ING1	3621	NM_198219	chr13	+	111365610	111373421
ING1	3621	NM_005537	chr13	+	111367359	111373421
ING1	3621	NM_001267728	chr13	+	111367784	111373421
LINC00346	283487	NR_027701	chr13	-	111516334	111522655
ANKRD10	55608	NM_017664	chr13	-	111530887	111567454
ANKRD10	55608	NR_104587	chr13	-	111530887	111567454
ANKRD10	55608	NM_001286721	chr13	-	111545039	111567454
ANKRD10	55608	NR_104586	chr13	-	111545039	111567454
ARHGEF7	8874	NM_001113511	chr13	+	111767624	111947542
ARHGEF7	8874	NM_001113512	chr13	+	111767624	111947542
ARHGEF7	8874	NM_145735	chr13	+	111767624	111947542
ARHGEF7	8874	NM_003899	chr13	+	111806061	111958081
ARHGEF7	8874	NM_001113513	chr13	+	111839173	111958081
TEX29	121793	NM_152324	chr13	+	111973015	111996594
SOX1	6656	NM_005986	chr13	+	112721913	112726020
SPACA7	122258	NM_145248	chr13	+	113030651	113089009
TUBGCP3	10426	NM_001286277	chr13	-	113139319	113242499
TUBGCP3	10426	NM_006322	chr13	-	113139319	113242499
TUBGCP3	10426	NM_001286278	chr13	-	113153121	113242499
TUBGCP3	10426	NM_001286279	chr13	-	113200796	113242499
C13orf35	400165	NM_207440	chr13	+	113301358	113338811
ATP11A	23250	NM_015205.2	chr13	+	113344643	113541482
ATP11A	23250	NM_032189.3	Chr13	+	113344643	113541482

SUPPLEMENTAL TABLE 1: ACCESSION NUMBERS OF GENES WITHIN THE 1-LOD-DROP INTERVAL. Entrez Gene IDs and associated RefSeq transcript accessions are provided for genes situated in the 1-LOD-drop interval on chr13q34. The 1-LOD-drop interval was determined within the FH(+) subset.

SUPPLEMENTAL FIGURE 3: SEQUENCE KERNEL ASSOCIATION TEST (SKAT) IN THE 1-LOD-DROP INTERVAL ON CHR13Q34. Using the sequencing data in 214 nonagenarians, analyses were performed with SKAT for associating groups of SNVs under the linkage peak with fT3. Four different window sizes (25kb, 50kb, 100kb and 150kb) were used for grouping neighbouring variants according to a half overlapping tile pattern. Grouped variants were then submitted to a joint association analysis with fT3 adjusted for age and sex. Obtained results are indicated by the four tracks and display high consistency with respect to genomic position and significance. The highest significance after correction for multiple testing (Bonferoni) was observed using the 150kb windows (p=0.00319, 37 tests) for the variants positioned at chr13:111,348,340-111,498,339.



SUPPLEMENTAL FIGURE 4: ASSOCIATION ANALYSIS WITH COMMON VARIANTS IN THE 1-LOD-DROP INTERVAL ON CHR13Q34. Data on fT3 serum levels was available for 208 out of the 214 sequenced nonagenarian genomes. Variants were filtered on the minor allele frequency (MAF ≥ 5%) and call rate (CR ≤ 5%) in these 208 genomes and associated with fT3 serum levels using a sex and age adjusted linear regression. A total of 5,997 variants within the 1-LOD-drop region, as determined in the FH(+) subset were tested.



SUPPLEMENTAL FIGURE 5: QQPLOT OF ABSOLUTE T.STATISTICS OBTAINED WITH SINGLE MARKER ASSOCIATIONS WITH FT3 ON VARIANTS WITHIN THE 1-LOD-DROP REGION ON CHR13Q34.

chrom	position	allele	beta	se	tstat	pval	df
chr13	110991189	G/A	-0.251	0.065	-3.871	1.46E-04	204
chr13	111011400	A/T	-0.282	0.072	-3.915	1.23E-04	204
chr13	111015628	C/T	-0.198	0.051	-3.871	1.46E-04	204
chr13	111015780	C/A	-0.198	0.051	-3.871	1.46E-04	204
chr13	111015877	C/T	-0.198	0.051	-3.871	1.46E-04	204
chr13	111016124	A/C	-0.198	0.051	-3.855	1.56E-04	202
chr13	111016153	C/T	-0.199	0.051	-3.875	1.44E-04	203
chr13	111017045	C/T	-0.198	0.051	-3.871	1.46E-04	204
chr13	111017784	G/A	-0.201	0.052	-3.899	1.31E-04	204
chr13	111018009	G/C	-0.186	0.052	-3.580	4.33E-04	198
chr13	111018072	G/A	-0.198	0.051	-3.871	1.46E-04	204
chr13	111018132	T/C	-0.198	0.051	-3.871	1.46E-04	204
chr13	111018163	T/G	-0.198	0.051	-3.871	1.46E-04	204
chr13	111018235	C/A	-0.198	0.051	-3.871	1.46E-04	204
chr13	111018666	G/A	-0.199	0.051	-3.871	1.46E-04	202
chr13	111018729	C/G	-0.199	0.052	-3.823	1.76E-04	200
chr13	111018752	A/G	-0.198	0.051	-3.859	1.53E-04	203
chr13	111018909	C/G	-0.190	0.051	-3.681	2.98E-04	203
chr13	111019083	T/C	-0.198	0.051	-3.871	1.46E-04	204
chr13	111019278	C/A	-0.204	0.051	-3.975	9.77E-05	204
chr13	111019472	T/C	-0.198	0.051	-3.871	1.46E-04	204
chr13	111019508	C/T	-0.196	0.052	-3.793	1.96E-04	202
chr13	111019568	A/G	-0.198	0.051	-3.871	1.46E-04	204
chr13	111019895	T/C	-0.198	0.051	-3.871	1.46E-04	204
chr13	111019976	C/A	-0.198	0.051	-3.871	1.46E-04	204
chr13	111020878	G/A	-0.204	0.051	-4.034	7.73E-05	204
chr13	111021623	C/G	-0.204	0.051	-4.034	7.73E-05	204
chr13	111025118	A/G	-0.191	0.053	-3.628	3.61E-04	204
chr13	111026734	G/A	-0.191	0.053	-3.628	3.61E-04	204
chr13	111028978	G/A	-0.188	0.052	-3.609	3.86E-04	204
chr13	111029923	G/A	-0.194	0.052	-3.760	2.22E-04	204
chr13	111031180	G/A	-0.194	0.052	-3.760	2.22E-04	204
chr13	111034542	T/C	-0.180	0.050	-3.571	4.45E-04	202
chr13	111315249	G/A	0.178	0.052	3.437	7.17E-04	199
chr13	112256430	C/G	-0.516	0.113	-4.562	8.76E-06	203
chr13	112256996	G/A	-0.509	0.111	-4.593	7.64E-06	204

chr13	112261984	G/A	-0.517	0.108	-4.775	3.43E-06	204
chr13	112263091	C/T	-0.517	0.108	-4.775	3.43E-06	204
chr13	112264614	C/T	-0.338	0.094	-3.592	4.12E-04	203
chr13	112265213	G/A	-0.517	0.108	-4.775	3.43E-06	204
chr13	112266639	G/A	-0.320	0.087	-3.677	3.02E-04	204
chr13	112267153	A/C	-0.320	0.087	-3.677	3.02E-04	204
chr13	112267402	G/A	-0.320	0.087	-3.677	3.02E-04	204
chr13	112268411	C/T	-0.320	0.087	-3.677	3.02E-04	204

Supplemental Table 2: SNVs in the 1-LOD-drop linkage region. The 1-LOD-drop region was determined in the FH(+) subset, on chr13q34 with p≤0.001 in the sex and age adjusted regression with fT3 serum levels.