Recruitment, Procedures, and Protocols for the ACTION Biomarker Study

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Abstract

The goal of ACTION project (Aggression in Children: Unraveling gene-environment interplay to inform Treatment and InterventiON strategies) was to identify precursors, features, and consequences of childhood aggression using epidemiological and omics-driven studies. A considerable effort within ACTION was the collection of new omics data in children with low and high aggressive behavior. To this end, the ACTION project included large-scale collection of biological samples in children from the Netherlands Twin Register (NTR), a twin cohort from the general population, and a cohort of children referred for psychiatric treatment (LUMC-Curium). Together these cohorts comprise the ACTION Biomarker Study. This documentation describes the study design and data collection for the ACTION Biomarker Study.

Keywords: childhood aggression, childhood behavior problems, mental health, buccal cells, urine, genetics, epigenetics, DNA methylation, biomarkers, metabolomics, amines, organic acids, steroid hormones, multi-omics

1 Background

ACTION (Aggression in Children: Unraveling gene-environment interplay to inform Treatment and InterventiON strategies) is a large collaborative endeavor, which includes genome-wide genetic and epigenetic association studies, biomarker discovery and epidemiological projects into the antecedents, characteristics, and consequences of childhood aggression[1,2]. ACTION was funded by the European Union Seventh Framework Program (FP7/2007-2013) under grant agreement no 602768 and includes researchers from Europe, Australia, and the United States. In ACTION new data collection of urine samples for biomarker discovery and buccal-cell samples for (epi)genetics in children was conducted in two Dutch cohorts, the Netherlands Twin Register (NTR)[3,4] and LUMC-Curium. Together these cohorts comprise the ACTION Biomarker Study[5].

The documentation for the ACTION Biomarker Study comprises three sections. First, we describe the recruitment and data collection procedures for the NTR and LUMC-Curium cohorts. Second, we provide information on the urine and buccal-swab collection protocols. Lastly, we describe how the genotyping, DNA methylation, biomarkers and metabolomics data were generated for the ACTION Biomarker Study and for an additional NTR dataset that has undergone epigenetic measurements as part of ACTION. Please note that the current documentation for the ACTION Biomarker Study is based on the previously published 'NTR ACTION urine and buccal DNA collection protocol'[6], the appendices of the doctoral dissertation of Fiona A. Hagenbeek[7], and various publications of the ACTION Consortium.

2 ACTION Biomarker Study recruitment and data collection procedures

2.1 Netherlands Twin Register

2.1.1 Background and overview

The NTR was established around 1987 by the Department of Biological Psychology at the Vrije Universiteit Amsterdam. Research at the NTR is aimed at elucidating the role of genetic and environmental influences in mental and physical health in children and adolescents (Young NTR; YNTR)[4,8] and in adults (Adult NTR; ANTR)[4,9]. The participants in the NTR-ACTION Biomarker Study were included in the YNTR. After registering, mothers received a first survey with items on pre- and perinatal items. When the twins are approximately 2, 3, 5, 7, 9/10 and 12 years of age the YNTR parents are invited to report on the health, lifestyle, and behavior of their twins. After parental consent, teachers are invited to provide similar observations when the twins are approximately 7, 10 and 12 years of age.

Twin pairs from the NTR were invited for participation in the biomarker study based on their longitudinal data on aggressive behavior at ages 3, 7, and/or 9/10 years. At, or around these ages, parents of twins completed the Achenbach System of Empirically Based Assessment (ASEBA) Child Behavior Checklist (CBCL) for pre-school children (1.5-5 years) or school-aged children (6-18 years) and teachers of twins completed the ASEBA Teacher Rating Form (TRF)[10]. A design was chosen that selected twin pairs who were concordant low-low, concordant high-high, or discordant low-high on indices of childhood aggression, with an oversampling of monozygotic (MZ) pairs. Study approval was obtained from the Central Ethics Committee on Research Involving Human Subjects of the VU University Medical Center, Amsterdam (NTR 25th of May 2007 and ACTION 2014.252), an Institutional Review Board certified by the U.S. Office of Human Research Protections (IRB number IRB00002991 under Federal-wide Assurance- FWA00017598; IRB/institute codes).

Collection of biological samples in the selected twin pairs for the NTR-ACTION Biomarker Study took place between December 2014 and October 2017. The collection of biological samples, often accompanied by additional phenotyping, consisted of four phases (selection criteria and collected materials in each phase can be found in **Figure 1**):

1. A '**practical pilot**' was conducted to test the protocols for urine collection. This step was done in non-twin children of the same age as the target sample of twins.
2. The first phase of data collection in twin pairs was referred to as the '*technical pilot*' and has been included in the NTR databases under the acronym '**ACTIONBB1**' which stands for ACTION Biobank 1. Here, first-morning urine and buccal-cell DNA was collected in twin pairs at two time points, with an interval of approximately two weeks. The aim was to estimate the temporal stability of the selected biomarker assays and metabolomics platforms in the NTR-ACTION Biomarker Study. No questionnaire data on aggression were collected. These twin pairs were unselected for phenotype information.
3. The '*biochemical study*' labelled as **ACTIONBB2**, consisted of the collection of first-morning urine, buccal-cell DNA, and questionnaires to assess i.e., parent-reported aggression in twin-children. These twin pairs were selected to be concordant or discordant for high or low aggression. ACTIONBB2 served as a pilot for the main collection wave in NTR-ACTION, here the validity of the biomarker assays and metabolomics platforms was tested further. In addition, based on the results of ACTIONBB2 seven biomarker assays were selected for study in the final wave of the ACTION Biomarker Study.
4. During the last wave of NTR data collection, also called the '*main study*' and labelled **ACTIONBB3**, the measures as described for ACTIONBB2 were collected in predominantly MZ twin pairs concordant or discordant for high or low aggression.

Family members living at the same address as the participating twin pairs were also asked for a buccal-cell DNA sample during ACTIONBB2 and ACTIONBB3.

Figure 1. Overview of the selection criteria and collected materials during each of the four phases of the ACTION Biomarker Study within the NTR.

Collection phase	Practical Pilot	ACTIONBB1	ACTIONBB2	ACTIONBB3
General selection criteria	• 6 non-twin children • 7-12 years of age • 50% girls	• 10 MZ or DZ twin pairs • 7-12 years of age • 50% girls	• 100 MZ or DZ twin pairs • 7-12 years of age • 50% girls	• 800 MZ twin pairs • 7-12 years of age • 50% girls
Phenotypic selection criteria	No phenotypic selection criteria	Below subclinical (T<65) mother-rated CBCL aggression scores at Y3 and/or Y7	• 100 children below subclinical (T<65) mother-rated CBCL aggression scores at Y3 and/or Y7 • 100 children above subclinical (T>65) mother-rated CBCL aggression scores at Y3 and/or Y7	• 200 concordant low aggression twin pairs • 200 concordant high aggression twin pairs • 400 twin pairs discordant for aggression
Urine collection	10 ml first-void morning urine	• 2x 12-24 ml first-void morning urine • 2 weeks between collection times	24-48 ml first void morning urine	24-48 ml first void morning urine
DNA collection	No DNA collection	• 2x 16 swabs buccal DNA • 2 weeks between collection times	16 swabs buccal DNA	16 swabs buccal DNA
Phenotyping at collection	No questionnaire collection	No questionnaire collection	• CBCL • Wellbeing • Health, medication and chronic disorders	• CBCL • Wellbeing • Health, medication and chronic disorders

Note: MZ = monozygotic; DZ = dizygotic; CBCL = Child Behavior Checklist from the Achenbach System of Empirically Based Assessment (ASEBA); Y3 = 3 years of age; Y7 = 7 years of age.

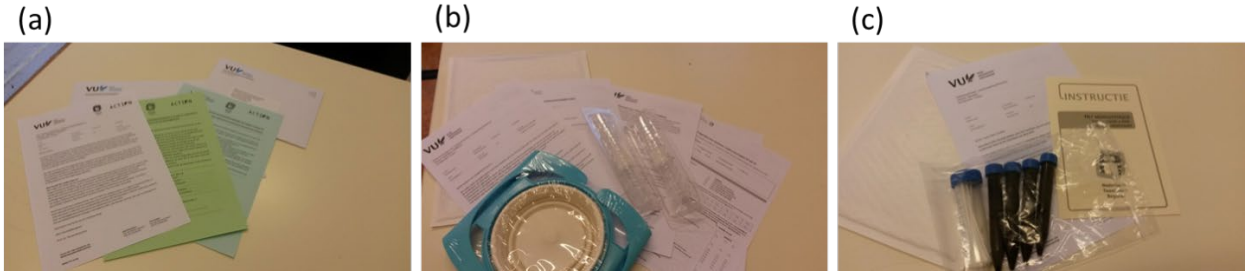
2.1.2 Recruitment and procedures Practical Pilot

The practical pilot consisted of 6 children (50% girls) of 7-10 years of age from 4 families from the Amsterdam region. For 5 out of 6 children consent was obtained from their parents to use their urine sample as a 'pool' sample (urine samples of the 5 children were combined and were used for quality control purposes) during the ACTIONBB1 part of the study.

Participating families received a collection package, including instructions for urine collection, a copy of the form to be filled out at the time of urine collection and all necessary materials (**Figure 2**). The parents were asked to follow '*Day 1*' of the first-morning urine collection protocol, which instructed parents on how to use an uritainer for urine collection and how much urine needed to be

transferred to storage tubes. At the end of the '*Day 1*' collection protocol parents were instructed to place the urine containing tubes in the freezer for storage. Parents only froze their children's urine samples if they had provided an informed consent, allowing the researchers to collect and use the urine samples as a 'pool' sample (urine samples of all children in the practical pilot were combined and used for quality control purposes) during the ACTIONBB1 part of the study. After urine collection, parents were asked how they experienced the urine collection, if they experienced any problems or had any comments regarding the instruction materials. Parents could either e-mail their experiences or tell them in person during collection of the urine samples. Their comments led to minor clarifications in the urine collection protocol as provided for parents from ACTIONBB1 onwards.

Figure 2. Letters, forms and materials for collecting urine and buccal-cell DNA samples in the NTR-ACTION Biomarker Study. **(a)**. Invitation letter and informed consent forms as used in ACTIONBB2 and ACTIONBB3. **(b)** Cover letter, instructions, survey booklet, urine collection form, uritainer, urine tubes (4x), and urine blisters (2x) as used in ACTIONBB2 and ACTIONBB3. **(c)** Cover letter, instruction booklet, sterile cotton swabs (16x), and collection tubes filled with buffer liquid (4x) as used in ACTIONBB1, ACTIONBB2 and ACTIONBB3.



2.1.3 Recruitment and procedures ACTIONBB1

Twin participants, for whom survey data were available at age three and/or age seven, in the age range of 10-13 years old with a low aggression score on the mother-reported ASEBA CBCL Aggression Syndrome Scale[10] were asked to participate in ACTIONBB1. Low aggression was defined as a T-score of less than 65 on the Aggression Syndrome Scale of the CBCL as measured at age 3 and/or age 7. NTR defined age and sex specific T-scores by multiplying a z-score by 10 and adding 50. Based on these criteria, 24 twin-children (12 twin pairs) from the Amsterdam region were invited to participate in ACTIONBB1 in December of 2014. Permission for participation in ACTIONBB1 was received from the parents of ten twin pairs (2 MZ, 6 dizygotic (DZ) same-sex and 2 DZ opposite sex [DOS] twin pairs) for participation in ACTIONBB1 (participation rate: 83.3%). The children had an average age of 11.6 (range: 10-13, *SD* = 0.9), and 40% of the children were girls.

The parents of selected twin pairs received a letter detailing the aim of the study and asking them to participate. This study was aimed at collection of longitudinal samples. Approximately one week after receiving the introduction letter, the parents were telephoned by a research assistant. During this call the parents received information regarding the study and were asked to indicate their willingness to participate in the study. After receiving verbal consent, the collection packages for the first day of data collection were sent within one to one-and-a-half weeks. The packages for the first day of data collection included all the necessary materials for urine and buccal-cell DNA collection for the first occasion including cover letters for each of the packages and the informed consent form. The completed informed consent forms needed to be returned by mail. Approximately one week after receiving the urine and buccal-cell DNA collection package, the parents were phoned by a research assistant to enquire if they had been successful in collecting the urine sample and buccal-cell swabs. Two weeks after receiving the first package with urine and buccal-cell DNA collection materials, the families received the second package with urine and buccal-cell DNA collection materials including cover letters for each package. Once urine samples and buccal-cell swabs had been collected at two time points with an interval of approximately two weeks, they were phoned to plan an appointment to collect the materials at a day and time of their convenience. Research assistants drove out to the families and collected all samples. The twin pairs received €20.- in gift certificates as a token for their participation, parents were asked to sign for receiving the gifts.

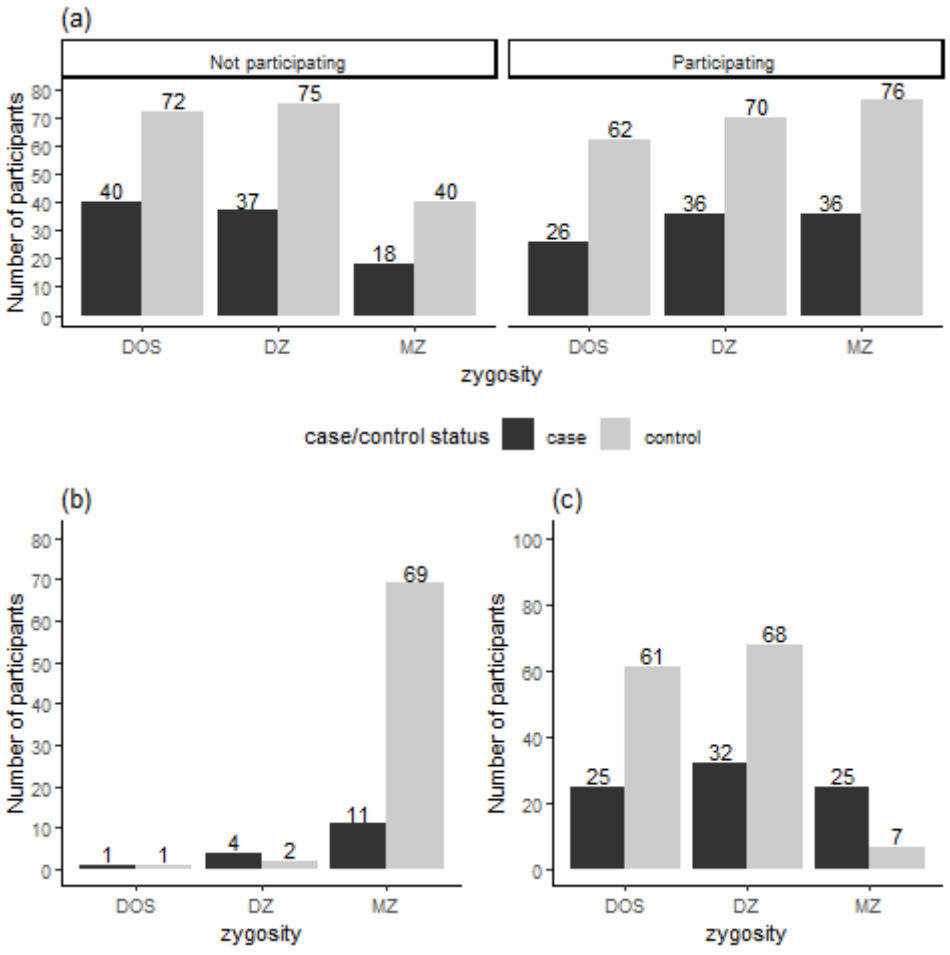
2.1.4 Recruitment ACTIONBB2

Twin pairs, for whom survey data from the CBCL were available at age three and/or seven, in the age range of 5-11 years old were invited to participate in ACTIONBB2. Participant selection for invitation was done at two time points. Twin pairs who were either concordant (high-high or low-low) or discordant (high-low) on the ASEBA CBCL Aggression Syndrome Scale were invited for participation. Scale scores were based on the mother reported ASEBA CBCL at age 3 or age 7. High aggression was defined as having an ASEBA CBCL Aggression Syndrome Scale T-score equal to or larger than 65, and low aggression as an ASEBA CBCL Aggression Syndrome Scale T-score of less than 65. Concordant low twin pairs were invited for participation after zip-code matching them to selected concordant high or discordant twin pairs.

In the period of the 15th of April to the 9th of November 2015 we invited 193 twin-children with high aggression scores (cases) and 395 twin-children with low aggression scores (controls) to participate in ACTIONBB2 across eight batches of invitations. All invited twin-children were invited as part of complete MZ or DZ twin pairs, based on their concordance or discordance on aggression. Family members of invited twin pairs were also asked for a buccal-cell sample. Across all invitation batches

we had a participation rate of 52%. The participation rate among the cases was 50.8%, of which 43.9% were girls. The mean age among cases was 9.2 years (range = 6-11 years; SD = 1.9 years; **Figure 3a**). The control group had a similar participation rate (52.7%), included 52.7% girls and had a mean age of 10.2 years (range = 6-11 years; SD = 1.2 years; **Figure 3a**). When the target was reached for this phase, we selectively forwarded 88 twin-children (44 twin pairs) to the next phase of the study, ACTIONBB3 (**Figure 3b**). These 88 twin-children had a mean age of 10 years (range = 6-11 years, SD = 1.3 years) and 46.6% were girls. In total, ACTIONBB2 included 218 children of 6 to 11 years of age (mean age = 9.9; SD age = 1.6, 51.2% girls) of which 82 were cases and 136 were controls. These 218 children belonged to 16 MZ, 50 DZ and 43 DOS twin pairs (**Figure 3c**).

Figure 3. Overview of the number of low- (control) or high-scoring (case) twin-children invited and participating in ACTIONBB2 by zygosity. **(a)** Number of low- (control) or high-scoring (case) twin-children participating versus not participating in ACTIONBB2 by zygosity. **(b)** Number of low- (control) or high-scoring (case) twin-children selectively forwarded to ACTIONBB3 by zygosity. **(c)** Number of participating low- (control) or high-scoring (case) twin-children included in ACTIONBB2 by zygosity.



Note: MZ = part of a monozygotic twin pair; DZ = part of a same-sex dizygotic twin pair; DOS = part of an opposite-sex dizygotic twin pair.

2.1.5 Recruitment ACTIONBB3

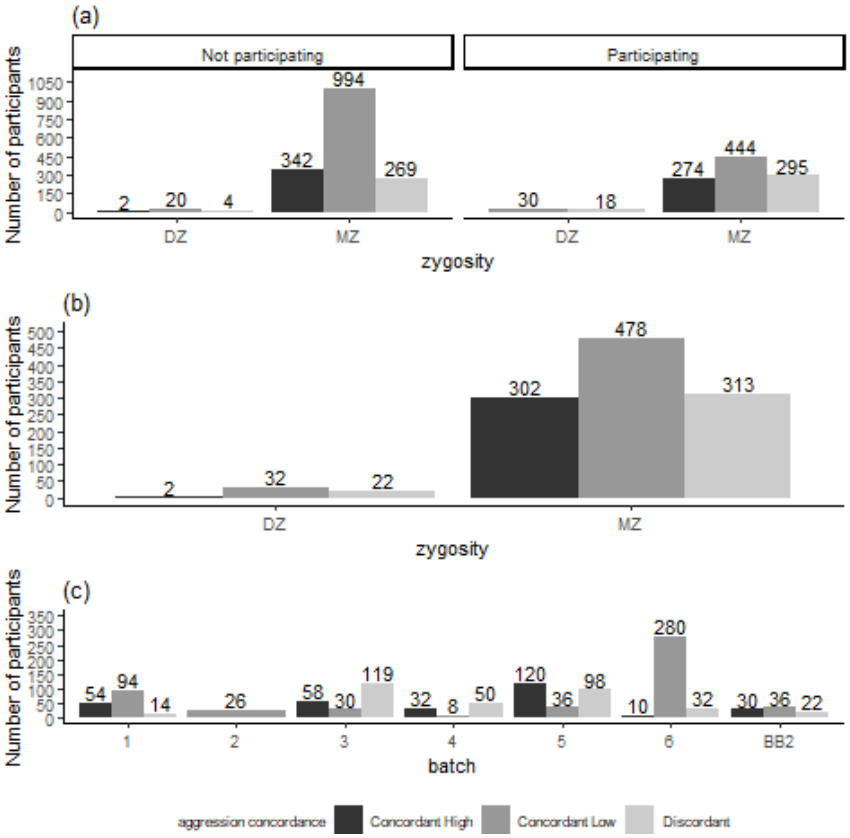
MZ twin pairs, for whom CBCL data were available at ages 3, 7, 9/10 and/or 12, in the age range of 5-13 years old at time of invitation, were selected for concordance (high-high or low-low) or discordance (high-low) for aggression. MZ twin pairs were invited if they were concordant high for aggression or discordant for aggression, in addition zip-code matched concordant low scoring (sum scores ≤ 4) MZ twin pairs were invited. Twin pairs were not invited if they had been previously included in ACTIONBB1 or ACTIONBB2, if they did not have a stable concordance status when longitudinal data was available, if our zygosity information for this pair was inconsistent, if our address information indicated that the twin pair was not living at the same address, the twin pair was living abroad, or if no contact information was available for the family. In addition, 44 twin pairs as initially invited and participating in ACTIONBB2 were forwarded for participation in ACTIONBB3. Whilst ACTIONBB3 aimed to include MZ twin pairs, DZ twin pairs were included for two reasons: 1) a selected MZ twin pair turned out to be a DZ twin pair after zygosity testing or 2) the DZ twin pairs were siblings of a selected MZ twin pair.

Invitations to participate in ACTIONBB3 took place across 6 batches of invitations. In the first two batches of invitations, all MZ twin pairs younger than 12 years of age with an Aggression Syndrome Scale score at age 3, age 7 and/or age 10, were selected based on their aggression concordances based on the subclinical threshold (T > 65). From batch 3 onwards we used age-specific cut-offs of the aggression score distribution as rated by mothers: aggression scores ≥13 at age 3, ≥5 at age 7 and ≥4 at age 9/10 were considered high aggression scores. For the sixth invitation batch, twins were again selected based on the age-specific cut-offs, but also on aggression scores as rated by teachers using the TRF, where the subclinical (T>65) threshold was used to determine concordance or discordance in aggression at age 7, 10 or 12. TRF scores above the subclinical threshold were considered high on aggression and TRF scores below the subclinical threshold were considered low on aggression.

In the period of the 8th of January 2016 to the 11th of May 2017 we invited 744 twin pairs concordant for low aggression, 309 twin pairs concordant for high aggression and 293 pairs discordant on aggression and their family members to participate in ACTIONBB3. Across all invitation batches we had a participation rate of 39.4% with 237 concordant low aggression twin pairs, 137 concordant high aggression twin pairs, and 157 discordant twin pairs participating in ACTIONBB3 (**Figure 4a**). The participation rate among twin pairs concordant on low aggression scores was 31.9%, while 44.3% of

the concordant high aggression twin pairs and 53.4% of the twin pairs discordant for aggression agreed to participate in ACTIONBB3. In ACTIONBB3 a total of 531 twin pairs agreed to participate. After including the 44 twin pairs originally recruited as part of ACTIONBB2, ACTIONBB3 totals 575 twin pairs of 6 to 13 years of age (mean age = 9.5; SD age = 1.9, 47.3% girls) of which 255 were concordant low aggression twin pairs, 152 were concordant high aggression twin pairs and 168 were discordant twin pairs. These 575 twin pairs belonged to 547 MZ and 28 DZ twin pairs, including 2 DOS twin pairs (**Figure 4b**). Of all twin pairs included in ACTIONBB3 24% were selected with use of the subclinical threshold of T>65 (e.g., ACTIONBB2 and batch 1 and 2 of ACTIONBB3), 48% were invited based on age- and sex-specific mother-rated sum score thresholds (batch 3-5 ACTIONBB3) and 28% was invited based on age- and sex-specific mother-rated sum score thresholds or teacher-rated T>65 scores (batch 6 ACTIONBB3; **Figure 4c**).

Figure 4. Overview of the number of concordant and discordant twin-children invited and participating in ACTIONBB2 by zygosity. **(a)** Number of concordant and discordant twin-children participating versus not participating in ACTIONBB2 by zygosity. **(b)** Number of concordant and discordant twin-children included in ACTIONBB3 by zygosity. **(c)** Number of concordant and discordant twin-children included in ACTIONBB3 by invitation batch.



Note: MZ = monozygotic twin; DZ = same-sex dizygotic twin; DOS = part of an opposite-sex dizygotic twin pair. Batch 1 - 6 = invited in invitation batch 1 - 6 for ACTIONBB3; BB2 = invited as part of ACTIONBB2 and forwarded for inclusion in ACTIONBB3.

2.1.6 Procedure ACTIONBB2 and ACTIONBB3

The parents of selected twin pairs received a letter detailing the aim of the study and asking them to participate (**Figure 2a**). With this letter, informed consent (IC) forms were included (**Figure 2a**). ICs for parents and siblings of twins were included for buccal-cell DNA collection (**Figure 2a**; printed on blue paper) and for buccal-cell DNA and urine samples in twins (**Figure 2a**; printed on green paper). Approximately one week after receiving the letter, a research assistant called the families to supply information regarding the study. Parents were then asked to indicate their willingness to participate in the study. After receiving the signed ICs or after verbal confirmation of participation via telephone (with agreement to mail signed ICs) collection packages for urine and buccal-cell DNA collection were sent to the families (**Figure 2b-2c**), including cover letters for each of the packages, instructions, a collection form (incl. questions about the collection process and current health and medication use) and a survey booklet with the ASEBA CBCL and a wellbeing and life satisfaction item.

Parents followed the instructions as provided to collect the urine and buccal samples and filled out the survey. Approximately one week after receiving the urine and buccal-cell DNA collection packages the parents were called to enquire if they had been successful in collecting the samples. Once urine and buccal samples had been collected, an appointment was made to collect the material by a research assistant who drove out to the families to collect the samples. The family received €20.- in gift certificates as a token for their participation, parents were asked to sign as proof of receiving these certificates.

2.1.7 Description of NTR-ACTION Biomarker Study participants

From December 2014 to May 2017, 3,304 twins were invited for the NTR-ACTION Biomarker Study, with 1,367 twins (41.4%) agreeing to take part. For epigenetic measures, 108 extra twins with buccal-cell samples and longitudinal aggression data were included from earlier sample collection in the NTR. Thus 1,495 twins (747 complete pairs), either with first-morning urine (*N* = 1,382) and/or buccal-cell swabs (*N* = 1,488), were included in the NTR-ACTION Biomarker Study. In total, 697 twins were included as part of a concordant low-scoring twin pair (348 pairs), 406 twins as part of a concordant high-scoring twin pair (203 pairs), and 392 twins (196 pairs) as part of a discordant twin pair (**Table 1**). Twins in the NTR-ACTION Biomarker Study were between 5.6 and 15.0 years of age at the time of urine and/or buccal-cell collection (mean age = 9.7; SD age = 1.8), 47.5% of the participants were girls (N = 710) and 82.6% belonged to an MZ twin pair (N = 1,234). At the time of

urine/buccal-cell collection, twin pairs selected for concordant high aggression had significantly higher CBCL Aggression Syndrome Scale scores as compared to twin pairs selected for concordant low aggression (β = 5.09; *SE* = 0.50; $p = 1.83 \times 10^{-24}$). Similarly, when comparing the discordant twin pairs, the high aggressive twins (*M* = 6.2, *SD* = 5.8) had significantly higher Aggression Syndrome Scale scores at the time of urine/buccal-cell collection than their low aggressive co-twins (*M* = 4.4, *SD* = 4.4; $t(185) = 5.73$, $p = 4.08 \times 10^{-08}$).

Table 1. Decriptives for all twin pairs with urine (*N* = 1,382) or buccal-cell DNA (*N* = 1,488) samples in the NTR-ACTION Biomarker Study (*N* = 1,495).

		Discordant		
	Concordant Low	Low (n = 196)	High (n =196)	**Concordant High**
N (N complete twin pairs)	697 (348)	392 (196)		406 (203)
Mean (SD) age sample collection	9.5 (1.9)	10.1 (1.7)		9.5 (1.8)
Range age sample collection	5.6 – 15.0	6.1 - 12.7		5.8 - 12.9
N (%) girls	363 (52.1%)	88 (44.9%)	82 (41.8%)	177 (43.6%)
N (%) MZ twins	544 (78.2%)	160 (81.6%)		370 (91.1%)
Mean CBCL (SD) aggression score[a]	2.7 (3.8)	4.5 (4.4)	6.3 (5.8)	7.5 (6.0)

[a] Mother-rated Aggression Syndrome Scale sum score of the ASEBA CBCL for school-aged children (6-18 years).

2.2 LUMC-Curium

The LUMC-Curium academic center for child and youth psychiatry (Oegstgeest, the Netherlands) provides inpatient and outpatient treatment programs and treats children with severe and complex mental health problems who need intensive care. Between February 2016 and June 2018, 6- to 13-year-old children referred to the LUMC-Curium for treatment were invited to participate in the ongoing biobank protocol of LUMC-Curium[11]. Study approval was obtained from the Medical Ethical Committee of the Leiden University Medical Center (B15.017, B17.031, B17.032 and B17.040). Written informed consent to participate in the ACTION Biomarker Study was provided by the participants' legal guardian(s) for all subjects. Parents of participating children collected first-morning urine and buccal cell swabs with the protocols as developed for the NTR-ACTION cohort and completed the ASEBA CBCL in a six-month timeframe surrounding the collection of the biological material[5]. As part of a standardized clinical assessment, primary DSM-IV classifications were obtained and physical measures, including height, weight, and resting heart rate were also collected[5]. In total, 809 parents and children were invited to participate in the study, of which 189 (23.4%) children agreed to participate (including eight sibling pairs and a sibling trio).

3 Urine and buccal swab collection protocols

3.1 Urine collection protocol

1. The first morning urine was collected as described in the 'urine collection instruction form' (**Appendix 1**). Urine should have been collected on the first morning on which the child had not been to the toilet at night, wet the bed, or was suffering from an infection, the flue or the common cold.
2. The uritainer was placed on the toilet seat as detailed in the instruction manual for the uritainer (**Appendix 2**).
3. The child urinated in the uritainer.
4. The parent noted the time and date of urination on the urine collection form (**Appendix 3**).
5. The parent transferred the urine from the uritainer to the urine sample tubes. For each child two urine tubes were provided in each collection round during ACTIONBB1 and four collection tubes were provided during ACTIONBB2, ACTIONBB3, and for LUMC-Curium participants. The tubes were not allowed to exceed the limit of 12ml of urine, with a minimum of 6ml of urine in ACTIONBB1 and with a minimum of 1ml of urine for 2 of the tubes and 10ml of urine for the other 2 tubes in ACTIONBB2, ACTIONBB3, and for LUMC-Curium participants.
6. The urine tubes were then placed in the protective blister. For each child, one protective blister was provided in ACTIONBB1 and two in ACTIONBB2, ACTIONBB3, and for LUMC-Curium participants. Each protective blister could hold up to two urine tubes.
7. The samples tubes in the protective blister were placed in the home freezer (-18^{o}C) as soon as possible.
8. The parent noted the time at which the urine samples were placed in the freezer on the urine collection form. Additionally, the parent filled out the remainder of the urine collection form, which consisted of information about the child, the type of freezer used, any comments with regards to the urine collection. In the NTR, additional questions for girls only with regards to their potential menarche were added in all three phases of data collection, and in ACTIONBB2 and ACTIONBB3 additional questions with regards to medication use, vitamin use and general health were included.

3.1.1 Urine transportation and storage

Urine samples were transported in a mobile freezer unit (-18^{o}C) to the laboratory at Good Biomarker Sciences (GBS) in Leiden for storage (-80^{o}C) until such time the analyses could begin. GBS also provided the partners at the Leiden Academic Centre for Drug Research (LACDR) with aliquots of the

urine samples for metabolomics analyses. Aliquots of the urine samples were prepared once all data for a collection wave had been collected.

3.2 Buccal swab collection

Buccal-cell DNA collection instructions for all phases of the study were identical and well established in earlier NTR studies[12,13]. Buccal-cell swabs were collected twice per day on two consecutive days. It was advised to collect buccal-cells once in the morning, prior to breakfast, and once in the evening, prior to dinner. Before buccal-cell collection participants were asked not to eat, brush their teeth, gargle or rinse their mouth. Each time, participants needed to use 4 cotton swabs, each cotton swab needed to be used for 10-20 seconds on a specific area of the mouth while applying light pressure:

1 cotton swab to be used on the inside of the upper lip and gums of the upper jaw

1 cotton swab to be used on the inside of the lower lip and gums of the lower jaw

1 cotton swab to be used on the inside of the left cheek

1 cotton swab to be used on the inside of the right cheek

After following the swabbing instructions, participants needed to place the cotton swabs tip-down in the provided tubes pre-filled with buffer liquid[14]. All four swabs needed to be placed in the same tube per collection time. Buccal-cell samples were stored upright, at room temperature, out of direct sunlight and kept at the Vrije Universiteit Amsterdam prior to transportation to Avera Institute for Human Genetics (Sioux Falls, South Dakota, USA) for DNA extraction and genotyping. Extracted buccal DNA was send to GBS and then shipped to the Human Genotyping facility (HugeF) of Erasmus University Medical Center, the Netherlands (http://www.glimdna.org/) for epigenetic testing (Infinium MethylationEPIC BeadChip).

4 Data generation

4.1 DNA extraction protocol from buccal swabs

1. **Thaw** 2 sample tubes (containing ~8 swabs) on bench top.
2. Lyse cells by adding 15µl of proteinase K (10mg/ml) per swab to each tube & centrifuge briefly. (8 swabs = 60µl proteinase K)
3. **Incubate** prepped tubes **overnight** in **a 56°C** water bath.
4. Place a 20 ml syringe (plunger discarded) into a labeled 50 ml Falcon tube.
5. Using a disposable forceps, place each cotton swab into the barrel of the syringe.
6. Centrifuge the 50 ml Falcon tube for **5 min at 1000 rpm**.

7. Carefully remove and discard the syringe and dry swabs.
8. Add the remaining contents of the original swab tubes to the 50 ml Falcon tube.
    a. You may now discard original 15 ml tubes.
9. Add 0.2 volumes of **8M KAc**. (3.5 ml= 700 µl; **4 ml=800 µl**; 4.5 ml= 900 µl; 5 ml=1000 µl)
10. Mix thoroughly by gently inverting the tube 10 times.
11. **Incubate** the tube in **ice** for **15-60 minutes**, mixing the tubes periodically.
12. Carefully add 1 volume (**~5 ml**) of **chloroform:isoamyl alcohol** (24:1).
13. Mix thoroughly by gently inverting the tube 10 times.
14. Place tube on a rotating platform **vortexer** for **30 minutes** at medium speed (4-5).
15. **Pre-spin** a 50 ml **phase-lock** heavy gel tube at **1500 x g** for **2 minutes** with a **brake set at 3**.
16. Pour the DNA sample mixture into the phase-lock tube.
17. Mix thoroughly by gently swirling the tube several times.
18. Centrifuge phase-lock tube at **1500 x g** for **5 minutes** with a **brake set at 3**.
19. Very carefully pour the upper aqueous layer (genomic DNA in solution) to a **new 50 ml Falcon tube**.
    a. You may now discard the phase-lock tube which contains solubilized cell precipitates under the gel.
20. Add 2 volumes (**~8 ml**) of **absolute ethanol**.
21. Mix thoroughly by gently inverting the tube 10 times.
22. **Incubate** the tube at **-20°C** for at least **30 minutes**. Set the temperature on the **centrifuge** to **4°C** during the **incubation step**.
    a. Incubation may be overnight if more convenient.
23. Centrifuge the tube at **4000 rpm** for **10 minutes** at **4°C**.
24. Being cautious not to disturb the pellet, carefully **discard the supernatant**.
25. Add **5 ml** of **70% ethanol** the tube.
26. Centrifuge the tube at **4000 rpm** for **10 minutes** at **4°C**.
27. Being cautious not to disturb the pellet, carefully **discard the supernatant**.
28. **Dry** the pellet for **30 minutes** to remove any residual ethanol.
29. Add **200 µl** of **TE buffer** to the tube.
30. **Vortex** the tube briefly to aid in the dissolution of the pellet.
31. **Incubate** the sample **overnight** at **4°C** to allow for complete resuspension of the DNA.
32. Vortex the sample and briefly centrifuge the tube.
33. Transfer the sample to an appropriately labeled tube.
34. Measure the yield and purity of the sample using the NanoDrop spectrophotometer.

4.2 Genotyping

Buccal cell samples were genotyped on the Affymetrix Axiom[15], or Illumina GSA[16] platforms by the Avera Institute for Human Genetics (Sioux Falls, South Dakota, USA). Genome-wide Single Nucleotide Polymorphism (SNP) data are available for 3,149 participants, including 1,702 parents or siblings of twins (AXIOM = 909, GSA = 2,240). Genotyping data were also analyzed to establish twin zygosity[17], of which the parents of the twins received the results.

4.3 DNA methylation

The epigenetic measurements were conducted in two batches. Batch 1 consisted of 13 plates, these batches included the ACTIONBB2 and ACTIONBB3 participants for whom DNA collection was finished before October 2017 as well as 108 additional epigenetic samples. All samples were randomized across plates. Batch 2 consisted of 3 plates of NTR-ACTION samples for whom DNA collection was completed after October 2017 and 2 separate plates of LUMC-Curium samples.

The 108 additional epigenetic samples consisted of 54 twin pairs from the NTR with previously collected buccal-cell samples for whom longitudinal parent and/or teacher-rated aggression data was available. These 54 twin pairs were age-matched on time of buccal-cell collection to approximate the mean age at biological sample collection of the NTR-ACTION Biomarker Study participants. The mean age of these additional twins was 9.9 years (SD = 2), 44.4% were girls and 98.1% were MZ twins. As these twin pairs were not initially selected on aggression concordance, they were assigned a concordance status based on their mother-rated Aggression Syndrome Scale score at age 3, 7 or 10. An age-specific sum score defined high-scoring children based on mother ratings as: age 3 ≥ 13, age 7 ≥ 5 and age 10 ≥ 4. To ensure all twin pairs could be assigned a concordance status, low aggression was defined as scores below the age-specific thresholds. In total, 30 twin pairs were assigned a concordant low-low aggression status, 4 twin pairs were assigned a discordant aggression status and 20 pairs a concordant high-high aggression status.

Genome-wide methylation data in buccal DNA samples were measured on the Infinium MethylationEPIC BeadChip kit (Illumina, San Diego, CA, USA)[18] by the Human Genotyping Facility (HugeF) of ErasmusMC (the Netherlands; http://www.glimdna.org/). The ZymoResearch EZ DNA Methylation kit (Zymo Research Corp, Irvine, CA, USA) was used for bisulfite treatment of 500 Ng of genomic DNA obtained from buccal swabs. The Infinium HD Methylation Assay was performed according to the manufacturer's specification. We applied a custom pipeline for quality control and normalization of DNA methylation data that was developed by the Biobank-based Integrative Omics Study (BIOS) consortium[19].

4.4 Biomarkers and metabolomics

4.4.1 Description of batch structure biomarker and metabolomics measurements

Biomarker and metabolomics measurements for ACTIONBB1, ACTIONBB2 and ACTIONBB3 were done separately. The longitudinal samples collected in ACTIONBB1 were measured simultaneously, ensuring any differences between the samples were due to the time of collection and not due to differences between analyses runs. Biomarker and metabolomics measurements for ACTIONBB2 were performed in a single analysis run. The biomarker measurements included 7 batches of approximately 35 samples per batch, and the metabolomics measurements included 4 batches of approximately 55 samples per batch. All samples were randomized over the batches, with twin-children randomized per pair across batches. In ACTIONBB3 biomarker and metabolomics measurements were performed in two analysis runs, including both the NTR ACTIONBB3 and all LUMC-Curium samples. The biomarker measurements included approximately 35 samples per batch, with 32 batches in analysis run one and 8 batches in analysis run two. Analysis run one for the metabolomics measurements also included all twins measured in ACTIONBB2, this analysis run consisted of 19 batches, analysis run two consisted of 5 batches, both runs included approximately 70 samples per batch. All samples were randomized across batches, with twin-children randomized per pair across batches. Batches were constructed in such a manner that the clinically referred children were spread across the batches and for analysis run one of the metabolomics measurements the ACTIONBB2 twin pairs were also spread across the batches.

4.4.2 Biomarker quantification

In each phase of the NTR-ACTION Biomarker Study the conducted biochemical measurements in the urine samples differed slightly. In **Table 2** the number of observations per biochemical measurement, per phase in the NTR-ACTION Biomarker Study, is given. Some measurements could not be obtained for all samples, most often this was due to insufficient remaining urine sample to perform the measurement, however, in the case of the obtained biomarkers, a missing measurement can also reflect biomarker levels below the detection level.

Enzyme-linked immunosorbent assay (ELISA) measurements of thiobarbituric acid reactive substances (malondialdehyde), beta-endorphin, cholecytokinin, leu-enkaphalin, dynorphin A, albumin, and neurotensin were only performed in ACTIONBB1, and ELISA measurements of procalcitonin, glucose, and the total antioxidant capacity were discontinued after ACTIONBB2. The following biochemical measurements were conducted in each phase of the NTR-ACTION Biomarker Study[5]:

1. a urine dipstick, (Siemens, Marburg, Germany) to screen for infections in urine and to measure leukocytes, nitrite, proteins, glucose, and blood presence in the urine. The dipstick was applied to the first thaw of the urine samples either by dipping in the residual urine volume after aliquoting or by dropping urine on the dipstick.
2. a density measurement with the Atago® refractometer (PAL-10S BLT/A+W, Atago, Tokyo, Japan). The refractive index is a ratio of the velocity of light in air to the velocity of light in solution, which is directly proportional to the number of dissolved solids in urine.
3. Creatinine was measured using a colorimetric assay kit according to manufacturer's instructions (Cayman, Ann Harbor, MI, USA).
4. and ELISA measurements according to manufacturer's instructions of neopterin (IBL International GmbH, Munich, Germany), C-peptide (IBL International GmbH, Munich, Germany), Substance P (Cayman, Ann Harbor, MI, USA), and oxidized guanine species (including 8-hydroxyguanosine, 8-hydroxy-2'-deoxyguanosine, and 8-hydroxyguanine) as a marker for oxidized DNA and RNA (Cayman, Ann Harbor, MI, USA.

Table 2. Number of participants with urine (*N* = 1,382) and buccal-cell DNA (*N* = 1,488) samples in the NTR-ACTION Biomarker Study (*N* = 1,495) per obtained biochemical measurement in each of the three phases of the NTR-ACTION Biomarker Study.

	ACTIONBB1_1	**ACTIONBB1_2**	**ACTIONBB2**	**ACTIONBB3**
Dipstick	18	18	217	1142
Creatinine	18	18	215	1141
Density	18	18	215	1142
Neopterin	18	18	217	1142
C-peptide	18	18	216	1142
Substance P	16	16	213	1142
Oxidized DNA/RNA	8	8	213	1142
Procalcitonin	18	18	218	0
Total Antioxidant Capacity	18	18	216	0
Glucose	18	18	216	0
Thiobarbituric acid reactive substances (malondialdehyde)	18	18	0	0
Beta-endorphin	15	18	0	0
Cholecytokinin	15	18	0	0
Leu-enkaphalin	15	18	0	0
Dynorphin A	13	18	0	0
Albumin	16	16	0	0
Neurotensin	14	11	0	0

Notes: ACTIONBB1_1, first measurement in ACTIONBB1; ACTIONBB1_2, second measurement in ACTIONBB1.

4.4.3 Metabolite quantification

As described in previous publications[5,20–22], the Metabolomics Facility of the University of Leiden (Leiden, The Netherlands) assessed metabolites in urine on three platforms: an ultra-performance liquid chromatography mass spectrometry (UPLC-MS) platform targeting amines, a gas chromatography mass spectrometry (GC-MS) platform targeting organic acids, and a UPLC-MS platform targeting steroids. Each measurement batch included a calibration line, quality control (QC) samples (every 10 samples), sample replicates, and blanks. The QC samples comprised pooled aliquots of all urine samples from all children. Using the pooled QC samples, the lab applied in-house developed algorithms to compensate for shifts in the sensitivity of the mass spectrometer across batches. Blank samples were used to determine if there was any interference from background signal. Relative Standard Deviation (RSD) of the Quality Control samples (RSDqc) were used to evaluate the performance and reproducibility of individual metabolites. The acceptance criteria for metabolite reporting were RSDqc <15% and background signal <20%, metabolites with RSDqc of 15–30% should be interpreted with caution. Metabolites were reported as 'relative response ratios' (target area/area of internal standard) after QC correction.

4.4.3.1 UPLC-MS Amine Platform

The amine method has been described in detail elsewhere[23].T In short, the amine metabolites were measured using ultra-performance liquid chromatography tandem mass spectrometry (UPLCMS/MS) employing an Accq-Tag derivatization strategy adapted from the protocol supplied by Waters (Waters, Etten-Leur, The Netherlands). Sample preparation consisted of protein precipitation by the addition of methanol to 5 µL of urine spiked with internal standards (see **Table 3**). The centrifuged supernatant was then evaporated using a speedvac prior to reconstitution in borate buffer (pH 8.5) with AQC reagent. Chromatic separation was done on an Accq-Tag Ultra column (Waters Chromatography B.V., Etten – Leur, The Netherlands) using a UPLC Agilent 1290 Infinity II LC system (1290 Multicolumn Thermostat and 1290 High Speed Pump, Agilent Technologies, Waldbronn, Germany) coupled to an AB SCIEX quadrupole-ion trap (SCIEX Qtrap 6500; Sciex, Framingham, MA, USA). Analytes were detected in the positive ion mode and monitored in Multiple Reaction Monitoring (MRM) using nominal mass resolution.

Table 3. Internal standards used in the UPLC-MS amine platform.

Asn_C13N15	Asp_C13N15	L-ornithine-3,3,4,4,5,5,-d6
L-NT-methyl-d3-L-histidine	Glu_C13N15	Lys C13N15

Ser_C13N15	Beta-alanine-2,2,3,3,-d4	Tyr_C13N15
Gln_C13N15	Thr_C13N15	L-Methionine_C13N15
Arg_C13N15	Ala_C13N15	Val_C13N15
Gly_C13N15	Phe_C13N15	Trp_C13N15
Histamine-α,α,β,β-d4 2HCl	L-2-aminobutyric acid-d6 acid	2-(4-hydroxy-3-methoxyphenyl) ethyl-1,1,2,2-d4-amine
L-Ile_C13N15	Leu_C13N15	

4.4.3.2 GC-MS Organic Acid Platform

The organic acid metabolites were measured using gas chromatography mass spectrometry (GC-MS). Sample preparation of 50 µL of urine spiked with internal standards (Succinic acid-2,2,3,3-d4, Fumaric acid-2,3-d2, and Citric acid-2,2,4,4-d4) consisted of liquid-liquid extraction with ethyl acetate to extract the organic acids and remove urea present in the urine. After collecting the organic phase, the samples were evaporated to dryness using a speedvac. Then, two-step derivatization procedures were performed on-line: oximation using methoxyamine hydrochloride (MeOX, 15 mg/mL in pyridine) as first reaction and silylation using N-Methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) as second reaction. After derivatization, 1 µL of sample was injected into the GC-MS, with helium as carrier gas (1,7 mL/min), chromatic separation was performed on a 30 × 0.25m ID column with a film thickness of 25m (HP-5MS UI). The mass spectrometer (MSD 5975C, Agilent Technologies, Waldbronn, Germany) with a single quadrupole using electron impact ionization (70 eV) was operated in SCAN mode (mass range 50–500).

4.4.3.3 UPLC-MS Steroid Platform

An UPLC-MS steroid platform was developed for the current study. Sample preparation comprised adding internal standards (Cortisone-2,2,4,6,6,9,12,12-d8, and Testosterone-16,16,17-d3) to 90 µL of urine and filtering the samples with a 0.2 µm PTFE membrane. Using an Acquity UPLC CSH C18 column, 130 Å, 1.7 µm, 2.1 mm x 100 mm, (Waters, Etten-Leur, The Netherlands), with a flow of 0.4 mL/min over a 15 min gradient, chromatographic separation was achieved by UPLC (Agilent 1290, San Jose, CA, USA). Samples were analyzed using a triple quadrupole mass spectrometer (Agilent 6460, San Jose, CA, USA) with electrospray ionization. By switching positive and negative ion mode, analytes were detected in MRM using nominal mass resolution.

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Appendix 1: Urine collection instruction form (adapted and translated from Dutch)

We would like to ask you to collection urine for our research project. This urine material can be used to study metabolites. Metabolites are metabolic products that may provide insight into the functioning of a person. As metabolite concentrations depend on the time of day, we ask you to collect your child's morning urine. Urine collection can be achieved in a straightforward manner by use of the enclosed 'uritainer'. The exact instructions follow below.

Necessary for urine collection:

1. uritainer; a toilet holder for collecting the urine
2. 4 small tubes; for the storage of the urine
3. 2 plastic blisters; to collect the urine tubes during transport
4. Urine collection form

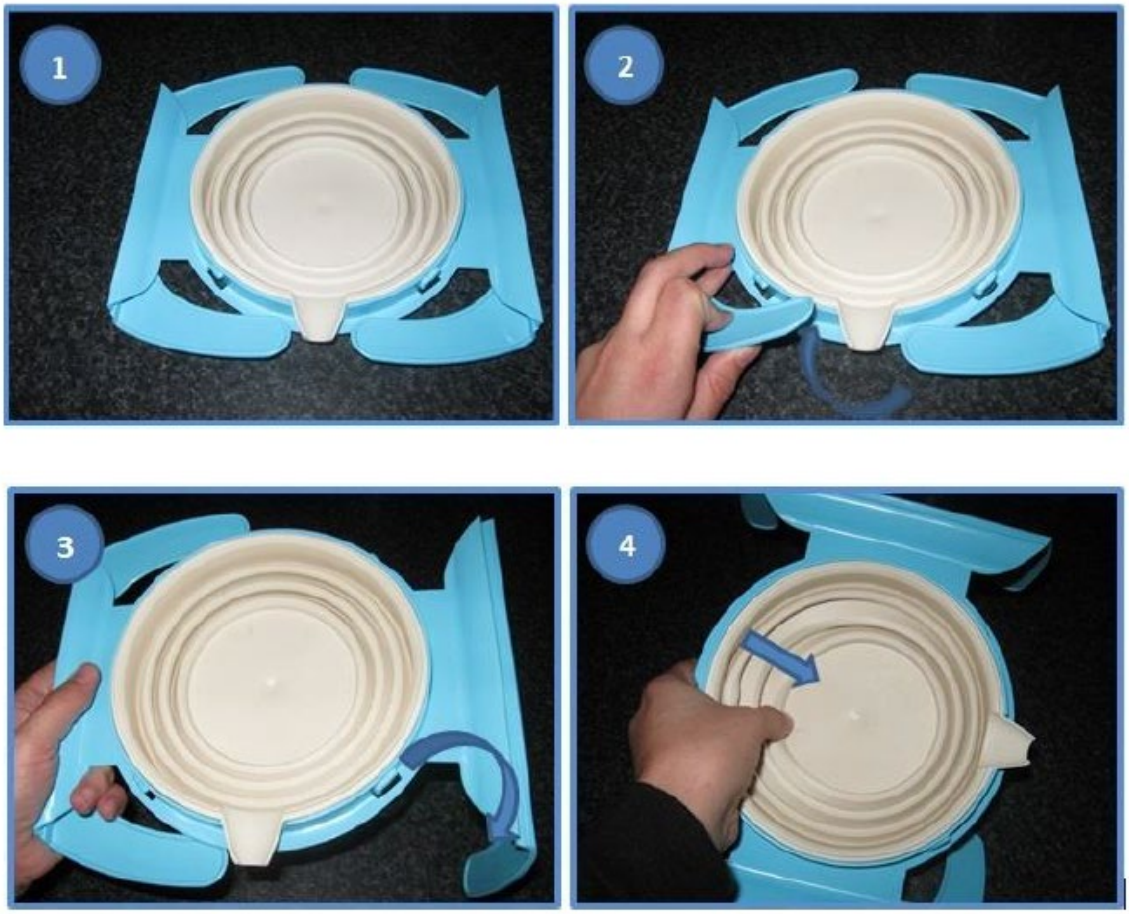
Collection:

1. Once your child has awakened in the morning you can collect the first morning urine. ***ATTENTION:*** collect the urine before your child has washed, soap residue and use of moist cloths could influence our measurements.
2. Take the uritainer from the plastic wrapper and place it on the toilet (**Appendix 2**).
3. Have your child urinate into the uritainer.
4. Take the uritainer from the toilet and pour the urine into the included small tubes (the uritainer has a pouring spout).
    a. We ask you to fill all the tubes. Please fill two tubes with a *minimum* of 10 ml and the other two tubes with a *minimum* of 1 ml (unable to fill the tubes with these minimums? Please contact us for a new collection kit!). ***ATTENTION:*** do not overfill the tubes (*maximum* of 12 ml per tube; e.g., the top line). Urine expands during freezing and overfilled tubes might crack or open under pressure.
5. Note the time of urine collection on the urine collection form (**Appendix 3**).
6. Place the urine tubes in the plastic blisters to prevent potential leaking or damage.
7. Store the collected urine in your freezer or the freezer compartment of your fridge!
8. Note the time at which the urine was placed in the freezer on the urine collection form and fill out the remainder of this form (**Appendix 3**).

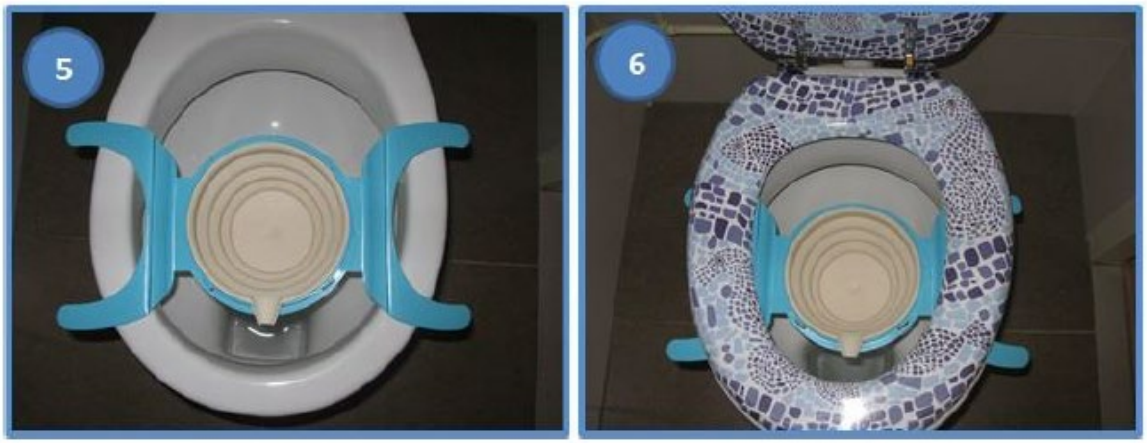
The remaining urine can be flushed and the uritainer may be recycled.

Appendix 2: instruction manual uritainer (translated from Dutch)

1. Take the uritainer out of the plastic wrapper (**Figure 1**)
2. Lift the brackets from the edge (**Figure 2**) and fold down the brackets (**Figure 3**).
3. Push the white drip tray down until it is fully extended (**Figure 4**).



4. Place the uritainer under the toilet seat. Pay attention to the placement of the uritainer; make sure it is placed just right for the collection of urine (**Figure 5**).
5. Clamp the uritainer between the toilet and the toilet seat to prevent shifting (**Figure 6**).



Appendix 3: urine collection form (translated from Dutch)

Urine collection:

Date (dd/mm/yyyy): ……/……/……

Time urine collection (hh/mm): ……/……

Time urine in freezer (hh/mm): ……/……

Remarks with regards to urine collection or freezing:

What type of freezer do you have?

☐ freezer as compartment in the fridge

☐ freezer separate from the fridge

Other remarks with regards to urine collection or freezing?

---

for girls:

	No	Yes
Is your daughter menstruating?	☐$_1$	☐$_2$
Is your daughter menstruating at the time of urine collection?	☐$_1$	☐$_2$

Health and medication use:

	No	Yes
1. Does the child have a chronical physical condition or physical disability?	☐$_1$	☐$_2$
2. Does the child have a condition or disability which **severely impacts daily** functioning, despite the potential use of aids or medication?	☐$_1$	☐$_2$
3. Does the child currently have any infections (e.g., toothache, infected eye, urinary tract infection)?	☐$_1$	☐$_2$
4. Does the child currently have a (childhood) disease such as (stomach)flue or chicken-pox?	☐$_1$	☐$_2$

5. If you answered question 3 or 4 with '*yes*', could u specify the type of infection or illness and when this started?

Type of infection and/or (childhood) disease	Started at (date [dd/mm/yyyy])

6. Does the child use any medication **at this moment**?	No	Yes
	☐$_1$	☐$_2$

7. If you answered question 6 with 'yes', could you specify the type of medication, how often this is used and how long this has been used?

Medicine	Condition	Frequency	Used since (date [dd/mm/yyyy])

8. Does the child use any vitamin supplements **at this moment**?

No	Yes
☐1	☐2

9. If you answered question 8 with 'yes', could you indicate the type of vitamin supplement, how often they are used and for how long they have been used?

Type supplement (e.g., brand name or description of contents)	Frequency	Used since (date [dd/mm/yyyy])

10. Could you indicate for each of the following conditions whether they are applicable to your child?

	No	Yes
a. asthma, chronic bronchitis or CARA	☐1	☐2
b. inflammation of the paranasal sinuses, forehead cavity or maxillary sinus	☐1	☐2
c. severe skin disease or eczema	☐1	☐2
d. severe bowel disorders, longer than 3 months	☐1	☐2
e. chronic cystitis	☐1	☐2
f. back disorder of persistent nature, longer than 3 months	☐1	☐2
g. epilepsy	☐1	☐2
h. serious heart defect	☐1	☐2
i. diabetes	☐1	☐2
j. malignant condition or cancer	☐1	☐2
k. liver disease or liver cirrhosis	☐1	☐2
l. severe kidney disease	☐1	☐2
m. joint inflammation or chronic rheumatism, lasting more than 3 months	☐1	☐2
n. deaf or very hard of hearing	☐1	☐2
o. blind or very visually impaired	☐1	☐2
p. spastic	☐1	☐2
q. disorder of the musculoskeletal system (orthopedic disorder)	☐1	☐2
s. allergies type of allergies:……	☐1	☐2
t. other **serious** congenital or **serious** long-term conditions description:……	☐1	☐2
11. Is the child treated for one of the disorders listed under question 10, or does the child have to check with a specialist at least once a year?	☐1	☐2

12If you answered any of the above health questions (**questions 1 to 11**) with '*yes*', could you give a brief explanation below? (e.g. a further explanation about the type of disorder or disability).

……

……