Serum insulin-like factor 3 levels are reduced in former androgen users suggesting impaired Leydig cell capacity

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Abstract

Background: Illicit use of anabolic androgenic steroids (AAS) is frequently observed in men and is associated with subsequent testosterone deficiency although the long-term effect on gonadal function is still unclear. Serum insulin-like factor 3 (INSL3) has been suggested to be a superior biomarker of Leydig cell secretory capacity compared to testosterone. The objective of this study was to investigate serum INSL3 concentrations in AAS users.

Methods: This community-based cross-sectional study included men aged 18 – 50 years, involved in recreational strength training and allocated to one of three groups: never-AAS users as controls (n=44), current (n=46) or former AAS users (n=42) with an average duration since AAS cessation of 32 (23;45) months.

Results: Serum INSL3 was lower in current AAS users and former AAS users than in controls, median (IQR), 0.04 (ND – 0.07) and 0.39 (0.24 – 0.62) versus 0.59 (0.45 – 0.72) µg/L, P<0.001. Former AAS users exhibited lower serum INSL3 levels than controls in a multivariate linear regression even after adjusting for serum total testosterone and other relevant confounders, (B) (95%CI), -0.16 (-0.29;-0.04) µg/L, P=0.011. INSL3 and total testosterone were not associated in the model, P=0.821. Longer accumulated AAS duration (log2) was associated with lower serum INSL3 in former AAS users, (B) (95%CI), -0.08 (-0.14;-0.01), P=0.022. Serum INSL3, but not inhibin B or testosterone, was associated with testicular size in a multivariate linear regression, (B) (95%CI); 4.7 (0.5 ; 8.9), P=0.030.

Conclusions: Serum INSL3 is reduced years following AAS cessation in men, independently of testosterone, suggesting persistently impaired Leydig cell capacity.

Keywords Anabolic androgenic steroids; androgens; Testosterone; Male hypogonadism; Male infertility; Insulin-like factor 3

Introduction

Anabolic androgenic steroids (AAS) are synthetic derivates of the key male sex hormone, testosterone but are chemically enhanced to exhibit a longer half-life, increased anabolic androgenic-ratio and higher affinity for the androgen receptor (1). Illicit AAS use has been widespread among body builders and elite athletes for decades but is now frequently observed among men in the broader population, although estimates of AAS prevalence among men differ among various populations and should be interpreted with care since the heterogeneity among the populations evaluated is considerable (1-4). A meta-analysis including most available studies worldwide estimated AAS prevalence rates of approximately 18% among individuals involved in recreational sports and 1% among non-athletes, while another study estimated the AAS prevalence among men in general in the United States to be approximately 2% (3,4).

In recent years, independent research groups have revealed various adverse effects of illicit AAS use in men, including implications for the reproductive system (5-13). It is indisputable that ongoing illicit AAS use causes hypogonadotropic hypogonadism due to inhibition of the hypothalamus-pituitary-gonadal (HPG)-axis by circulating supraphysiological androgen levels (9-11). However, the extent of recovery of the HPG-axis following AAS cessation in former AAS users is still unclear. In clinical studies, we and others noted reduced serum testosterone levels and lower testicular sizes in former AAS users following a mean elapsed duration since AAS cessation of more than two years (9,11). In contrast, a recent Australian study reported fully recovered serum total testosterone concentrations in former AAS users, although their testicular sizes remained reduced; additionally, the study reported an even greater numerical difference in serum total testosterone between former AAS and never-AAS users than the two former studies, but the authors argued for that reductions in serum sexual

hormone-binding globulin (SHBG) in former AAS users lead to proportionate reductions in serum testosterone rather than implying androgen deficiency (10,14,15).

A novel and promising biomarker of Leydig cell secretory capacity has emerged in serum insulin-like factor 3 (INSL3). In men, INSL3 is secreted exclusively by Leydig cells and acts uniquely on the relaxin family peptide receptor 2 (RXFP2) which is found in various tissues (16). The impact of INSL3 in adult men has not been fully elucidated but at least two physiological roles have been identified. INSL3 seems to be involved in the regulation of bone metabolism, as young men with the T222P mutation in the RXFP2 gene exhibited osteoporosis (17). Furthermore, INSL3 appears to be implicated in spermatogenesis, as post-meiotic germ cells express RXFP2; moreover, increased residual spermatogenesis was associated with higher serum INSL3 levels in a male contraception study (18,19).

Serum INSL3 was undetectable in men with anorchism and with hypogonadotropic hypogonadism but increased in response to long-term stimulatory hCG therapy in the latter group (16). Thus, serum INSL3 levels appear to reflect the number of functional Leydig cells in adult men (16,20). INSL3 secretion is dependent on LH stimulation, but synthesis and secretion exhibit far less daily variation than testosterone and are not related to body composition (20). Thus, serum INSL3 could be of significant clinical value as a biomarker of Leydig cell secretory capacity. The objective of this study was to assess the impact of illicit AAS use on serum INSL3 concentrations as a biomarker of Leydig cell capacity.

Methods

Study design and participation criteria

We conducted a cross-sectional study enrolling current AAS users, former AAS users with an elapsed duration since AAS cessation of more than three months and never-AAS users as controls, thereby imitating the nature of a prospective cohort study before, during and after illicit AAS use.

We included men 18 – 50 years of age who were involved in recreational strength training. To minimize risk of selection bias by inclusion, we did not draw up any participation criteria regarding extent or duration of illicit AAS use among current and former users, nor did we put forward any participation criteria on the extent of resistance training or body composition. Allocation of participants among the groups was based on self-reported AAS history and supported by measurement of several biochemical parameters known to be influenced by exogenous androgen supplementation, including gonadotropins, SHBG and hematocrit (21). Exclusion criteria were as follows: established male hypogonadism due to causes other than illicit AAS use including pituitary diseases, congenital or acquired male hypogonadism, cryptorchism, abnormal pubertal development and history of medically prescribed androgen therapy. The participants were included over two periods of time as two separate cohorts. Cohort I, from November 2014 to December 2015, which has been described previously (5-9,13) and Cohort II, from February 2017 to August 2017, using identical participation criteria as Cohort I.

Ethics

Oral and written informed consent were obtained from each participant. Ethical approval was granted by the Capital Regional Committee on Health Research Ethics (H-3-2014-127) and the Danish National Committee on Health Research Ethics (H-16030778). Participants were explicitly guaranteed anonymity, and we did not perform any procedures that could incriminate participants including conventional AAS urine doping tests. The Danish Data Protection Agency approved the handling of personal data in the study.

Procedures

All procedures were performed during one visit only in the morning hours following overnight fasting. We drew blood through a cannula placed in an antecubital vein following 30 minutes of supine rest. Medical records, a detailed history of strength training and illicit AAS use were obtained in a structured interview performed by JJR or MNF. Testicular size was evaluated using an orchidometer. Body composition was assessed using dual-energy X-ray absorptiometry (Hologic Discovery, QDR, MA, USA).

Laboratory analyses

Serum INSL3 was measured using liquid chromatography mass spectrometry (LCMS) by an in-house assay of which the development has previously been described (22). The interday relative standard deviation was < 15%. This method received accreditation status according to the ISO 15189 standard for medical laboratories (20). Serum total testosterone, inhibin B and SHBG levels were measured using LCMS, a three-step sandwich ELISA and a chemiluminescence-based immunoassay, respectively, which have all been described elsewhere (9). Free testosterone in serum was calculated using the method described by Bartsch (23).

Statistical analyses

Normal distribution of numerical variables was evaluated by assessing linearity of residuals in quantile plots. Normally distributed variables are presented as means (SDs) and were compared using analysis of variance with Tukey-Kramer post hoc adjustment for multiple comparisons. Nonnormally distributed numerical variables were log-transformed and are presented as geometric means (95%CIs). If a normal distribution was not achieved using log-transformation, we presented data as medians (25^{th} – 75^{th} percentiles) and compared these among the groups using the Kruskal-Wallis test. Categorical variables were compared using the Chi-square test or Fisher's exact test as appropriate. The Kruskal-Wallis test and tests for comparisons of categorical variables were adjusted for multiple comparisons with Bonferroni post hoc tests. We assessed recovery of reproductive hormones using linear regressions in former AAS users with an elapsed duration since AAS cessation equal to or shorter than 60 months only, as we speculated that AAS-induced alterations in reproductive hormones persisting longer than 60 months would be permanent. We created a multivariate linear regression model with serum INSL3 as the dependent variable to evaluate whether a potential difference in this reproductive hormone between former AAS users and controls withstood adjustment for relevant confounders. Data were generally complete, but testicular size was only assessed in Cohort I. P-values < 0.05 were considered statistically significant. All analyses were performed using SAS version 9.4 (SAS Institute, Cary, North Carolina, USA).

Results

Participant characteristics

Thirty-seven current AAS users, 33 former AAS users and 30 never-AAS users were enrolled in Cohort 1, while 9 current AAS users, 9 former AAS users and 14 never-AAS users were enrolled in Cohort 2. Thus, in total, the study consisted of 46 current AAS users, 42 former

AAS users and 44 never-AAS users as controls (Table 1). None of the participants displayed obvious abnormal pituitary hormone levels, although the group of current AAS users had higher plasma prolactin levels than the other two groups (Table 2). Current users exhibited apparent biochemical markers of supplementation with exogenous androgens including reduced serum SHBG and plasma HDL cholesterol, suppressed plasma levels of gonadotropins, elevated hematocrit, and increased liver enzymes (Tables 1 and 2). In contrast, we did not observe any of these biochemical parameters of exogenous androgen use in former AAS users or controls. Current and former AAS users did not differ with respect to accumulated duration of AAS use, geometric mean (95%CI), 126 (97 ; 164) weeks versus 93 (68 ; 128) weeks, P = 0.140 (Table 1). Elapsed duration since AAS cessation was, geometric mean (95%CI), 32 (23 ; 45) months among former AAS users, and only six (14%) reported AAS use within the last year of inclusion in the study (Table 1). The three groups did not differ with respect to age; mean age (SD) was 32 (7) years. Participants in all three groups exhibited a lean body composition, but current AAS users displayed a lower mean body fat percentage and approximately 9 kg higher lean body mass than former AAS users and controls (Table 1).

Reproductive hormones

Serum INSL3 levels were lower in current and former AAS users than in controls, median (25^{th} - 75^{th} percentiles), 0.04 (ND – 0.07) µg/L and 0.39 (0.24 – 0.62) µg/L versus 0.59 (0.45 – 0.72) µg/L; current users versus controls, P < 0.001, and former users versus controls, P = 0.005 (Table 2). Former AAS users also exhibited decreased serum total and calculated free testosterone compared with controls; medians (25^{th} – 75^{th} percentiles), 15 (12 – 20) nmol/L versus 20 (17 – 24) nmol/L, P = 0.008; 423 (320 – 472) pmol/L and 491 (443 – 579) pmol/L versus, P < 0.001 (Table 2). Serum SHBG did not differ between former users and controls.

We noted a lower INSL3/LH ratio in former AAS users than in controls, P = 0.003, but the TT/LH ratio did not differ significantly between the two groups, P = 0.086.

In a linear multivariate regression model using serum INSL3 as the dependent variable, including former users and controls only; serum INSL3 remained lower in former AAS users than in controls, mean difference (95%CI), -0.16 (-0.29 ; -0.04) µg/L, P = 0.011, following adjustment for serum total testosterone, LH, SHBG, age, body fat percentage, smoking and use of other illicit drugs (Table 3). Serum INSL3 and total testosterone were not associated in the multivariate model, P = 0.821 or in the analysis of covariance, P = 0.073.

In a linear regression adjusted for elapsed duration since AAS cessation, longer accumulated duration of AAS use (log2) was associated with lower serum INSL3 levels in former AAS users, (B) (95%CI), -0.08 (-0.14;-0.01), P = 0.022 (Figure 1).

In an age-adjusted multivariate linear regression model among former AAS users and controls, using testicular size as the dependent variable and including serum INSL3, total testosterone and inhibin B as covariates, only serum INSL3 was associated with testicular size, (B) (95%CI); 4.7 (0.5 ; 8.9), P = 0.030, while total testosterone, P = 0.308, and inhibin B, P = 0.055, were not.

Recovery of reproductive hormones

In univariate linear regressions, we noted recovery of serum inhibin B levels among former AAS users reaching the mean level of controls after an elapsed duration since AAS cessation of approximately 21 months; (B) (95%CI), 2.2 (0.7 ; 3.7) months, P = 0.006 (Figure 2B). In contrast, we did not note any recovery of serum levels of either marker of Leydig cell function, serum INSL3 (P = 0.541) or total testosterone (P = 0.861) among former AAS users

(Figure 2C and 2D). Plasma LH levels already appeared recovered within 12 months after AAS cessation (Figure 2A).

Discussion

The key finding of the current study is that serum INSL3 levels were lower in former AAS users than in never-AAS users, even years after AAS cessation, and the reduction in INSL3 concentrations was independent of well-established confounders, including serum total testosterone. The present clinical study provides novel data suggesting persistently impaired Leydig cell capacity in many illicit AAS users. Furthermore, we noted that the accumulated duration of AAS use was associated with lower serum INSL3 levels in former AAS users, indicating that a clinically relevant dose-response relationship may play a role. The theory of hampered Leydig cell secretory capacity in past illicit AAS users is supported by two previous studies (24,25). First, a minor study demonstrated that serum testosterone response to hCG stimulation, by a conventional 72-hour test, was attenuated in men with AAS-induced male hypogonadism compared to men with idiopathic hypogonadotropic hypogonadism (24). Second, a recent Danish register-based study found a 15- and 21-fold increased risk of developing a need for testosterone supplementation therapy and receiving a diagnosis of testicular dysfunction, respectively, among men who were positive for urine AAS doping tests compared to age-matched controls (25).

In healthy adult men, serum INSL3 has been suggested as a better marker of Leydig cell capacity than testosterone (20). Indeed, a recent study investigating eugonadal men and men suspected of testicular disorders demonstrated that baseline serum INSL3 concentrations were more closely associated with the hCG-induced increases in serum testosterone concentrations than baseline testosterone per se (20). Consistently, we noted that serum

INSL3, but not inhibin B or total testosterone, was associated with testicular size, which is a well-established clinical sign of gonadal function.

Serum INSL3 levels have not previously been assessed in illicit AAS users, but the current findings are in accordance with our and others' previously reported data demonstrating prolonged hypogonadism due to lower serum testosterone in former AAS users (9,11).

In contrast to Leydig cell biomarkers, we noted recovery of gonadotropins and serum biomarkers of spermatogenesis in former AAS users with estimated recovery of serum inhibin B levels to occur within two years. Recovery of semen quality in previous AAS users was not assessed in the present study. A recent Australian study reported recovery of gonadotropins in former AAS users within one year from AAS cessation and recovery of serum inhibin B levels within approximately 32 months (10). Semen samples in that study showed that sperm concentration and motility did not differ between former AAS users and never-users while sperm morphology was not reported (10). Furthermore, a recent register-based study suggests comparable birth-rates and use of assisted human reproduction between individuals who tested positive for AAS in the years following doping sanction compared to randomly selected age-matched controls (26). Nonetheless, newer advanced semen analyses, including assessment of acrosome reaction and sperm DNA fragmentation, showed that these variables were strongly correlated with male fertility but have never been assessed in AAS users (27,28). Therefore, the recovery of semen quality and fertility potential in previous AAS users needs further investigation.

This study has several limitations that should be addressed. The nature of a prospective cohort study would have been superior as a design to assess the impact of illicit AAS use on reproductive function in men, observing a cohort of men before, during and after illicit AAS use, but such a study would not be ethically sound. Instead, we used a cross-sectional study

design, which exhibits apparent limitations implicitly inherent to a retrospective approach. On the other hand, the cross-sectional study also offers advantages in terms of exposure and outcome assessment when exposures are well-defined such illicit AAS use (29). We relied on self-reported personal data, including history of illicit AAS use, and participants may therefore have been incorrectly allocated among the three study groups. Nevertheless, none of the former AAS users or controls displayed biochemical parameters of exogenous AAS use, and a recent study demonstrated that distinction between current and former AAS users is highly accurate using the same biochemical parameters of ongoing AAS use as we measured (10). In line with this notion, the results of the present study demonstrate that the combination of suppressed circulating INSL3 levels and increased testosterone should raise concern among physicians of exogenous androgen use if a patient has not informed of illicit AAS use.

In conclusion, serum INSL3 levels are lower in former AAS users than in never-users of AAS even years after AAS cessation, suggesting that impaired Leydig cell secretory capacity could persist for years in former AAS users. The implications of these findings need further investigation such as a trial investigating the effect of stimulation therapy to recover Leydig cell capacity in past AAS users.

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Data Availability Statement: Restrictions apply to the availability of some or all data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

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Figure Legends

Figure 1: Linear regression between accumulated AAS duration (log2) and serum INSL3 levels in former androgen users.

Dashed line indicates mean serum INSL3 levels in controls.

AAS, anabolic androgenic steroids; **INSL3,** insulin-like factor 3; **S-,** serum;

Figure 2: Elapsed duration since androgen cessation and reproductive hormones in former AAS users.

Dashed lines indicate mean serum or plasma levels in controls.

AAS, anabolic androgenic steroids; **INSL3,** insulin-like factor 3; **LH,** luteinizing hormone; **P-,** plasma; **S-,** serum; **TT,** total testosterone

Table 1: Participant characteristics

	Controls	Current AAS users	Former AAS users	P-value
	n = 44	**n = 46**	**n = 42**	
Age (years)	31 (7)	31 (8)	34 (7)	0.118
B-hemoglobin (mmol/L)	8.9 (0.6)	10.3 (0.8)	9.3 (0.6)	<0.001[a]
B-hematocrit (%)	43 (2)	50 (4)	44 (2)	<0.001[a]
P-ALAT (U/L) ¶	37 (34 ; 41)	77 (63 ; 94)	42 (37 ; 48)	<0.001[a]
P-HDL cholesterol (mmol/L)	1.1 (0.2)	0.6 (0.2)	1.1 (0.2)	<0.001[a]
P-creatinine (µmol/L)	88 (13)	90 (11)	87 (14)	0.637
Body composition				
Height (cm)	182 (7)	179 (6)	181 (5)	0.076
Weight (kg)	90 (12)	97 (11)	93 (14)	0.017 [b]
BMI (kg/m^2)	27 (3)	30 (3)	28 (4)	<0.001[a]
Body fat percentage (%)	17 (4)	14 (2)	19 (4)	<0.001[a]
Lean body mass (kg)	71 (8)	80 (9)	71 (9)	<0.001[a]
Fat mass (kg)	15 (5)	13 (3)	17 (5)	<0.001[c]
Smoking (n (%))				0.002 [d]
Never	32 (73)	24 (52)	18 (43)	
Previously	10 (23)	7 (15)	15 (36)	
Currently	2 (4)	15 (33)	9 (21)	
Use of other illicit drugs (n (%))				0.007 [d]
Never	24 (55)	13 (28)	13 (31)	
Previously	20 (45)	25 (54)	24 (57)	
Currently	0 (0)	8 (17)	5 (12)	
Illicit AAS use				
Accumulated duration on AAS (weeks) ¶	-	126 (97 ; 164)	93 (68 ; 128)	0.140
Duration since AAS cessation (months)	-	-	32 (23 ; 45)	-
AAS cessation within 6 months, n (%)	-	-	1 (2)	-
AAS cessation within 6 – 12 months, n (%)	-	-	5 (12)	-
AAS cessation 12 – 24 months, n (%)	-	-	12 (29)	-
AAS cessation > 24 months, n (%)	-	-	24 (57)	-

Numerical variables are presented as means (SDs) unless otherwise stated. ¶ Geometric mean (95%CI).

a difference between current AAS users and the other two groups; **b** difference between current AAS users and controls; **c** difference between former AAS users and current AAS users; **d** difference between controls and the other two groups

AAS, anabolic androgenic steroids; **ALAT,** alanine transaminase; **B-**, blood; **BMI,** body mass index; **HDL,** high density lipoprotein; **P-,** plasma.

Table 2: Reproductive and pituitary hormones among study participants

	Controls n = 44	Current AAS users n = 46	Former AAS users n = 42	P-value
Reproductive hormones				
S - INSL3 (µg/L)	0.59 (0.45 - 0.72)	0.04 (ND - 0.07)	0.39 (0.24 - 0.62)	<0.001[a]
S - TT (nmol/L)	20 (17 - 24)	88 (41 - 123)	15 (12 - 20)	<0.001[a]
S - cFT (pmol/L)	491 (443 - 579)	3548 (1488 - 5498)	423 (320 - 472)	<0.001[a]
P - estradiol (pmol/L)	21 (1 - 53)	100 (9 - 350)	33 (1 - 53)	0.004 [b]
S - SHBG (nmol/L) •	35 (15)	10 (9)	33 (17)	<0.001[b]
S - inhibin B (pg/mL) •	174 (49)	82 (46)	178 (61)	<0.001[b]
S - AMH (pmol/L) ¶	44 (38 ; 51)	21 (17 ; 27)	44 (38 ; 52)	<0.001[b]
INSL3/LH-ratio ¶	0.17 (0.14 ; 0.20)	-	0.09 (0.06 ; 0.13)	0.003[c]
TT/LH-ratio ¶	5.9 (5.0 ; 6.8)	-	4.8 (4.1 ; 5.7)	0.086
Pituitary hormones				
P - FSH (U/L)	4.4 (3.5 - 6.0)	0.3 (0.1 - 0.5)	4.1 (3.3 - 6.0)	<0.001[b]
P - LH (U/L)	3.5 (2.6 - 4.4)	ND (ND - 0.1)	3.6 (2.2 - 4.1)	<0.001[b]
P - IGF1 (µg/L) •	161 (36)	171 (49)	151 (35)	0.078
P - prolactin ($x10^{-3}$ U/L) •	134 (38)	201 (106)	118 (32)	<0.001[b]
P - TSH ($x10^{-3}$ U/L) •	2.0 (1.0)	1.9 (1.0)	1.8 (0.8)	0.589
P - corticotropin (pmol/L) •	6.0 (2.1)	6.8 (3.8)	5.7 (2.3)	0.199

Numerical variables are presented as medians (25^{th} – 75^{th} percentiles) unless otherwise stated. • mean (SD) ¶ Geometric mean (95%CI).

a sifference among all three groups; **b** difference between current AAS users and the other two groups **b** difference between former AAS users and controls

AMH, anti-Müllerian hormone; **cFT,** calculated free testosterone; **FSH,** follicle-stimulating hormone; **IGF1,** insulin-like growth factor 1; **INSL3,** insulin-like factor 3; **LH,** luteinizing hormone; **ND,** nondetectable; **P-,** plasma; **S-,** serum; **SHBG,** sexual hormone-binding globulin; **TSH,** thyroid-stimulating hormone; **TT,** total testosterone.

Table 3: Multivariate linear regression model using serum INSL3 as the dependent variable among former AAS users and controls

	(B) (95%CI)	P-value	Former AAS users versus controls	P-value
Unadjusted			-0.16 (-0.28 ; -0.05)	0.006
Multivariate-adjusted			-0.16 (-0.29; -0.04)	0.011
Age (per 10 year increment)	-0.10 (-0.18 ;-0.02)	0.016		
S-TT (per nmol/L increment)	0.002 (-0.13 ; 0.02)	0.821		
P-LH (per U/L increment)	0.01 (-0.02 ; 0.05)	0.497		
S-SHBG (per 10 mmol/L increment)	0.04 (-0.02 ; 0.09)	0.178		
Body fat % (per % increment)	0.01 (-0.01 ; 0.02)	0.412		
Current smoking (ref = never/previously)	-0.09(-0.26 ; 0.09)	0.322		
Current use of illicit drugs (ref = never/previously)	0.40 (0.17 ; 0.64)	0.001		

AAS, anabolic androgenic steroids; **INSL3,** insulin-like factor 3; **LH,** luteinizing hormone; **P-,** plasma; **S-,** serum; **SHBG,** sexual hormone-binding globulin; **TT,** total testosterone

Figure 1

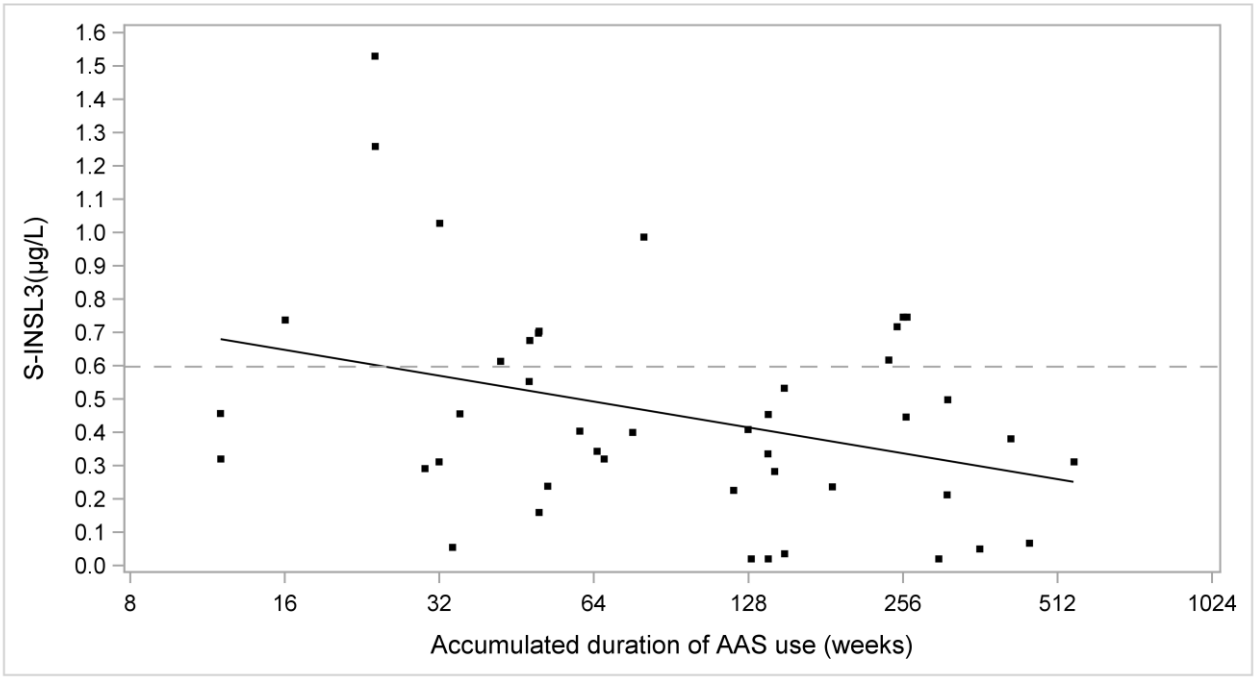

Figure 2